Studies of the cytosolic thymidine kinase in human cells and comparison to the recombinantly expressed enzyme

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Studies of the cytosolic thymidine kinase in human cells and comparison to the recombinantly expressed enzyme

PhD Thesis

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Papers I - IV:

I. T. Kristensen, H.K. Jensen and B. Munch-Petersen (1994) Quantitation of TK1 mRNA in patients with chronic lymphatic leukemia. To be published in *Advances in Experimental Medicine and Biology*.

II. T. Kristensen, H.K. Jensen and B. Munch-Petersen (1994) Overexpression of human thymidine kinase mRNA without corresponding enzymatic activity in patients with chronic lymphatic leukemia, *Leukemia Research*. **18**, 861-866.

III. H.K. Jensen and B. Munch-Petersen (1994) Altered kinetic properties of recombinant human cytosolic thymidine kinase (TK1) as compared to the native form. To be published in *Advances in Experimental Medicine and Biology*.

IV. H.K. Jensen and B. Munch-Petersen (1994) Human cytosololic thymidine kinase expressed in *E.coli* occurs solely as the high affinity tetramer form with the low K_m value. Manuscript.

PREFACE.

My experimental work during the last 3 years is gathered in this thesis to complete my Ph.D study. The work is carried out at Roskilde University financed by a University scholarship (Kandidat Stipendium) and under supervision of associate professor Birgitte Munch-Petersen.

The task for this thesis has been concentrated on:

"Studies of the cytosolic thymidine kinase (TK1) in human cells and comparison to the recombinantly expressed enzyme".

The experimental work is divided into two different approaches to the problem. The first approach was to develop a sensitive method (competitive PCR) to quantitate TK1 mRNA and to compare TK1 mRNA to the enzyme activity. A model system of normal lymphocytes stimulated to growth was investigated by this method together with leukemic cells, chronic lymphatic leukemia (CLL) cells. The other approach was to construct an expression vector for TK1 in E.coli. The recombinant TK1 was purified and characterized as compared to native TK1 purified from human lymphocytes.

The experimental work is presented as well as a review over the field of regulation af TK1. Four papers are included, where two are original articles and two are conference proceedings from a meeting on Purine and Pyrimidine metabolism (Indiana, may 1994). Paper I and II is carried out together with PhD-student Tina Kristensen, though Tina wrote paper I. Paper III and IV are my own work, though my superviser Birgitte Munch-Petersen has been tightly connected in the process of all the papers.

Roskilde University, den 12. december 1994

Helle Koch Jensen

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I want specially to thank Staffan Eriksson (Karolinska Institutet in Stokholm) who invited me to work at his laboratory for three weeks. Here also special thanks to Anita Herrström who helped me find my way around both inside and outside the laboratory. Also I want to thank the rest of the laboratory staff who made me feel part of the group during my stay.

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A very special thank to my supervisor Birgitte Munch-Petersen who has supported me on all levels through this work.

ABBREVIATIONS

ara-T: 2′-arabinosol-thymidine
ATP: Adenosine triphosphate
AZT:3′-azidothymidine
BSA: Bovine serum albumine
CTP: Cytidine triphosphate
CLL: Chronic lymphatic leukemia
DTT: Dithiothreitol
FCS: Fetal calf serum
HIV: Human immunodeficiency virus
HSV: herpes simplex virus
IPTG: Isopropyl-ß-D-thiogalactopyranoside
PCR: Polymerase chain reaction
PHA: Phytohemagglutinin
PMSF: Phenylmethylsulphonyl fluoride
TK: Thymidine kinase
TK1: Cytosolic thymidine kinase
TK2: Mitochondrial thymidine kinase
TTP: Thymidine triphosphate

INTRODUCTION.

In the work of developing new drugs against different diseases, the easy part is to develop a drug affecting the disease. It is much more complicated to find a drug that only cure or inhibit the disease without causing sideeffects.

Nucleoside analogs are used as drugs in the treatment of cancer and virus infections. If the nucleoside analogs are phosphorylated by nucleoside kinases in the cell, they interfere with the DNA synthesis of the cell and is toxic to the cell. Therefore it is important to have a specific knowledge of the involved enzymes and in which cells they are located. Thymidine kinase (TK) is a vital nucleoside kinase phosphorylating many of the nucleoside analogs used in therapy.

If the cancer cell or virus infected cell (target cell) carries a nucleoside kinase specific for the nucleoside analog and the normal cell is without this enzyme, the nucleoside analog works as a specific drug. This is the case in the treatment of Herpes Simplex Virus (HSV) infections (Fyfe et al, 1978; Elion, 1985). HSV is a DNA virus carrying a gene for TK. HSV TK can phosphorylate the nucleoside analog acyclic guanidine which is not a substrate for TK in normal cells (Elion et al, 1977). Therefore this analog is a very good drug at HSV infections.

It is otherwise with the use of the nucleoside analog 3'-azidothymidine (AZT) in Human Immunedeficiency Virus (HIV) infections. AZT was found to inhibit the infectivity and cytopathic effect of HIV in vitro (Mitsuya et al, 1985). AZT

inhibits the reverse transcriptase of the virus more efficiently than the DNA polymerase (Cheng et al, 1987; Ono et al, 1989). On the other hand AZT is also a good substrate for the human TK1 dominating in dividing cells (Eriksson et al,1991). Therefore this analog is affecting both the virus and the normal cells and thereby causing sideeffects, such as anemia and bone marrow depression (Langtry & Campoli-Richards, 1989).

TK is present as two isoenzymes in mammalian cells, TK1, a cytosolic TK strictly regulated with the cell cycle, and TK2, a mitochondriel constitutively expressed TK (Lee & Cheng, 1976a; Munch-Petersen & Tyrsted, 1977). Therefore the TK level is high in dividing cells due to TK1 and low in quiescent cells due to TK2. Anyhow altered TK enzymes is found in cancer cells (Munch-Petersen & Tyrsted, 1985; Munch-Petersen & Tyrsted, 1986; Munch-Petersen, 1988; Munch-Petersen, 1990). A further characterization of these isoenzymes would open for developing specific nucleoside analogs and could therefore be valuable in diagnosis and therapy. Furthermore, TK1 is a strictly cell cycle regulated enzyme with documented regulation on several levels of expression (Coppock & Pardee, 1987; Gudas et al, 1988; Groudine et al, 1984; Ito & Conrad, 1990). Thereby it is also a very good model system to investigate regulation of gene expression in general.

The first part of this thesis is to develop a sensitive method to quantitate TK1 mRNA (competitive PCR) and to clarify the coherence between TK1 mRNA and enzyme activity during PHA stimulation of human lymphocytes. Chronic lymphatic leukemic

(CLL) cells was included in this work because CLL cells are non-dividing cells and therefore they should have no TK1 enzyme activity. On the other hand they are leukemic cells and generally cancer cells are characterized by a high TK1 activity level. Furthermore Munch-Petersen and Tysted (1986) found that the dominating TK activity isolated from a CLL patient displayed an enzyme kinetic pattern similar to that observed with TK1.

In the present work with CLL cells, it was surprisingly found that the TK mRNA level was very high without any corresponding enzyme activity. These results are presented as proceedings (paper I) and published in Leukemia Research (paper II). This study was to investigate the expression of TK1 both on the mRNA and enzyme level in quiescent and dividing normal cells and CLL cells. Parallel with this, we wanted to establish an expression system enabling us to express recombinant TK1 enzyme. Thereby it would be possible to examine the relation between the gene and the enzyme. Mutations could be created in the gene and the effects investigated on the enzyme level. TK1 is a low expressed enzyme and it is very difficult to achieve large amounts of the enzyme by purification from a human source (Ellims et al, 1982; Gan et al, 1983; Munch-Petersen et al,1991; Sherley & Kelly, 1988a). Therefore an expression system would make it possible to achieve sufficient amounts of enzyme to perform protein structure studies e.g. crystalizing the enzyme for x-ray studies.

The work with the expression system is presented in the second part of the thesis. An expression system in *E.coli* for

direct expression of unmodified human TK1 is developed and the recombinant TK1 is compared with the native TK1 purified from human lymphocytes. These results are presented as proceedings in paper III and in paper IV as a manuscript.

The recombinant TK1 revealed some interesting characteristica. The idea was to express the recombinant TK1, purify it and examine if the enzyme was identical to the native TK1 isolated from human lymphocytes. The recombinant TK1 was very similar to the native enzyme, with respect to specific activity, substrate specificity and K_m values for the two substrates, thymidine and ATP. But when investigating for the ATP dependend shift of TK1 resulting in two apparent forms with different molecular weights recently found by Munch-Petersen et al (1993), it was showed that recombinant TK1 only appear in one form, as the high affinity tetramer. The low affinity dimer form of the native TK1, observed in the absence of ATP, was not seen with recombinant TK1. Further the recombinant TK1 were 10 fold more sensitive towards TTP than the native enzyme. This lead us to suggest, that the native enzyme most likely is post-translational modificated in some way by procedures not present in *E.coli*.

In this thesis the general background for the work is outlined followed by the aims of the present investigation. The methods are presented as flowdiagrams with explaining text. The results are presented and discussed followed by the conclusions of the work. The four papers are enclosed after the references.

GENERAL BACKGROUND.

Thymidine kinase - a salvage pathway enzyme in the DNA precursor metabolism:

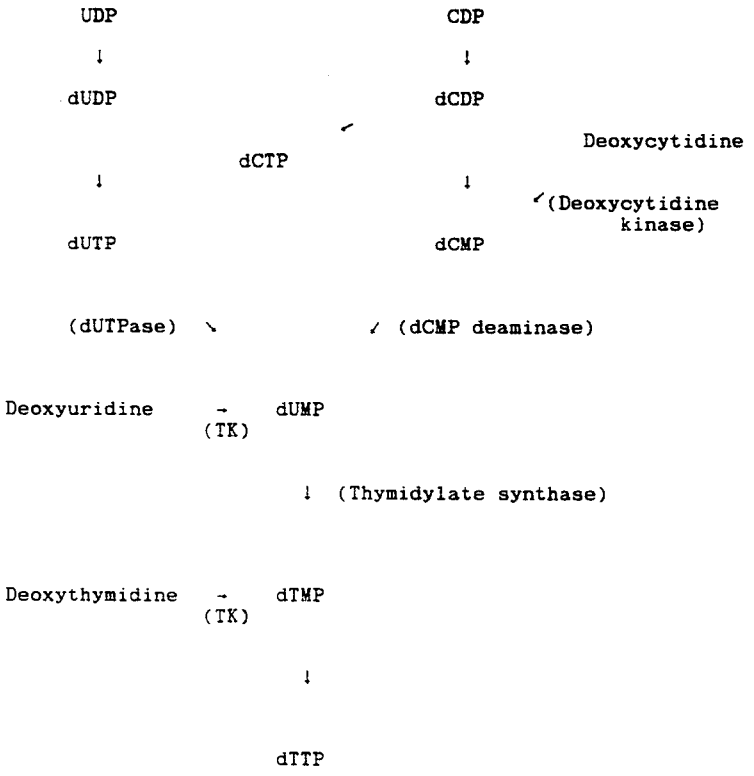
Thymidine kinase (ATP: thymidine 5'phosphotransferase E.C. 2.7.1.21) is a pyrimidine nucleoside salvage pathway enzyme represented by two isoenzymes; TK1 and TK2, also called the cytosolic and the mithochondrial TK, respectively. Both enzymes catalyze the first phosphorylation step of thymidine in the salvage pathway of TTP synthesis. TK1 is the dominating form in dividing cells and TK2 is the only form present in non-dividing cells, but in low amounts (Munch-Petersen & Tyrsted, 1977; Eriksson et al, 1991b). The two isoenzymes have characteristic differences in their enzyme kinetic pattern and subunit molecular weights (Munch-Petersen, 1984; Munch-Petersen et al, 1991). TK1 is cell-cycle regulated and the enzyme level is low or undetectable in quiescent (G_0) cells, but increases dramatically when the cells enter the S-phase (Coppock & Pardee, 1987; Stuart et al, 1985; Bello, 1974; Sherley & Kelly, 1988b).

Imbalances of the DNA precursor pool:

The regulation of the pathways resulting in the DNA precursor pool also called the deoxynucleotide triphosphate (dNTP) pool are performed by the catalyzing enzymes. Different

routes lead to the same final product. For instance dTTP is formed from two pathways, a *de novo* and a *salvage* pathway (figure 1). In the *de novo* pathway dTTP is synthesized from inorganic compounds. In the *salvage* pathway components such as thymidine and deoxyuridine are re-used from the breakdown of DNA in dying cells or from damaged DNA excised in the DNA repair process. The regulating enzyme for the *salvage* pathway of dTTP is TK and even though dTTP can be synthesized by the *de novo* pathway, TK seems to be very important for the cell as nearly all living organisms have a TK.

FIGURE 1: Flowdiagram of the *de novo* and the *salvage* pathway of dTTP metabolism.



The pools of the four dNTP's fluctuates with the DNA synthesis and the cell cycle. dTTP is the most cell cycle regulated nucleoside. dTTP is the key regulator of the enzyme catalyzing reduction of ribonucleotides to deoxyribonucleotides and therefore it is likely that the regulation of dTTP is essential for the balance between the four dNTP's (Reichard, 1987).

Production of dTMP is the critical step in the regulation of dTTP. dTMP is produced either by thymidylate synthetase methylation of dUMP or by TK phosphorylation of thymidine. dUMP can be produced by TK phosphorylation of deoxyuridine or by dCMP deaminase from dCMP. This gives TK a central role in the regulation of dNTP's.

Dysfunction of the regulation of the dNTP pool is known to give severe damage of the cell by increased mutation rate. Severe immune diseases as a result of deficiency or malfunction of adenosine deaminase is well known (Carson & Seegmiller,1976; Carson et al,1977; Giblett et al,1972). ATP and dATP are accumulated as a consequence of disturbances in the breakdown of adenosine. dATP downregulates the activity of ribonucleoside reductase, responsible for reduction of ribonucleotides to deoxyribonucleotides in the de novo pathway. Therefore accumulation of ATP and dATP will deplete the cells for the four dNTP's.

Organisms deficient in TK have problems of regulating the dNTP pool with the following increased mutation rate. These organisms also have increased sensitivity towards UV radiation (McKenna & Hickey, 1981). UV radiation is known to induce

dimers of thymine in the DNA and the dimers are cut out and repaired. When the dNTP pools are disregulated in TK deficient cells, it is likely that the repair functions repair with the increased mutation rate, in the same way as DNA polymerase seen with adenosine deaminase deficiency.

Isoforms of TK1 in cancer cell:

It is generally accepted that there is a close correlation between the TK1 activity and the proliferative state of the cell (Littlefield,1966; Bello,1974; Kit,1976; Munch-Petersen & Tyrsted,1977; Johnson et al,1982). Cancer cells are characterized with a high proliferation and therefore they have a high level of TK1 activity. On behalf of this, increased levels of TK in the cells are used as indication of tumoractivity of some cancers e.g. breastcancer (O'Neill et al, 1992; Robertson et al, 1990; Schwartz, 1992), non-Hodgkin's lymphoma (Hallek, 1992) and acute lymphatic leukemia (Russo et al, 1987).

In normal human cells two isoenzymes of TK is present, TK1 and TK2. They differ in molecular weight, substrate specificity and specific activity (Lee & Cheng, 1976b; Munch-Petersen, 1984; Munch-Petersen et al, 1991). Different isoenzymes are found in leukemic cells (Munch-Petersen & Tyrsted, 1988). At least three other isoenzymes were identified. TK1-onc (oncogenic form of TK1) has thymidine substrate kinetics and TTP inhibition kinitics similar to TK1

from profilerating lymphocytes whereas a lower molecular weight and altered ATP kinetics were found. This isoenzyme was found in samples from three patients with acute myelocytic leukemia and from a patient with chronic lymphatic leukemia. The isoenzymes TK3 and TK4 were very different from TK1 and TK2 and were found in a patient with acute monocytic leukemia and in a patient with acute lymphatic leukemia.

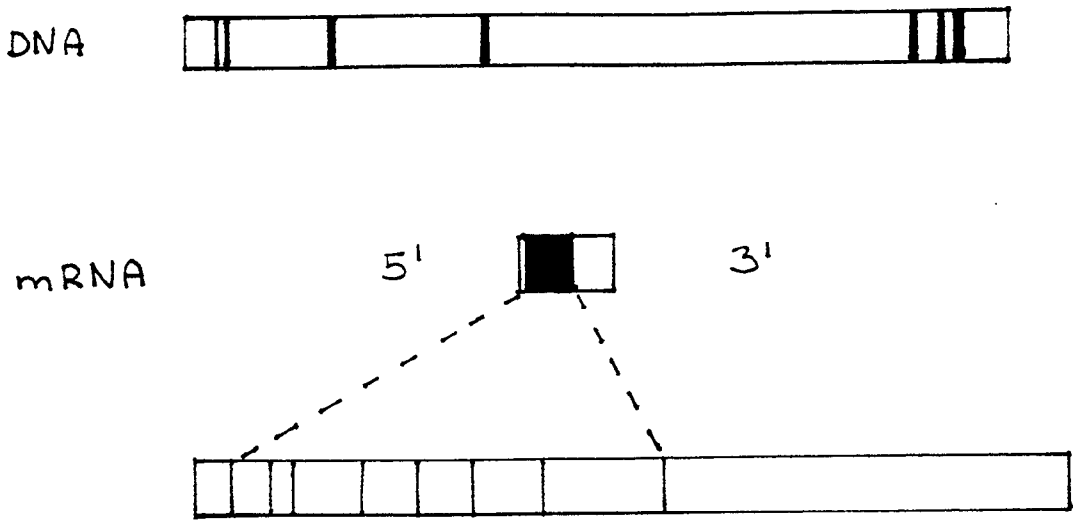
If the appearence of altered TK's in cancer cells is a consequence of a halt on a developmental stage of the cancer cell or the altered TK induces the cell to a cancer phenotype is not yet clarified.

The fact that many studies of TK has been performed on cell lines which in at least one way are abnormal cells, can give rise to speculations about whether the findings are a picture of a normal TK or an isoform, related to the phenotype of the cell line.

Regulation of TK1 expression and enzymatic activity during the cell cycle:

Human TK1 is coded by a 13 kb gene on chromosome 17. It consist of 7 exons and large areas of introns (Flemington et al, 1987). After transcription and splicing, the mRNA is 1421 basepair (bp) with an open reading frame of 702 bp encoding a 234 aminoacid protein (Bradshaw & Deininger, 1984)(figure 2).

FIGURE 2: In top of the figure the DNA sequence of TK1 is illustated. The black areas are the seven exons and the white areas are introns. Below is the TK1 mRNA illustrated. The seven exons of the gene are spliced together with a small untranslated part in the 5'end and a large untranslated part in the 3'end.



The regulation of TK1 is found on many different levels of expression which indicate that it is very important for the cell to have a highly controlled TK activity. The precise role of the stringent regulation of expression of human TK1 is not yet elucidated but the fact is, that nearly all living organisms have a cell cycle regulated TK.

In the following I will present the different levels of regulation found by investigating human cells or human minigenes of TK expressed in TK deficient cells. In the investigations performed with human cells different kind of

cells are used and the results are not in every case similar. This is probably caused by the origin of the different cells. Cell lines are widely used and it is known that cell lines, being immortal, are deficient in some of the regulatory mechanisms possessed by normal cells, which are unable to grow in culture as immortal cell lines.

Promotor control

The transcription of genes is regulated by promotor sequences. The promotor for TK1 has been identified to be the sequence region spanning -456 to +32 bp. There is a TATA-like sequence, two inverted CCAAT sequences and GC-rich sequences (Flemington et al,1987). Kim & Lee (1991) have demonstrated that the upstream sequence of the TK1 promotor, spanning between -64 and -133 bp, designed as CCRU (cell cycle regulatory unit), is responsible for the G_1/S control of the transcription initiation. This region contains one distal inverted CCAAT sequence and a GC element. Chang & Cheng (1993) found by binding experiments of nuclear proteins to this sequence that there is a strong binding activity to the CCAAT box. This binding activity is constitutively present in a variety of tumor cell lines but not in quiescent fibroblasts. The fibroblasts developed binding activity after serum stimulation whereas the binding activity in tumor cell lines were independent of serum stimulation. They conclude that the constitutive interaction of a CCAAT binding factor with the promotor is consistent with the loss of stringent cell growth regulation associated with a tumorigenic phenotype. Pang & Chen (1993) found similary results with fibroblasts; the quiescent

fibroblasts had no binding activity, but 24 hours after serum stimulation a strong binding activity was seen. Chang & Chen (1988) have shown that old fibroblast cells have a 8 fold lower expression of TK1 mRNA 24 hours after serum stimulation as compared to young fibroblast cells. Pang & Chen (1993) found that old fibroblast cells had a 30 fold lower binding activity after serum stimulation as compared with young fibroblast cells. Flemmington et al (1987) were right when they providently wrote: "These repeats with their inverted CCAAT sequences are very likely to be an important element of the human TK promotor, since they are present within a broader homology region upstream from a number of cell cycle regulated genes, it is also possible that they play a role in that regulation".

Introns

Some reports concentrates on the role of introns in the regulation of TK1 expression. Lipson et al (1989) have studied expression of human minigenes under control of different promotors. They found that the two first introns increased the amount of TK1 mRNA but had no effect on the S-phase regulation of TK1 mRNA. In mouse TK gene, nuclear protein binding sites were found in intron 2 (Rotheneder et al,1991). These binding sites did not contribute to growth regulation of TK expression but were found to positively modulate the activity of the TK promotor. Also hamster TK gene expression is stimulated by intron sequences (Lewis 1986). Gross et al (1987) reports that the expression of chicken TK gene is not dependent on transcriptional regulatory elements located within the introns.

From these results it is not likely that introns do have a major role in the regulation of TK expression during the cell cycle.

Transcriptional control

Coppock and Pardee (1987) investigated TK1 mRNA during the cell cycle. They studied the mammalian cells BALB/c3T3. They found that TK1 mRNA increased about 20 fold between G_0 and S-phase, that the rate of *run on* TK1 transcription increased 2-4 fold between G_0 and S-phase and that halflife of TK mRNA was about 8-12 hours in S and M-phase and decreased as cells entered quiescence. They suggested that the appearence of TK1 mRNA in the beginning of S-phase is regulated not only by the rate of gene transcription but also by decreased rate of TK1 mRNA degradation. Similar results were found by Stewart et al (1987) where the mRNA level increased 10 - 20 fold during the S-phase. Sherley & Kelly (1988b) reported that in HeLa cells the level of TK1 mRNA only increased less than 3 fold between G_0 and S-phase in contrast to a 15 fold raise in TK protein. They proposed that post-transcriptionally mechanisms to accounted for the fluctuation in TK protein in contrast to Coppock & Pardee (1987) where the fluctuation of mRNA was explained as a result of changes in transcription rate and TK1 mRNA stability.

Translational control

It is shown that there is a regulation during the cell cycle both on translational rates and the rates of protein degradation. Ito & Conrad (1990) have investigated expression of human TK1 cDNA under control of different promotors in TK

deficient rat cells. Under control of the human TK1 promotor, the level of TK1 mRNA and enzyme was low and after serum stimulation both TK1 mRNA and enzyme increased. When they inserted a constitutive promotor, the TK1 mRNA was high through G_1-phase but the enzyme and protein levels low until about 10 hours after serum stimulation. This demonstrated a regulation mechanism on the translational level. Similar results were found by Kauffman et al (1991) by human minigenes expressed in murine TK deficient cell lines. When a growth independent promotor was substituted for the natural TK1 promotor, TK1 mRNA was expressed in quiescent cells, without any expression of TK1 polypeptide. The normal increase in TK enzyme was obserced at S-phase after serum-stimulation. Sherley & Kelly (1988b) have shown that the TK1 polypeptide was around 10 fold increased in the S-phase while the TK1 mRNA at the same time only increased about three times. This indicated that the TK1 protein was most efficiently translated in the S-phase. In the same work they also showed that the halflife of the protein in most of the cell cycle is about 40 hours but in a discrete interval of the early G_1-phase the halflife is less that one hour. Kaufmann & Kelly (1991) report that the last 40 aminoacids of TK1, encoded by exon 7 seems to be necessary for cell cycle regulation of protein levels. In experiments with TK1 minigenes expressed in TK deficient mouse cells they showed that deletion of the carboxyl-terminal 40 aminoacids complete abolishes cell cycle regulation and stabilizes the protein throughout the cell cycle. When the cells were arrested in metaphase by nocodazole (an inhibitor of microtubules), immunoblots showed that 90% of

the TK polypeptide was cleared within an hour corresponding to a halflife of about 20 min.

Post-translational control

Posttranslational modifications are reported as a regulation mechanism for TK1 activity. Chang & Huang (1993) have demonstrated that TK1 becomes phosphorylated and that fluctuation of TK activity is related to the extent of phosphorylation of seryl residues of the TK1 polypeptide. It is important to mention that this work is performed with un-differentiated HL60 cells were the TK1 expression is not stringently controlled except for a very moderate activation of TK in respons to growth stimulation. In a recent work Chang et al (1994) have investigated TK1 phosphorylation in proliferating and M-phase arrested HeLa cells. They show that TK1 is hyperphosphorylated during the M-phase and have a 10 fold lower affinity for thymidine (K_m=15.5 μM) than in poliferating cells (K_m=1.5 μM). They suggest that TK1 is phosphorylated by a mitotic kinase(s) and thereby changed to a less active TK1 to prevent unnecessary synthesis of TTP at the time of mitosis.

A regulation mechanism on the enzymatic level is found by Munch-Petersen et al (1993) with pure TK1 from human lymphocytes. A reversible ATP-dependent transition between two forms of TK1 with different enzymatic properties is reported. The pure substrate-free TK1 showed non-hyperbolic thymidine substrate kinetics with an K_m of 15 μM. Incubation with ATP at 4^0C induced a timedependent transition to an enzyme form with hyperbolic kinetics and a K_m value of 0.7 μM. The shift between

the two forms is reversible and the maximum velocity for these two forms of TK1 were identical. These two forms of TK1 did also differ in molecular weight when eluted from a superose column. The substrate-free TK1 appeared as a dimer of 50 kDa and TK1 incubated with ATP appeared as a tetramer of 100 kDa. This indicate that the shift between the two forms of TK1 with different activities is a result of conformational changes of the enzyme.

The results of Chang et al (1994) and Munch-Petersen et al (1993) show that there are two forms of TK1, one with a low K_m (1.5 μM and 0.7 μM, respectively) and one with a high K_m (15.5 μM and 15 μM, respectively). Chang et al (1994) achieved the kinetic results with crude enzyme extracts and suggested that mitotic kinases are responsible for the phosphorylation and thereby alter the enzyme affinity. Munch-Petersen et al (1993) have performed these investigations with pure enzyme and showed that the transition between the two forms occurs at 4^0C and in a pure enzyme preparation and that these two forms also have different native molecular weight. Whether these two works complement each other or it is just a coincidence of results looking similar but owned to different factors, is not known yet. However one thing can be stated, the possibility of protein kinases associated with the pure lymphocyte TK1 is very little. Such a kinase must have the samme sub-unit molecular weight and co-purified on the affinity column and the carboxy methyl sepharose along with the enzyme. Furthermore the ATP dependent transition between the two forms of TK1 is carried out at 4^0C and it is implausible that a human enzyme are active

at this temperature. It may be that TK1 can carry out autophosphorylation, but then the reversible transition also requires a phosphatase activity, and this is impossible for a small enzyme like TK1 (24 kDa).

Recombinant enzymes:

Recombinant expression of enzymes started as a way to achieve large amounts of a specific enzyme. This is now used widely in the industry e.g. to produce washpowder with enzymes such as proteinases and lipases. In scientific research the method is widely used as an approach to investigate the relation between the gene sequence and the protein. When an enzyme is recombinantly expressed it is possible to create specific mutations and study the effects on the enzyme. Further, expression of recombinant enzymes is a method to achieve sufficient amounts of low expressed enzymes to perform structural studies of the protein e.g. NMR (Nuclear Magnetic Resonance), mass spectroscopy and crystalizing the protein for x-ray studies.

Expression of recombinant enzymes can be performed in bacteria or eucharyotic cells. Expression in bacteria is simple and the growth rate is high as compared to eucharyotic cells. Bacteria can not perform post-translational modifications such as phosphorylation or glycosylation. Simple eucharyotic cells, such as yeast can phosphorylate and glycosylate recombinant protein but in many cases it is performed at different sites as

compared with the native protein. Expression of recombinant enzymes in *E.coli* is therefore a very common choice also due to the high yield of recombinant enzyme.

The delicate problem in expression of proteins is, that the expressed protein can be toxic to *E.coli*. Even though the recombinant protein is not toxic, an accumulation of recombinant protein inside *E.coli* do interfere with the metabolism of the bacteria, and the bacteria degrades the foreign proteins to survive. Therefore it is convenient to use an inducible promotor, which is that the promotor only can bind the polymerase when it is induced e.g. with an increase in temperature or addition of IPTG. The expression bacteria can then be transformed and grown without expression of recombinant protein and the expression can be induced at a specific timepoint.

Two main strategies are used in expression of recombinant enzymes. Either a direct expression of the protein or expression of a fusion protein, where the cDNA coding for the protein of interest is spliced together with cDNA coding for another protein. A proteinase digestion site is created between the two cDNA's. In this way the protein of interest can be cleaved from the fusion protein.

Expression of fusion proteins is preferable in many cases. The fusion part of the protein is normally a bacterial protein and therefore the fusion protein is more likely to be accepted in the bacteria. Futhermore the fusion part can be used in the purification of the protein. The fusion protein can be fixed on an affinity column and by washing the column

with a relevant proteinase, the protein of interest will be cleaved from the fusion protein and elute in pure form. A number of fusion protein expression vectors are now commercially available and can be purchased along with a purification kit consisting of the relevant affinity column matrix and the proteinase.

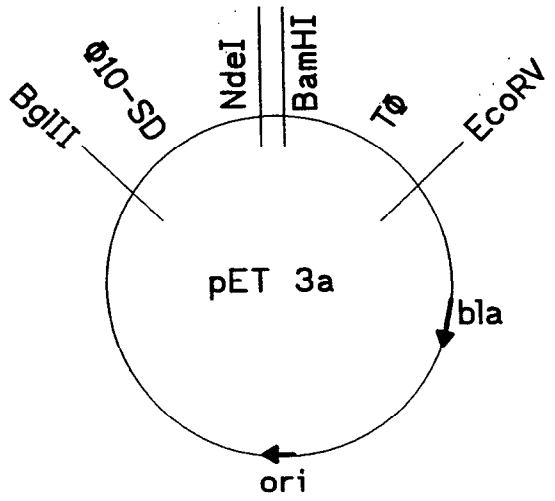
Direct expression have the advantage that the protein of interest can be expressed from cDNA with exactly the same base sequence as the cDNA for the native protein. With fusion proteins, it is in many cases necessary to modify the cDNA at the junctions between the cDNA for the two proteins to create a proper proteinase site. This results in a slightly modified protein. Another advantage of direct expression is that the protein can be purified directly by the same purification technics applied for the native protein.

The expression of TK1 in the present investigation is a direct expression. The aim was to produce sufficient amounts of protein for structural studies, as the structure is not known for TK1 even though a computer model is suggested (Folkers et al,1991). Further, it was the purpose to study the gene sequence in relation to the protein and the enzyme activity. The strategy was to express a enzyme identical to the native enzyme. At the time of planning this, there were no reports on known phosphorylation or glycosylation in TK1. The first report on phosphorylation of TK1 was from Chang & Huang (1993). Further the purification of the recombinant TK1 would not be a problem as we rutinely purify TK1 in our laboratory.

The expressionsystem used to express recombinant TK1.

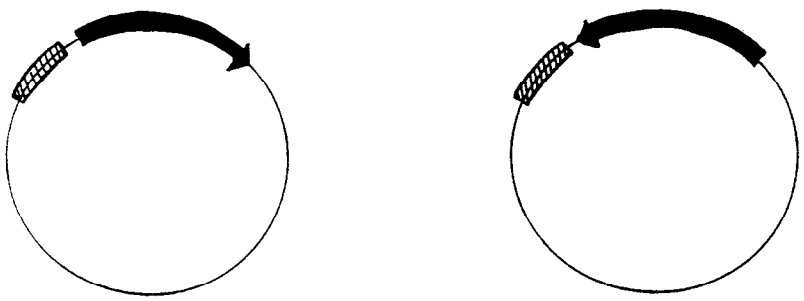
In the expression system I have chosen, there is a high direct expression of enzyme in *E.coli* (Studier et al, 1990). The vector used, pET3a, carry two cloning sites, BamH1 and Nde1. When the aminoacid coding region of TK1 is inserted at these two restriction sites, the expressed protein will be translated identical to the native protein. pET3a is constructed from the high copy plasmid, pBR322 and carry therefore an ampicillin resistens gene. The tetracyclin resistens gene of pBR322 is destroyed at the construction of pET3a. The vector carries a promotor for T7 RNA polymerase and a Shine Dalgarno (SD) sequence upstream the insert and a transcriptional termination sequence downstream the insert. The pET3a vector is shown in figure 3.

FIGURE 3: The figure shows the pET3a vector. The area between the BglII and EcoRV restriction sites are the area altered as compared to the original plasmid, pBR322. Φ10 is the T7 RNA polymerase promotor and SD is the translational signal. NdeI and BamHI are the cloning sites and TΦ is the region for transcriptional termination.



The expression bacteria is a K12 strain of *E.coli*, BL21(DE3). In this bacteria the gene for T7 RNA polymerase, under control of the IPTG inducible promotor, *lac*UV5, is inserted in the chromosomal DNA. BL21(DE3) can be more tolerable against toxic gene products by transformation with lysE or lysS plasmids. These plasmids carry the gene for T7 lysozyme, which inhibit the T7 RNA polymerase and ease the lysis procedure after harvesting the cells. The difference between lysE and lysS is shown in figure 4. In lysE, the T7 lysozyme is expressed from the promotor and there is accumulated substantial levels of lysozyme causing high suppression of the T7 RNA polymerase. In the lysS plasmid the gene is orientated in the opposite direction from the promotor resulting in lower levels of lysozyme and only a limited suppression of the T7 RNA polymerase. I found that BL21(DE3)lysS was the most suitable strain for expression of the TK1-pET3a vector.

FIGURE 4: The difference between the lysE and lysS plasmids. The lysE is the left and the lysS is the right.



AIMS OF THE PRESENT INVESTIGATION

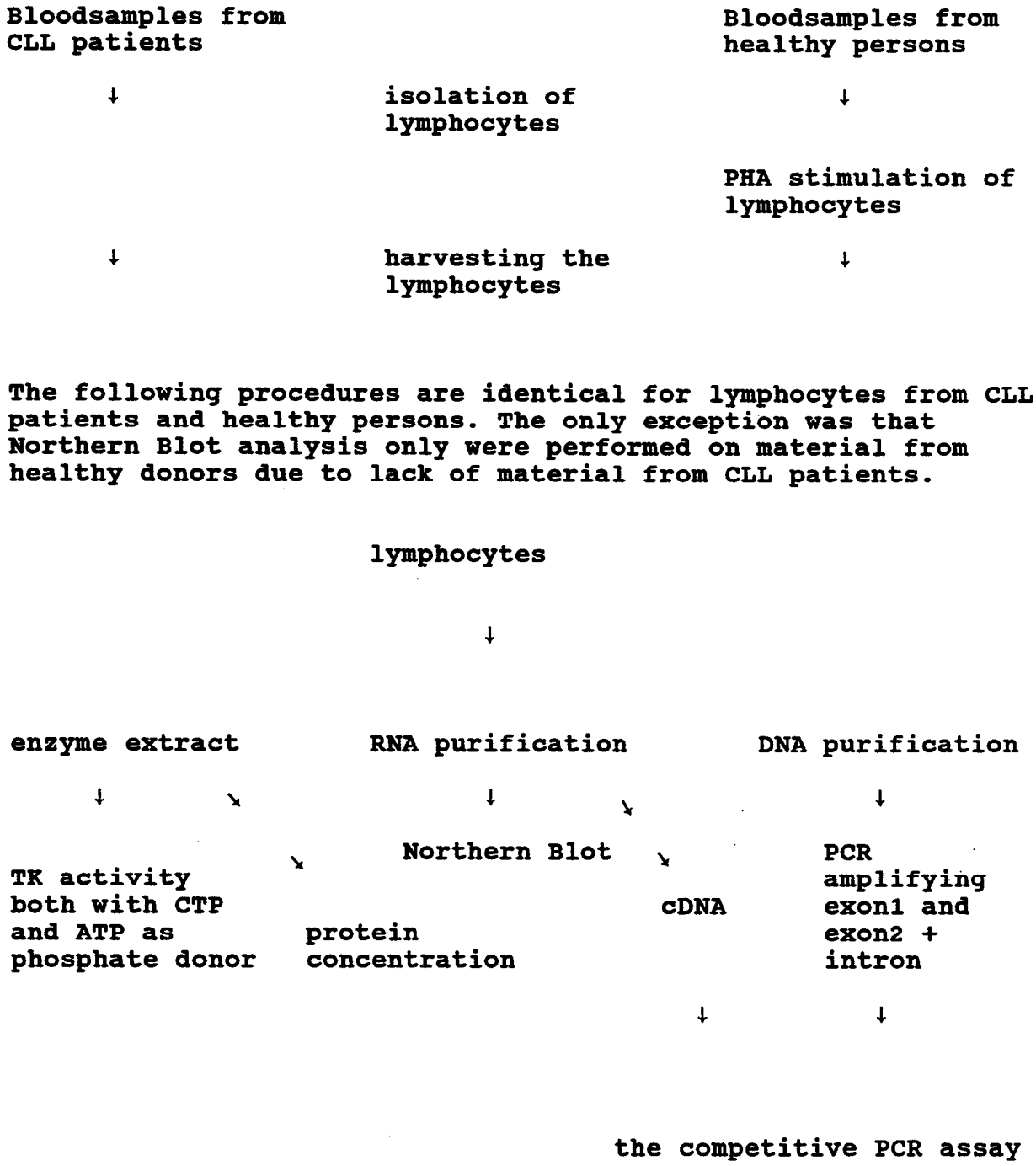
In the development of new nucleoside analogs in treatment of cancer and virus infections, it is very important to have a detailed knowledge about the nucleoside analog phosphorylating enzymes. It is important to know in which cells the phosphorylating enzyme is active, the substrate specificity and affinity of the enzyme and in what degree the enzyme fluctuate with the cell cycle.

Therefore I wanted to develope a sensitive test for TK1 mRNA (competitive PCR) and thereby clarify the relation between expression of TK1 mRNA and TK1 activity in normal mitogen stimulated lymphocytes and in white blood cells from patients with chronic lymphatic leukemia (CLL).

The structure of the enzyme is important in the prediction of new nucleoside analogs. To clarify the protein structure large amounts of protein is required and this is impossible to achieve from natural sources, as TK1 is a low expressed enzyme. An expression system would solve this problem and further the relation between the gene and the enzyme could be investigated by mutagenisis.

Therefore I wanted to construct an expression system for direct expression of unmodified human TK1 to produce high amounts of TK1 for structure studies. To ensure that the properties of the rich source of TK1 was identical to the native TK1, the recombinant TK1 was to be characterized and compared with the native TK1. Another goal was to evaluate eventual differences with concern to the possible regulation mechanisms in human cells as compared to *E.coli*.

Flowdiagram of the methods used in paper I and II



Flowdiagram of the methods used in paper III and IV

PCR amplifying the aminoacid coding region of human TK1 from pTK11

↓

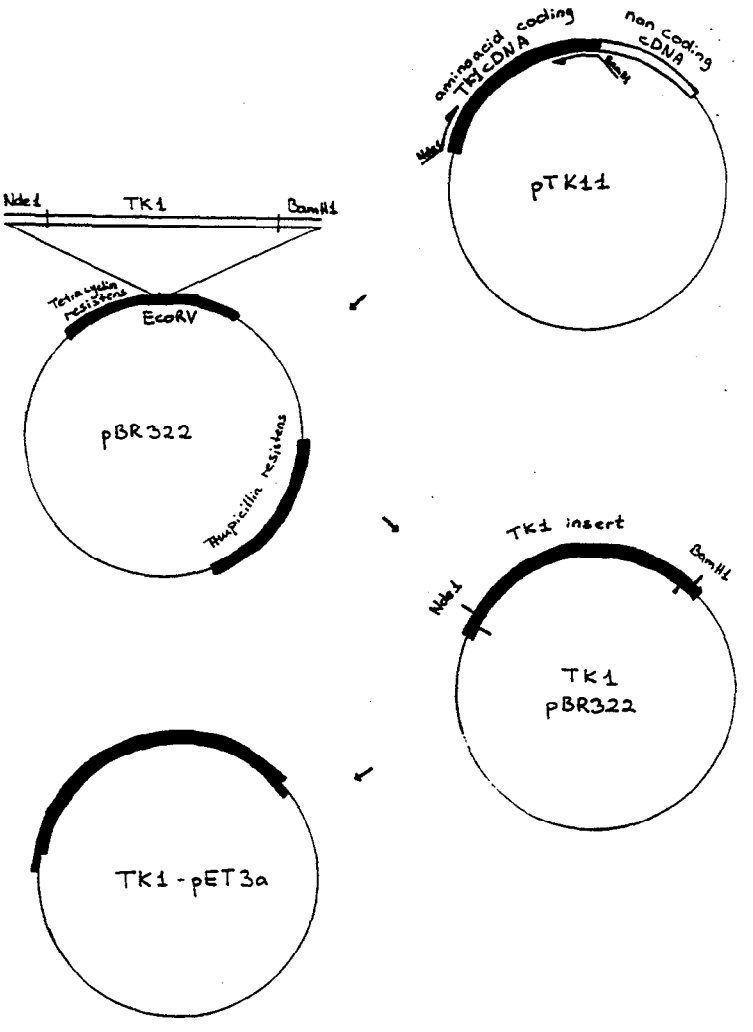
subcloning the PCR product into pBR322

↓

cutting the insert out and ligate into pET3a

↓

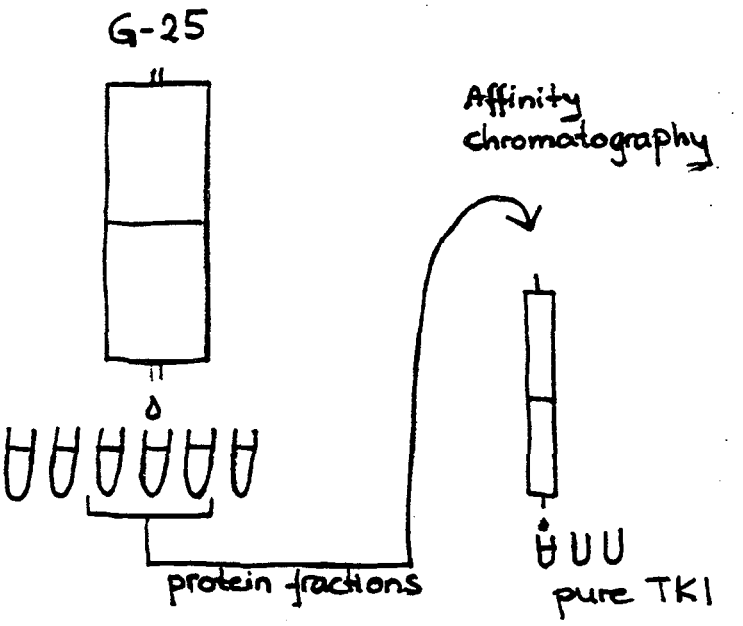
transforming three different bacteria



from a large volume of
induced culture, the bacteria
are harvested and lysed

↓

gelfiltration on G-25 and
chromatography on
3'-TMP-sepharose



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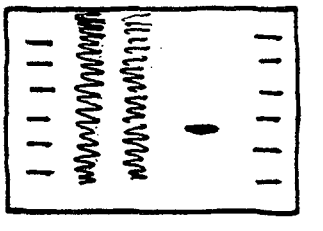
analysis of recombinant TK1
by SDS-PAGE

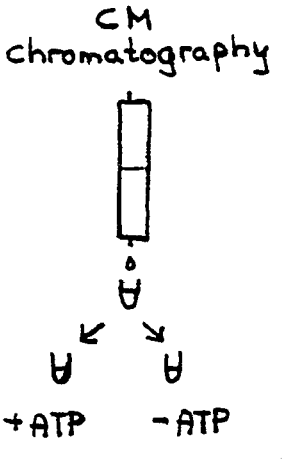


↓

removal of thymidine and
bacterial TK and
concentration of TK1
on CM-sepharose

↓

division of the enzyme in
two aliquots:
one with ATP (+ATP form) and
one without ATP (-ATP form)



↓

elution on G-200 sephadex to
determine the molecular mass

and

examination of the kinetic properties
with the substrates thymidine
and ATP, the inbitor TTP and
two substrate analogs

METHODS.

The methods used in this thesis are described in paper II and IV. The detailed procedure will not be repeated here, but the principle in the procedures will briefly be described according to the flowdiagrams.

Flowdiagram (p 28) of the methods used in paper (I and) II: Overexpression of thymidine kinase mRNA without corresponding enzymatic activity in patients with chronic lymphatic leukemia.

Cells

Bloodsamples from healthy persons were collected in Heparin vacuum tubes from six volenteers at the institute. Bloodsamples from five untreated CLL patients were collected in the same way at Roskilde Hospital. This were in agreement with the physician in charge and the patients.

Lymphocyte isolation

Within few hours after the sampling, the lymphocytes were isolated by a Ficoll-Isopaque gradient centrifigation technic (Munch-Petersen et al, 1973). The cells were counted and all the CLL cells and 20% of the normal lymphocytes were stored as pellets at -70°C.

PHA stimulation

Immidiately after the lymphocyte isolation, the remaining 80% of the normal lymphocytes were suspended in RPMI medium + 10% fetal calf serum and stimulated to growth by PHA at 37°C. The cell concentration were 10^6 cells/ml. Four bottles were

prepared for each donor and the cells were harvested 2,3,4 and 7 days after PHA stimulation. By flowcytometry it was shown, that after 24 hours about 30% of the cells were in S-phase. This is similar to previous observations (Kofoed et al, 1986). The harvested cells were counted and stored at -70°C in the same way as the unstimulated lymphocytes.

Enzyme extracts

Enzyme extracts were prepared by suspending the cell pellet in lysisbuffer and disrupting the cells by sonication. The lysate was centrifuged to remove cell debris and the supernatant was used for TK activity measurement and total protein determination. The protein content was determined by Coomassie brilliant blue according to Bradford (1976).

TK activity assay

The TK activity were determined as initial velocities of the enzyme reaction, phosphorylating thymidine to TMP at 37°C. ^{3}H-thymidine was used as substrate in the TK activity assay. Samples were applied to DEAE filters 5, 10 and 15 min after starting the reaction by addition of enzyme. The filters were washed by ammoniumformiate to remove unphosphorylated ^{3}H-thymidine and the radioactivity determined by scintillation counting.

Both ATP and CTP were used as phosphate donor in the TK activity assay. We wanted to distinguish whether the TK activity was due to TK1 or TK2 using the pronounced differences in substrate specificity. Both isoenzymes utilize ATP efficiently as phosphate donor, but TK2 can also use CTP efficiently, whereas CTP is a poor substrate for TK1 (Lee &

Cheng, 1976b; Ellims et al, 1981). The maximun activity was obtained by ATP as co-substrate. The activity obtained, when CTP was used as co-substrate, were calculated as % of the maximum activity.

RNA purification

The RNA were prepared according to the guanidine thiocyanate method (Chomczynski & Sacchi, 1987). By this method total RNA is isolated and the RNA is highly protected against RNases due to guanidine thiocyanate. Sharp ribosomal RNA bands were achieved, when the RNA were analysed by agarose gel electrophoresis indicating a high quality of undegraded RNA.

Northern blot

The RNA preparations were denatured and electrophoresed through a formaldehyde containing agarose gel. The gel were transferred to a Hybond N^+ membrane as described (Sambrook et al, 1989). The probe were human TK1 cDNA from plasmid pTK11 (Bradshaw & Deininger, 1984) labelled by ^{32}P-CTP. The hybridization were performed with high stringency washing and the results were detected by x-ray film exposure.

cDNA preparation

RNA was transcribed to cDNA by a reverse transcriptase (RT) assay with random oliginucleotides (hexamers), dNTP's and reverse transcriptase. The reaction was terminated after 2 hours at 37°C. In each experiment a parallel assay was performed with ^{3}H-TTP instead of TTP to control the efficiency of the reverse transcription reaction. The incorporated ^{3}H-TTP were messured by applying a sample on a 3MM Whatmann filter, the free ^{3}H-TTP was removed by washing the filter, and

remaining radioactivity was determined by scintilationcounting.

Competitive PCR

The principle in the competitive PCR method is a co-amplification of target cDNA concurrently with the corresponding genomic DNA (Gillian et al, 1990). Thus, the two templates compete for the same substrates and primers ensuring equal efficiency of amplification. The genomic DNA serves as internal standard. In our experiments we have chosen exon 1 and 2 with intron 1 from the TK1 gene as internal standard and exon 1 and 2 as the target cDNA fragment. The fragments were amplified using a pair of primers identical to those reported by Lipson and Baserga (1989b). The sizes of the resulting fragments are 138 bp with cDNA as template and 248 bp with genomic DNA as template. The internal standard of 248 bp genomic DNA is prepared by PCR with DNA as template and using the primers mentioned above. The product is quantified by agarose gel electroforesis together with different known amounts of DNA. The unknown amount of cDNA, is estimated from a set of PCR-reactions performed on a dilution series with known amounts of the genomic DNA. The PCR products are separated by agarose gel electroforesis. The amount of cDNA (in grams) in the sample is estimated as that amount (in grams) of genomic DNA giving equal intensity of the two amplification products. The number of TK1 cDNA copies is calculated from the amount of cDNA, by division with the molecular weight of the 138 bp cDNA fragment (average molecular weight/base = 308). The number of copies of TK1 cDNA is taken as being representative for the number of copies of TK1 mRNA.

In our experiments with competitive PCR, contamination have been the main problem. The level of TK1 mRNA in quiescent cells are very low and in these cDNA samples contaminating genomic DNA interfered with the competitive PCR. We used genomic DNA as internal standard and therefore it is critical if any genomic DNA is present in the RNA samples. Even though we did perform DNase treatment of our RNA samples our detection limit at 0.006 copy/cell was most likely determined by residual DNA contamination. Another explanation of this problem could be unspliced RNA in the RNA preperation. Gudas et al (1990) have suggested an order for the splicing of TK1 mRNA, where the intron between exon 1 and exon 2 are the last intron to be spliced away in the mRNA splicing.

These problems with contamination descibed above can be described as internal contamination problems. Another general problem when performing PCR is external contamination. This is contamination with DNA from the laboratory. The primary source is amplified DNA from previous PCR reactions. Precausions can be taken such as handling amplified PCR with seperate pipettes and in a seperate room, but the risk of contamination is increased when the same primers have been used for a period in the laboratory.

Despite these problems, we were able to detect levels of TK1 mRNA that could not be detected by the Nothern Blot technic.

Flowdiagram (p 29-30) of the methods used in paper (III and) IV: Human cytosolic thymidine kinase expressed in *E.coli* occurs solely as the high affinity tetramer form with the low K_m value.

TK1-pET3a vector

The plasmid pTK11 containing a complete cDNA sequence of human TK1 was used to create the insert coding for the human TK1. pTK11 was template in a PCR amplification with two primers flanking the aminoacid coding region. These two primers had restriction sites in the 5'ends, BamH1 and Nde1 and they were corresponding to the cloning sites in the expression vector, pET3a.

It was not possible to cut the amplification product by the two restriction enzymes, therefore the amplification product was sub-cloned into pBR322. The PCR product was blunt-ended by Klenow polymerase and ligated into the blunt-end restriction site, EcoRV of pBR322. From this construction the insert was cut with BamH1 and Nde1 and inserted into the pET3a vector. This TK1-pET3a vector was sequenced (Sanger et al, 1977) to ensure no mutation had occurred during the initial PCR.

TK expression

Three different *E.coli* expression strains was transformed by the TK1 vector. A TK deficient strain, KY895, could not be transformed by the vector, indicating that the direct expression of TK1 were toxic to the cell. Therefore the next attempt was to transform a strain adjusted to expression of

toxic genes (BL21(DE3)lysE), as the transcription of the vector was suppressed. After induction, this resulted in a yield of 50 µg/liter culture. The cells were harvested 24 hours after IPTG induction at 25°C. The third attempt was to transform a strain, where transcription of the vector only were slightly suppressed (BL21(DE3)lysS). The yield of this system were about 1 mg/liter culture. This system was used for the expression of recombinant TK1.

Purification of recombinant TK1

A large scale volume of the culture were induced to expression and the cells were harvested. The bacteria were lysed by sonication in lysisbuffer and the enzyme extract was cleared by centrifugation. Using sonication to lyse the cells have the advantage compared to e.g. the freeze/thaw method, that the DNA is disrupted as well and the lysed cells have a much lower viscosity.

The enzyme extract was gelfiltrated on G-25 sepharose to remove small molecules and chromatographed on a 3'-TMP-affinity column (Munch-Petersen et al, 1991). The thymidine eluted protein from the affinity column was purified to more that 95% homogeneity shown by SDS-PAGE. To remove thymidine and bacterial TK, the fractions were chromatographed on CM sepharose. In contrast to the recombinant TK1, the bacterial TK do not bind the negative CM groups (Okazaki & Kornberg, 1964)

After the CM sepharose chromatography, the purified enzyme was divided in two aliquots and to one of these ATP was added to 2.5 mM. These two aliquots were referred to as +ATP enzyme and -ATP enzyme, respectively. The reason for preparing

a +ATP and -ATP enzyme was related to previous results showing, that the lymphocyte TK1 have different enzymatic properties depending on the presence or absence of ATP (Munch-Petersen et al, 1993). All the experiments to characterize the recombinant TK1 were performed with both the +ATP and -ATP enzyme.

Determination of the molecular mass

The molecular mass of the active enzyme and thereby its ability to oligomerize were determined by elution on a G-200 sephadex column. The elution volume was determined for protein standards with a molecular mass of 12, 29, 66, 150 and 200 kDa, respectively. A straight line was obtained by plotting the logarithm to the molecular mass as a function of elution volume. Both the +ATP and -ATP recombinant TK1 were determined and compared to the lymphocyte +ATP and -ATP enzyme. Before the +ATP enzyme were eluted from the column, the column was equilibrated with 2.5 mM ATP.

Enzyme kinetics

The kinetic properties were examined for the substrates, thymidine and ATP and for the inhibitor TTP. The substrate specifity were investigated for the nucleoside analogs, AZT and ara-T. The kinetic properties of the substrates were investigated by varying one of the substrates. In this way thymidine kinetics were determined by varying thymidine at a constant ATP concentration. The TTP inhibition was investigated by varying the TTP concentration at a fixed thymidine and ATP concentration. The substrate specifity was determined as the enzyme activity with the nucleoside analog, compared to the enzyme activity with thymidine as the substrate.

The substrate kinetic data were plotted in a Hofstee plot (v versus v/s) and the K_m values and the cooperativity determined. The TTP inhibition data were examined in a reverse plot (1/v versus TTP) and the I_{50} was determined. I_{50} is the inhibitor concentration were the enzyme is 50% inhibited.

RESULTS AND DISCUSSION.

TK1 mRNA and enzyme activity in PHA stimulated lymphocytes:

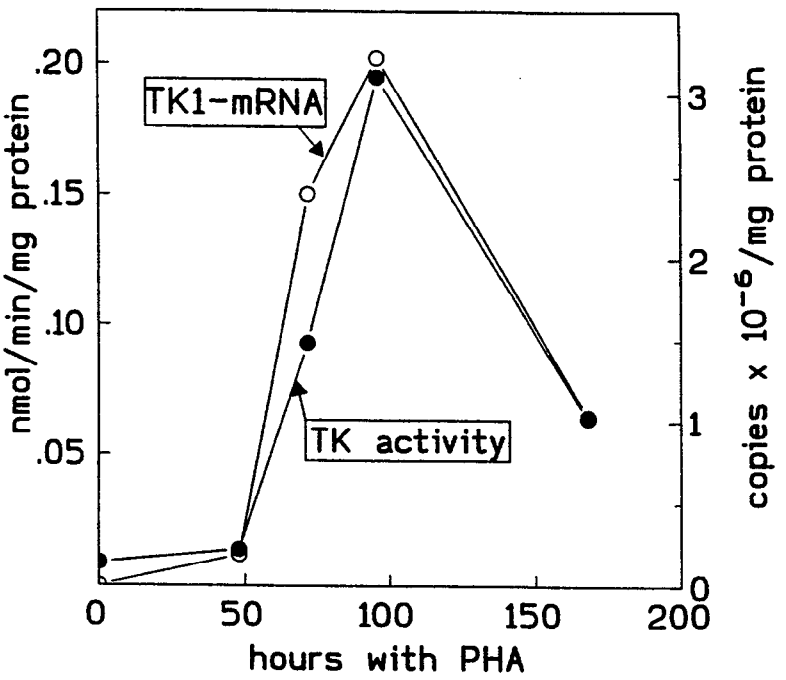
Lymphocytes stimulated to growth by the mitogen PHA is used as a model system of quiescent cells entering the cell cycle. This system is previous investigated with respect to TK activity, DNA synthesis and cell division (Munch-Petersen & Tyrsted, 1977; Tyrsted & Munch-Petersen, 1977; Loeb et al, 1970; Barlow & Ord, 1975). In this study the TK1 mRNA, the total TK activity and the proportional amounts of TK1 and TK2 is investigated.

It is generally accepted that the activity of TK1 increases with the onset of DNA synthesis. Investigation of TK1 mRNA levels in quiescent and dividing cells show that TK1 mRNA is virtually absent in G_0/G_1 cells but, increases with the onset of DNA synthesis. However, the results differ depending on which cell system is used. Sherley & Kelly (1988b) show that the increase of TK1 mRNA in HeLa cells is too limited to account for the high increase in TK1 enzyme activity and suggest that translational regulation mechanisms is involved. They measured a 3 fold increase in TK1 mRNA and a 15 fold increase of TK1 protein. Coppock & Pardee (1987) describe a 20 fold increase of TK1 mRNA corresponding to the increase of TK1 enzyme activity.

In dividing lymphocytes, the level of TK1 mRNA was increased about 100 fold when compared to quiescent lymphocytes (paper II). The total TK enzyme activity increased

concomitantly with the increase of TK1 mRNA (figure 5).

FIGURE 5: The TK1 mRNA and the TK activity followed through PHA stimulation. The filled circles represent the TK activity of the left y-axis and the open cicles represent the TK1 mRNA of the right y-axis.

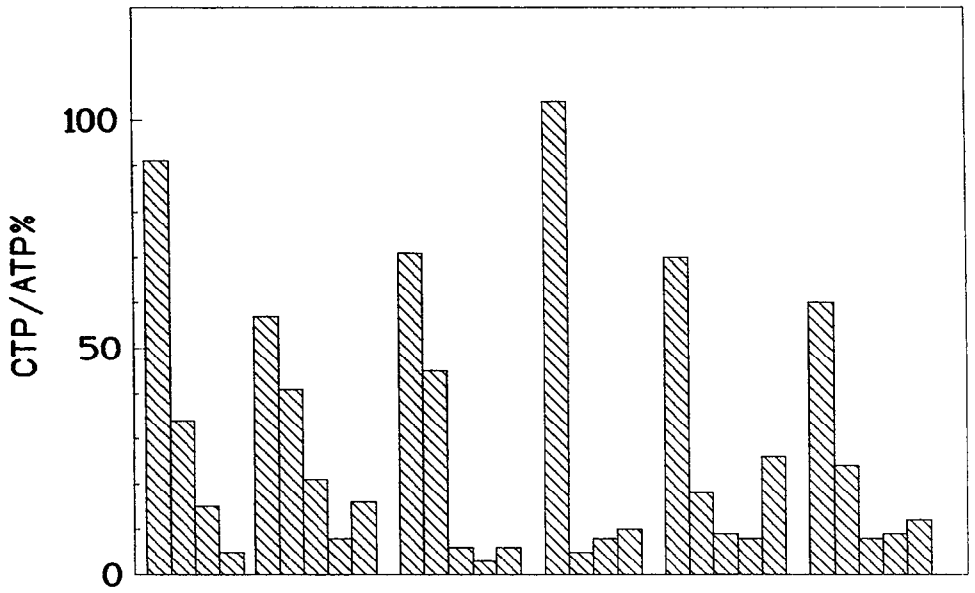


To ensure that the enzyme activity was due to TK1 mRNA we characterized the TK enzyme with respect to phosphate donor specificity. It is shown (Lee & Cheng, 1976b; Ellims et al, 1981) that TK1 uses CTP very poorly as phosphate donor compared to ATP, whereas TK2 uses CTP as phosphate donor nearly as efficient as ATP.

As shown in paper II, the TK enzyme in quiescent lymphocytes were due to TK2. When the total TK activity was followed through the PHA stimulation for seven days, the capacity to utilize CTP decreased, indicating the increasing dominance of TK1 with a peak at the fourth day (figure 6). This correlates with previous results, where TK1 is the dominating TK enzyme in dividing cells and TK2 is the dominating in

quiescent cells, though at a low level (Munch-Petersen & Tyrsted, 1977; Munch-Petersen, 1984).

FIGURE 6: The ability to use CTP instead of ATP as phosphate donor is illustrated. Samples from six donors have been meassured during PHA stimulation (0 - 7 days).



TK1 mRNA and enzyme activity in chronic lymphatic leukemia cells:

Chronic lymphatic leukemic (CLL) cells are characterized as non dividing cells. CLL is a lymphoproliferative disorder with lymphocytosis around 200 - 600 x 10^6 white blood cells/ml blood as compared to a normal level of 4 - 11 x 10^6 cells/ml blood. Ellims et al (1981) found in a study of 12 CLL patients, using the CTP/ATP ratio to discriminate between the isoenzymes, that in 8 patients the dominating enzyme was TK2. The

morphological characteristics of these cells were as small mature lymphocytes. In the other 4 Cll patients TK1 seemed to be the dominating enzyme and the morphology showed a pleomorphic subpopulation resembling prolymphocytes and lymphoblasts. They characterized this pattern as indolent and aggressive CLL cells. It is reported that the serum level of TK is in correlation with the stage of the disease (Källander et al,1984) and that there is a correlation of serum TK and the spontaneous ^{3}H-thymidine incorporation in the non proliferating CLL cells (Källander et al,1987). Further Munch-Petersen et al (1986) found that a TK1 isoenzyme was the dominating enzyme in a sample from a CLL patient.

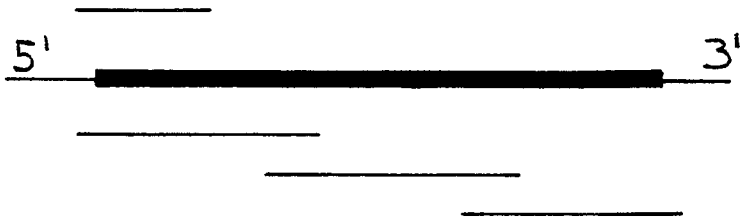
Because of the quiescent stage of CLL cells it was expected to find a low TK1 mRNA level and a low TK activity level, due to the constitutively expressed TK2. However, according to the above reports, an increased level of TK enzyme in some of the CLL samples may be found. In the samples from five CLL patients, it was shown (paper II) that the TK enzyme activity was low and at the same level as in quiescent lymphocytes. When the TK enzyme was investigated for phosphate donor substrate specificity, it was shown that the TK enzyme was TK2. Surprisingly, a 100-fold higher level of TK1 mRNA as compared to the level in quiescent lymphocytes was found. Apparently the CLL cells TK1 mRNA was not translated into active enzyme as the only TK present, was TK2 with an activity level similar to that of quiescent cells. It should be noted that our samples from CLL patients were taken just after diagnosis and before any treatment was initiated. Therefore the

samples most likely represent early stages of disease.

The high level of TK1 mRNA without corresponding TK enzyme activity could be explained if the TK1 mRNA was defect in some way, and thereby not translated or translated into a defect enzyme without activity. Therefore a group of graduate students investigated the TK1 mRNA from CLL samples (Laursen et al, 1994). There aim was to study possible differences in the TK1 mRNA as compared to human lymphocytes. However, they did not find any differences by investigating the CLL TK1 mRNA. They used a method where the TK1 mRNA is reverse transcribed to cDNA and PCR amplificated. The PCR product is denaturated and electrophoresed on a polyacrylamid gel. This method, developed by Orita et al (1989) is called Single Stranded Conformational Polymorphism (SSCP) and should reveal down to single nucleotide deletions or substitutions. Anyhow, they discovered the interesting detail that it was considerably more difficult to PCR amplify the 3'end of the TK1 mRNA as compared to the 5'end. The reason for this is not yet clarified. It could indicate a difference in the 3'end of the TK1 mRNA resulting in a translational error not detectable by SSCP. On the other hand it could indicate that the very high level of TK1 mRNA we found in CLL by the competitive PCR for some reason is truncated. As we performed the competitive PCR by primers flanking exon 1 and exon 2 in the 5'end, we only determine an increased level of this region and therefore it cannot be excluded that the TK1 mRNA is truncated. Further investigations have to be made to clarify this discrepancy. In figure 7 is shown the areas amplified to the SSCP analysis and the areas amplified in the

competitive PCR.

FIGURE 7: The TK1 cDNA is illustrated as the thick line. Above the cDNA is the area that is PCR amplified by competitive PCR. Below the cDNA the three amplification products used in SSCP are showed.



Expression of recombinant TK1:

In the efforts to create an expression vector for TK1, a strategy was followed as previously used for the expression of deoxycytidine kinase (Chottiner et al,1991). The mRNA was transcribed to cDNA and PCR amplified with primers including restriction-sites in the 5'end. In this way the resulting PCR product is flanked by two restriction-sites and can by those be cloned into the vector. I chose another vector than Chottiner et al (1991) but also a vector from the pET vector system developed by Studier et al (1990). The reason for this was, that the cloning restriction-sites for the primers have to be

restriction sites, which are not present in the mRNA sequence.

It was not possible to cut the PCR fragment into sticky ends. The reason for that, may be, that the restriction-sites were too close to the end of the PCR product. Therefore the PCR product was subcloned into pBR322 by blunt-end ligation. From this clone, it was very easy to cut out the insert by the two restriction enzymes and clone it into the expression vector (figure 8).

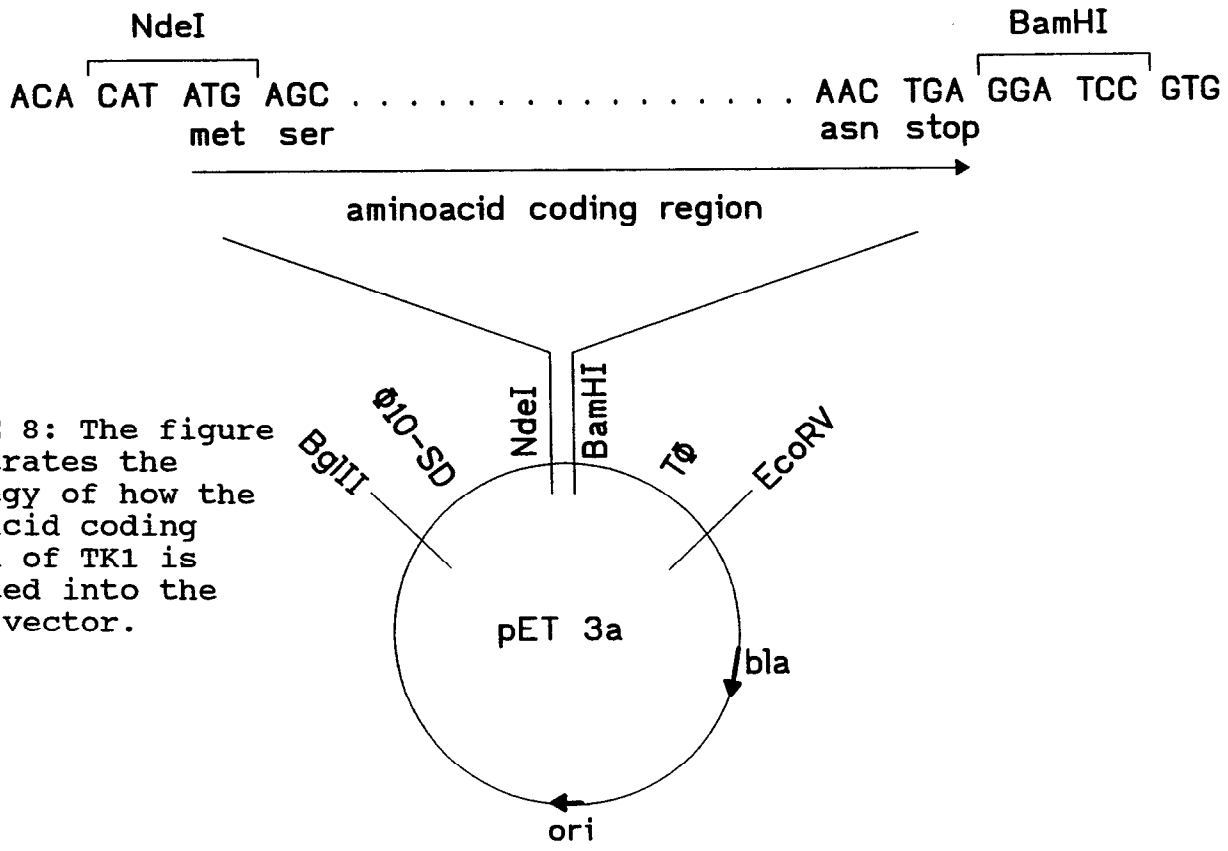
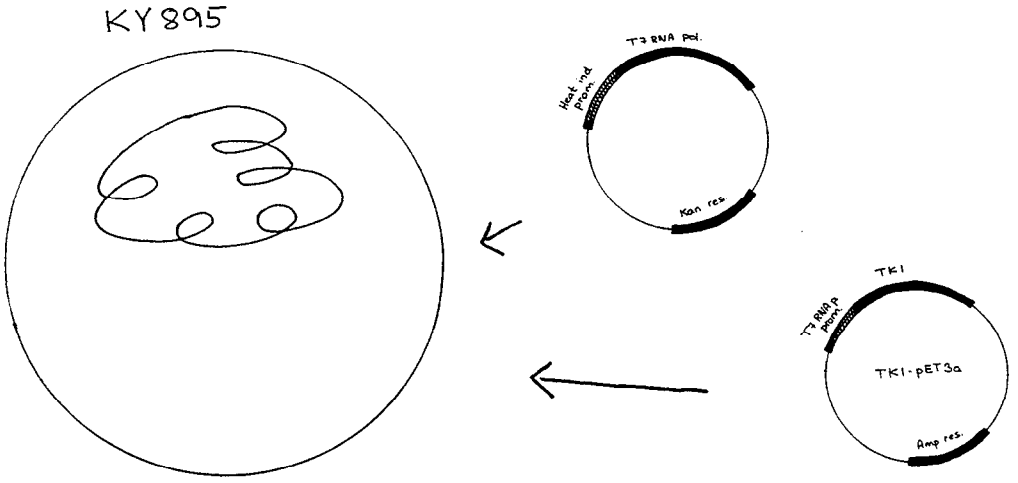


FIGURE 8: The figure illustrates the strategy of how the aminoacid coding region of TK1 is inserted into the pET3a vector.

The initial strategy for expression of the enzyme was to use a TK minus *E.coli* strain, KY895 (Igarashi et al,1967). I wanted to transform this strain with a heat inducible T7 RNA polymerase plasmid along with the expression vector under control of the promotor for T7 RNA polymerase (figure 9).

FIGURE 9: The figure shows the unsuccesful strategy to express the TK1-pET3a vector in the TK deficient *E.coli*, KY895. The heat inducible plasmid for T7 RNA polymerase is shown to the left and the TK1-pET3a vector is shown to the right.



Obvious the TK1 vector was toxic to KY895 as it was not possible to achieve any transformants. This in spite of that, KY895 was transformed with the TK1 vector without the plasmid for T7 RNA polymerase. It seemed like the background transcription alone was sufficient for the toxic effect. In other respects there were no problems with the transforming procedure. KY895 was easily transformed with the T7 RNA polymerase plasmid. This increased only slightly the doubling time of the bacteria growth as normally seen, when a bacterie carries a plasmid.

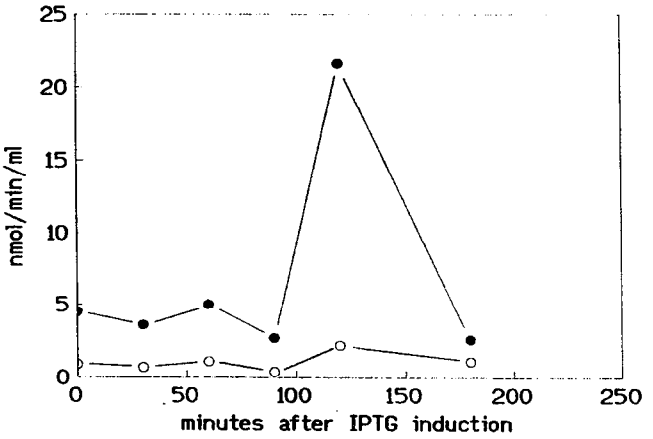
The next strategy was to transform an expression bacteria suitable for expression of toxic genes, BL21(DE3)lysE. This strain has T7 RNA polymerase inserted in the chromosomal DNA under control of the IPTG induceble UV5 promotor. The lysE plasmid transcribes a lysozyme gene from the promotor and this enzyme inhibit the T7 RNA polymerase. Besides, the lysozyme interferes with the cell membrane which make the cells easier

to lyse after harvesting. Using BL21(DE3)lysE, TK1 was expressed with a yield of 50 μg/liter culture which is a poor yield even for a directly expressed enzyme. As the aim was to achieve high amounts of TK1 enzyme to be able to do structure studies, this was not sufficient and acceptable.

The final strategy was to transform BL21(DE3)lysS which is very similar to BL21(DE3)lysE. The only difference is, that in the lysS plasmid the lysozyme gene is orientated reverse to the promotor and this results in a lower transcription of lysozyme. Therefore the T7 RNA polymerase is inhibited less than by the lysE plasmid. In this system the yield was about 1 mg/liter culture.

The conditions for expression of the enzyme were 25°C and harvesting 24 hours after IPTG induction. Initially I expressed the system at 37°C. It resulted in a peak of expressed TK1 two hours after IPTG induction (figure 10).

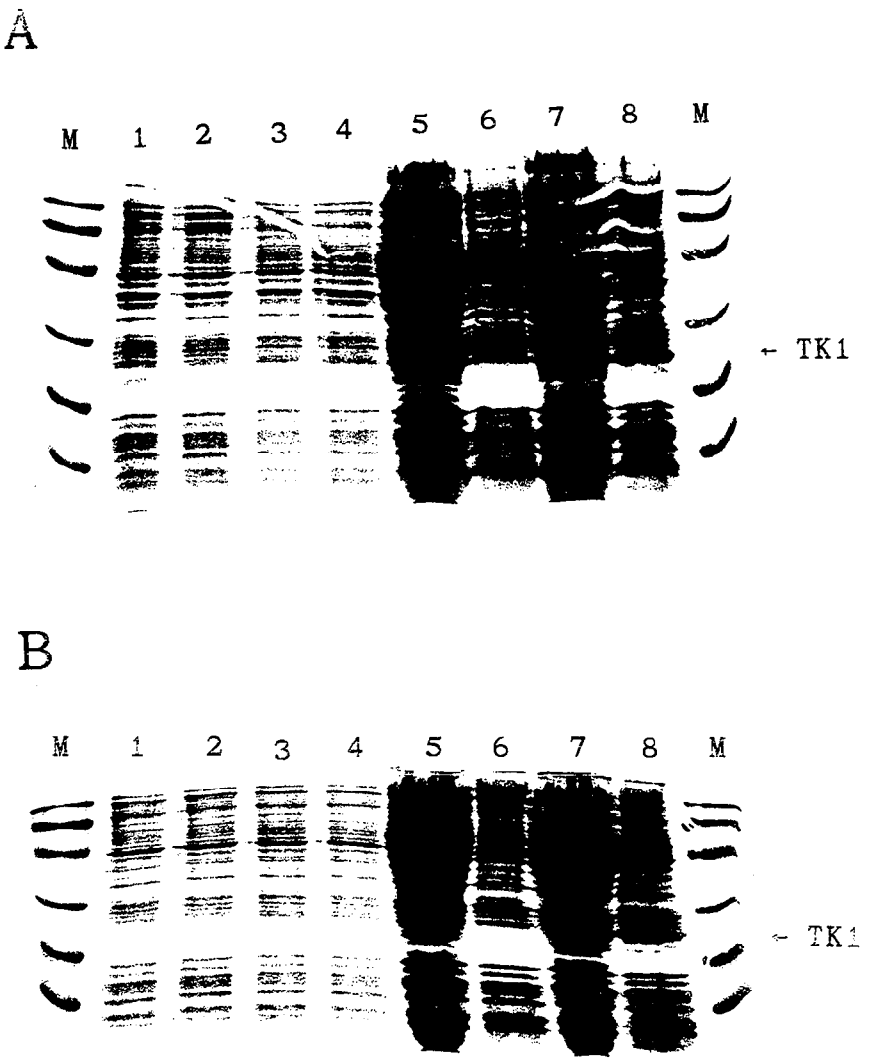
FIGURE 10: The TK activity is followed after IPTG induction. The filled circles are the TK1-pET3a vector and the open circle are the pET3a vector used as control.



For some reason the TK1 enzyme decreased rapidly after this peak point. The culture was pulse labelled with ^{35}S-methionine and samples collected over time and analysed by SDS-PAGE and x-ray film exposure. The results showed that the recombinant TK1 produced during the pulse were degraded. Most likely, this was due to proteinases of the bacteria metabolism. Anyhow, it was not convenient to work with a system were the point of harvesting was so critical. When inducing large volumes of bacteria it is very difficult to harvest the cells at a certain timepoint. Furthermore this peakpoint could easily be moved either way as a result of the airing intensity of the culture. When expression was carried out at 25°C the peak point of expression had a duration of several hours even though the same picture of decreased TK1 enzyme level after the peak was observed.

Another reason for choosing 25°C as expression condition was a report of Fetzer & Folkers (1992) describing a fusion protein expression of Herpes Simplex Virus TK in KY895. They report, that TK form inclusion bodies at 37o°C but not at 25°C. Inclusion bodies are aggregated protein that can be produced in expression systems. They could not get these inclusive bodies into solution. In fact, I did investigate possible TK1 aggregation after expression at 37°C, and found by SDS-PAGE analysis that TK1 was soluble both at 37°C and 25°C. This is shown in figure 11. If inclusion bodies were formed, the TK1 band would be seen with the bacteria lysate but not in the lysed and centrifuged samples because such inclusion bodies is pelletted during centrifugation.

FIGURE 11: SDS-PAGE of the proteins expressed by TK1-pET3a and pET3a in BL21(DE3)lysS. (A) The outer lanes are molecular markers (97, 66, 45, 31, 21, 14 kDa). Lane 1 - 4 are lysed bacteria 30 min after induction and lane 5 - 8 are lysed bacteria 18 hours after induction. Lane 1 and 5 are uninduced pET3a, lane 2 and 6 are induced pET3a, lane 3 and 7 are uninduced TK1-pET3a and lane 4 and 8 are induced TK1-pET3a. (B) The samples on this SDS-PAGE are the same as in A. The only difference is that the cells are centrifugated after lysis by sonication and the supernatants are applicated the SDS-PAGE.

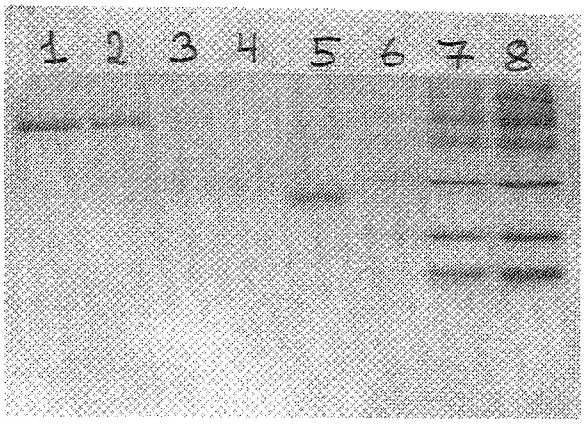


Recombinant TK1 as compared with the native TK1:

Before producing large amounts of recombinant TK1 for structural studies e.g. mass spectromotry studies and crystalizing the enzyme to x-ray studies, it was important to ensure that the recombinant TK1 was identical to the native TK1. This was done by sequencing the vector, by sub-unit molecular mass determination of pure TK1 enzyme on SDS-PAGE, examining the substrate specifities, studying the enzyme kinetics and the molecular mass of the active enzyme.

The sequence of the TK1 vector was determined to ensure that no mutation had occurred during the initial PCR and it was found that the cDNA sequence of TK1 vector was identical to the published sequence for human TK1 (Bradshaw & Deininger, 1984). The recombinant TK1 was purified by the same procedures used for the native TK1 (Munch-Petersen et al,1991), but with a few procedure modifications. Proteinase inhibitors were added to all buffers, since in preliminary experiments it was found that the enzyme were very unstable. After gelfiltration on G-25 the enzyme extract of recombinant TK1 was chromatographed directly on a 3'TMP affinity column and the enzyme was eluted in pure form by thymidine. To remove thymidine and bacterial TK the fractions were chromatographed by CM sepharose. Bacterial TK in contrast to human TK1 does not bind to the negative groups of CM (Okazaki & Kornberg,1964). The CM fractions were used in the kinetic experiments. Recombinant TK1 was purified to more than 95% homogeneity as shown by SDS-PAGE (figure 12). The sub-unit molecular weight was similar to the lymphocyte TK1.

FIGURE 12: Coomassie stained SDS-PAGE of pure recombinant TK1. Lane 1 - 3 are 1 μg, 0.5 μg and 0.1 μg BSA, respectively. Lane 4 - 5 are 1 and 10 units of TK1. Lane 6 - 8 are 0.1, 0.5 and 1 μg of standard molecular markers (97, 66, 45, 31, 21 and 14 kDa). 1 unit of TK1 represent the enzyme activity converting 1 nmol of thymidine to TMP per min.



By TK enzyme activity assay it was found, that the specific activity was similar to that of the native enzyme. Also, the substrate specificity towards AZT and ara-T were similar to that of the lymphocyte TK1, 40% and 0%, respectively as compared to thymidine as substrate (Table 1). This was identical with previous investigations with the lymphocyte TK1 (Munch-Petersen et al, 1991; Eriksson et al, 1991).

As described in GENERAL BACKGROUND (p 20-21) Munch-Petersen et al (1993) found a hithero unknown regulation mechanism of lymphocyte TK1. When stored without ATP, the enzyme was a dimer with a low affinity for thymidine (K_m = 12-15 μM) and when stored with ATP, the enzyme was a tetramer with a high affinity for thymidine (K_m = 0.5 - 0.7 μM). Therefore the purified recombinant TK1 from CM chromatography was divided in two, one with ATP and one without ATP. Surprisingly, there was no difference between the two forms of recombinant TK1 (paper IV). The recombinant TK1 appeared only in one form, whether it has been incubated with or without ATP. This was

shown by investigating the enzyme kinetics with thymidine and ATP. With both substrates the enzyme kinetics were similar to that obtained with lymphocyte TK1 incubated with ATP (table 1). The thymidine enzyme kinetics is shown in figure 13.

FIGURE 13: Hofstee plots of the thymidine enzyme kinetics with (A) lymphocyte TK1 and (B) recombinant TK1. The filled circles are the -ATP form of the enzyme and the open circles are the +ATP form of the enzyme.

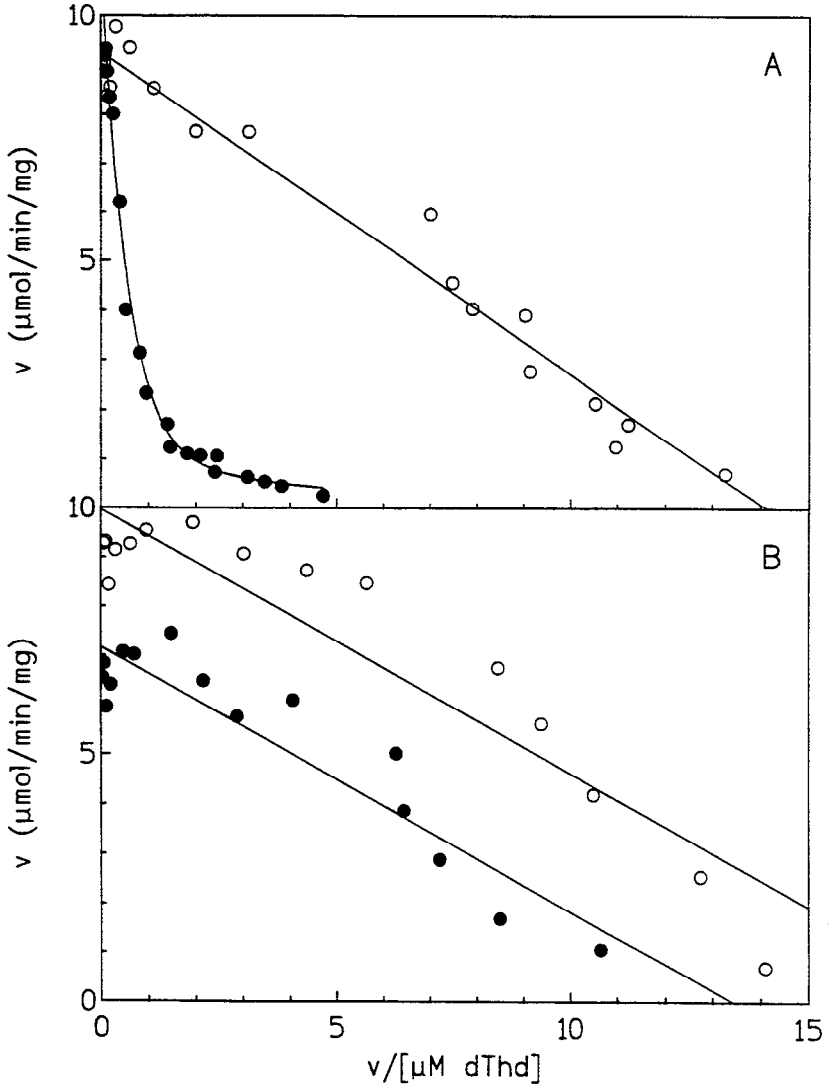


Table 1. Comparison of the properties of lymphocyte TK1 and recombinant TK1.

		Lymphocyte TK1	Recombinant TK1
Sub-unit molecular mass (kDa)		24	26
Molecular mass (kDa)	+ATP	175	155
	-ATP	75	155
Thymidine Spec.act. nmol/min/mg		9,500	10,000
[a]AZT as substrate	+ATP	40%	46%
	-ATP		38%
[a]ara-T as substrate	+ATP	0%	0%
	-ATP		0%
[b]ATP K_m (μM)	+ATP	14*	31
	-ATP	140*	27
[b]Thymidine K_m (μM)	+ATP	0.5-0.7	0.4
	-ATP	12-15	0.4
[c]thy. Hill coefficient	+ATP	1.2	1.5
	-ATP	0.7	1.4
[d]TTP inhib. I_{50} (μM)	+ATP	9-10*	1.5
	-ATP	9-10*	1.5

[a]The substrate analogs AZT and ara-T were determined in % of the activity with thymidine as substrate.

[b]K_m values for thymidine and ATP were calculated from Hofstee plots (v versus v/s).

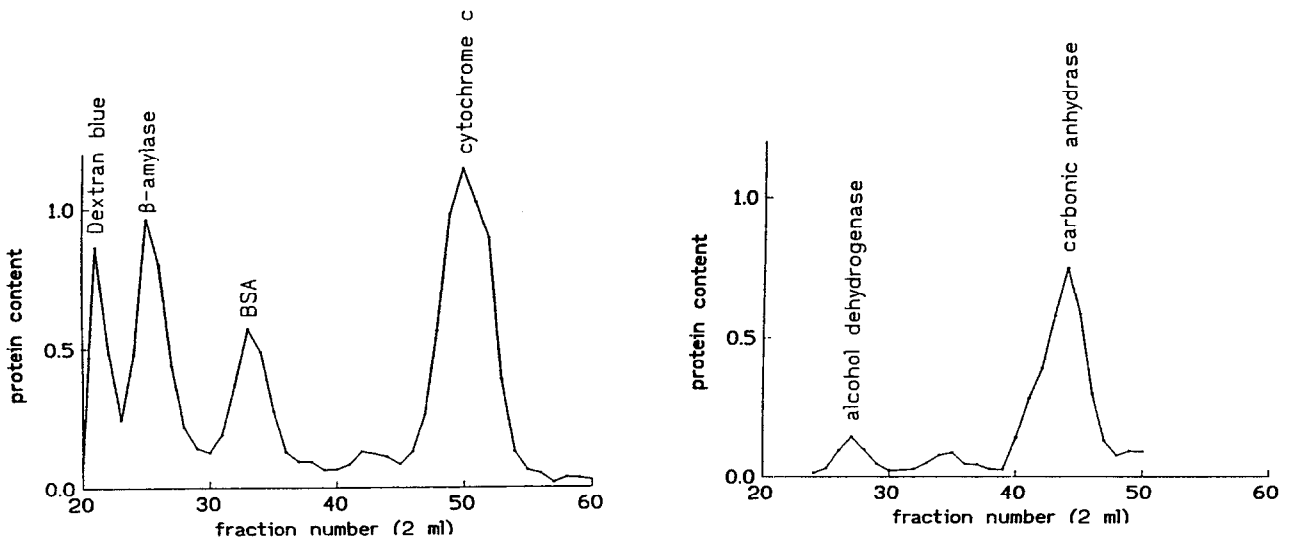
[c]The thymidine Hill coefficient were determined from Hill plots (log v/V_{max}-v versus log s).

[d]I_{50} for TTP is the inhibitor concentration at 50% inhibition.

* Munch-Petersen et al (1995b)

The different thymidine substrate kinetics of the -ATP and +ATP forms of the lymphocyte TK1 were related to the different molecular mass of the two forms. Thus the molecular mass of the lymphocyte TK1 as determined by elution on Superose 200 was a dimer (50 kDa) in the absence of ATP and a tetramer (100 kDa) in the presence of ATP. This indicates that ATP induces a tetramerization of the 24 kDa TK1 from human lymphocytes (Munch-Petersen et al,1993).

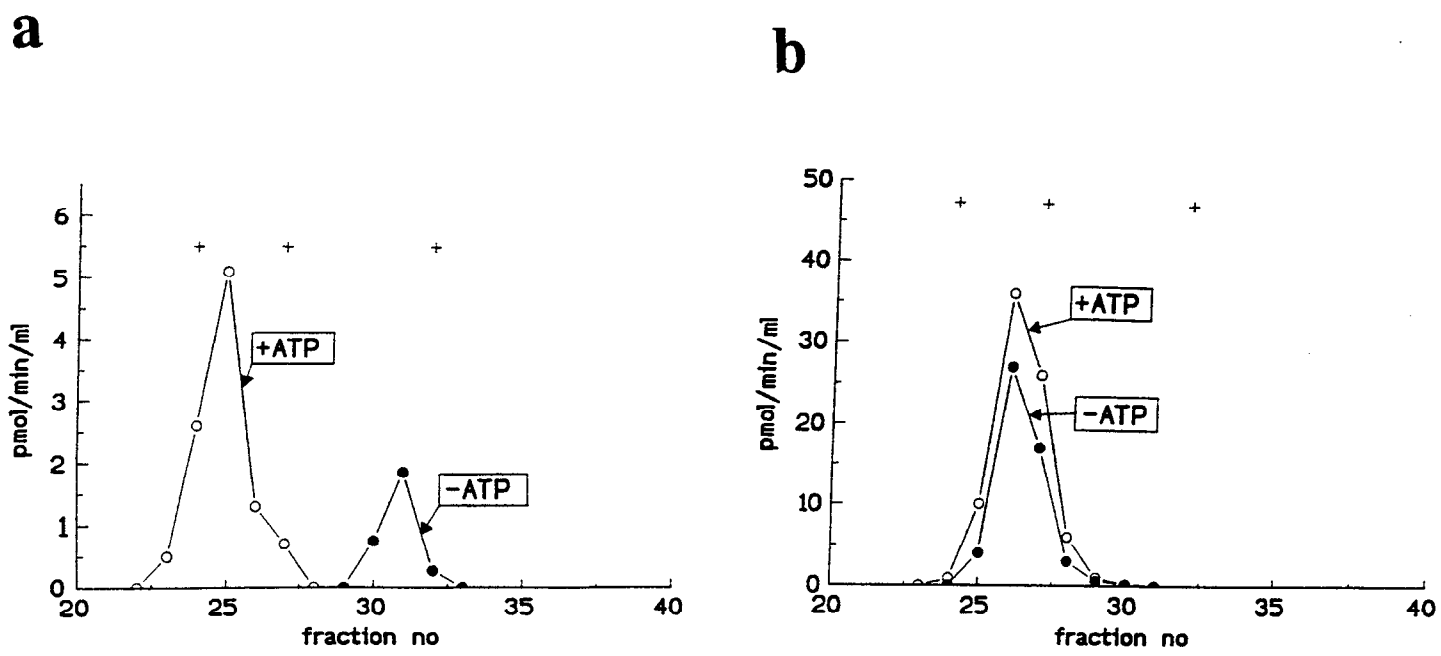
FIGURE 14: Elution of standard proteins on G-200 sephadex.



In the present work we have determined the apparant molecular mass of the lymphocyte and recombinant TK1 by elution on G-200 sephadex (paper IV). The elution of standard proteins are shown in figure 14. In absence and presence of ATP the apparent molecular mass of the lymphocyte TK1 were 75 kDa and 175 kDa respectively. The recombinant TK1 however, exclusively appeared with the high molecular mass. The apparent molecular mass in absence and presence of ATP were identical and about

155 kDa. This indicates that the ability to dissociate from a tetramer structure to a dimer structure was lost in the recombinant TK1 (figure 15).

FIGURE 15: Elution of (a) lymphocyte TK1 and (b) recombinant TK1 on G-200 sephadex.

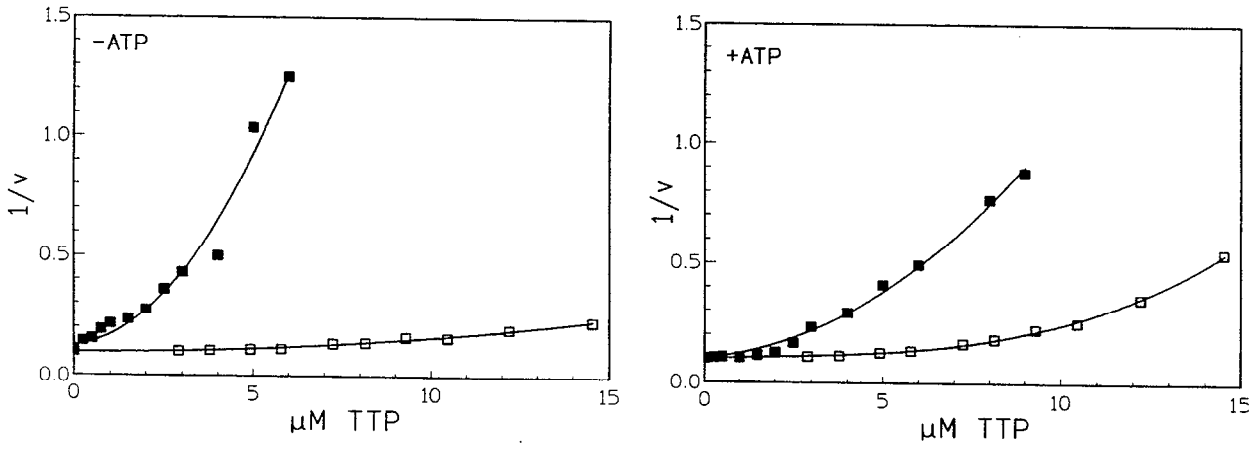


In this work the apparant molecular weights of the lymphocyte TK1 by elution on G-200 sephadex was higher, than in the previous elution experiments with the same enzyme preparation on superose 12 (Munch-Petersen et al.1991; Munch-Petersen et al.1993). This may be due to a non-globular shape of the proteins resulting in different filtration on the two column matrices. Similarly, the slightly lower molecular mass of recombinant TK1 (155 kDa) as compared to the lymphocyte TK1 (175 kDa) may be explained by an altered conformation of the tetramer. As the recombinant TK1 can not dissociate to a dimer, a tighter association of the four sub-units is probable.

Uptil now I had only found differences between recombinant TK1 and lymphocyte TK1 because I have included examination of enzyme without ATP. No differences has been found between the two enzymes as long as ATP was present.

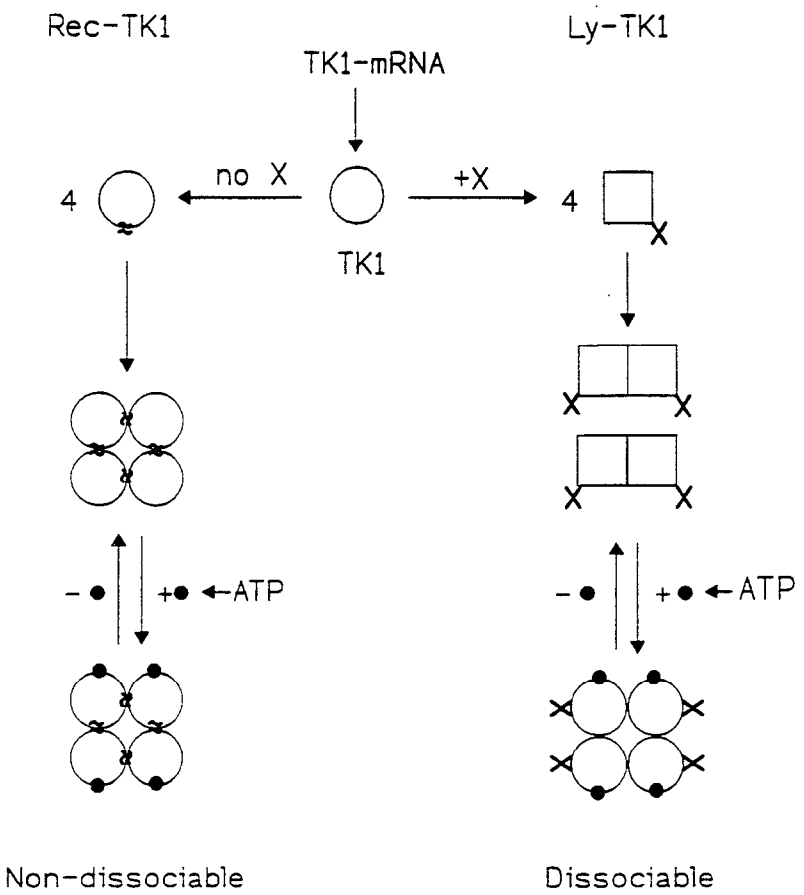
Finally the recombinant TK1 was examined with respect to TTP inhibition. The lymphocyte TK1 has a I_{50} value of 9-10 μM TTP (Munch-Petersen et al, 1995b). Surprisingly, the recombinant TK1 had a I_{50} value of about 1.5 μM TTP. Therefore recombinant TK1 is about 10 times more sensitive towards TTP than lymphocyte TK1. For both enzymes the sensitivity towards TTP was not dependend on incubation with or without ATP. Figure 16 illustrates the TTP kinetics for the recombinant TK1 and the lymphocyte TK1.

FIGURE 16: Reverse plots of of the TTP inhibition kinetic of recombinant TK1 and lymphocyte TK1. The open squares are lymphocyte TK1 and the filled squares are recombinant TK1.



The fact that recombinant TK1 only appears in one form in contrast to lymphocyte TK1, lead us to suggest a model for post-translational modifications of lymphocyte TK1 (Munch-Petersen et al, 1995a)(figure 17): After translation of TK1, the protein is post-translational modificated preventing the enzyme from immediate formation of a tetramer. The enzyme forms a dimer and can change formation to a tetramer after binding of ATP. Recombinant TK1 is not post-translational modified and form therefore a tetramer immediately after translation.

FIGURE 17: A model of the effect of the proposed post-translational mechanism modifying lymphocyte TK1 in contrast to recombinant TK1.



CONCLUSIONS.

The sensitive test for quantitating TK1 mRNA (competitive PCR) detects very low amounts of TK1 mRNA, down to 0.006 copies/cell and is many fold more sensitive than Northern blot.

The expression of TK1 mRNA and TK enzyme activity in normal lymphocytes stimulated to growth by PHA is low or undetectable in quiescent cells and increases around 100 fold with the DNA syntesis and cell division. This result is in agreement with other results with stimulated serumstarved cells. The only difference is that PHA stimulated cells have a delayed entry to S-fase after addition of PHA in contrast to stimulated serumstarved cells. This delayed entry to S-fase is in agreement with, that lymphocytes are true G_0 cells.

The expression of TK1 mRNA in CLL cells were remarkably high, at the same level as in dividing cells. The TK enzyme activity was very low, at the level found in quiescent cells. When the phosphate donor specifity of the enzyme was investigated is was shown that the TK activity in the quiescent cells was due to TK2.

An expression system for direct expression of TK1 was constructed in *E.coli*. The yield was about 1 mg/l culture and it can be purified to more than 95% homogeneity.

The recombinant TK1 and TK1 purified from lymphocytes, had similar sub-unit molecular weight (24 - 26 kDa), specific activity (about 10 μmol/min/mg), K_m for thymidine (0.4 μM), K_m for ATP (30 μM), substrate specifity towards AZT (40%) and AraT (0%) and molecular weight of the active enzyme (about 155 kDa)

as a tetramer.

The recombinant TK1 and the lymphocyte TK1 were different with respect to TTP as inhibitor. The recombinant TK1 was about 10 fold more sensitive towards TTP.

The effects of removal of ATP on lymphocyte TK1 was not seen with recombinant TK1. When ATP was removed from lymphocyte TK1, the enzyme changes reversibly from a high affinity tetramer to a low affinity dimer. Recombinant TK1 only appear at the high affinity form and only as a tetramer. The missing ability of the recombinant TK1 to appear as a low affinity dimer may be due to the lack of or changed post-translational modifications.

SUMMARY.

Thymidine kinase (TK) is a key enzyme in the salvage pathway of the nucleoside metabolism catalyzing the first phosphorylation step in TTP synthesis. Human cytosolic TK (TK1) is highly cell cycle regulated. TK1 is regulated on many different levels of expression and isoforms with altered enzymatic properties are found in cancer cells. Investigation of these factors offers possibilities to understand the molecular background for TK1 expression including to clarify general regulation patterns. It also gives valuable information for constructing new nucleoside analogs for the therapy of cancer and virus infections.

In the first part of the present investigation a sensitive test for quantitating TK1 mRNA (competitive PCR) is developed and the results show that PHA stimulated lymphocytes reveal the same pattern concerning expression of TK1 mRNA and TK1 enzyme activity as serum-stimulated cells. This pattern is a low level of TK1 mRNA and TK1 enzyme activity in quiescent cells and a high level of TK1 mRNA and TK1 enzyme in dividing cells. In chronic lymphatic leukemic cells, that are non dividing cells, the results surprisingly showed that the TK1 mRNA level was high and at the same level as found in dividing lymphocytes. The high level of TK1 mRNA is not translated into active enzyme as the TK enzyme activity was low as in quiescent lymphocytes. These results indicate, that there is a defect in the regulation of TK1 in chronic lymphatic leukemic cells. To investigate this phenomenon more closely I decided by

recombinant technics to examine the relation between the TK1 gene and the TK1 protein.

In the second part of this investigation a direct expression system for human TK1 in *E.coli* was developed to produce a source of high amounts of TK1, to be able to examine the structure of TK1. The resulting recombinant TK1 was similar to native TK1 purified from human lymphocytes with regard to sub-unit molecular weight, specific activity, K_m for thymidine, K_m for ATP, substrate specifity towards AZT and ara-T and native molecular weight as a tetramer.

This indicate that the recombinant and native TK1 are identical but further investigations showed some interesting differences. Recombinant TK1 is about 10 fold more sensitive towards TTP as inhibitor. Furthermore the effect of removal of ATP from the native TK1 on the enzyme kinetics and native molecular weight was not found for recombinant TK1. Native TK1 shifts reversibly between a low affinity form - a dimer when ATP is absent from the enzyme, to a high affinity form - a tetramer when ATP is present. Recombinant TK1 exclusively appear at the high affinity form and only as a tetramer also in the absence of ATP.

This indicate that TK1 is post-translationally modified in human cells and that this modification can not be performed in *E.coli*.

DANISH SUMMARY.

Thymidin kinase (TK) er et nøgle enzym i salvage pathway (genbrugsvejen) af nucleosid metabolismen, der katalyserer den første fosforylering i syntese af TTP. Human cytoplasmatisk TK (TK1) er et stringent celle cyklus reguleret enzym. TK1 bliver reguleret på mange forskellige niveauer under ekspressionen og forskellige isoformer er fundet i cancer celler. Undersøgelse af disse faktorer giver mulighed for at forstå den molekylære baggrund for ekspressionen af TK1 foruden at få generelle regulerings mønstre belyst. Det vil også give værdifulde oplysninger i forbindelse med konstruktion af nye nukleosid analoger, der bliver brugt til behandling af cancer og virus infektioner.

I første del af denne undersøgelse er en følsom test til kvantitering af TK1 mRNA (competitive PCR) blevet udviklet og resultaterne viser at PHA stimulerede lymfocytter udviser det samme mønster som serum-stimulerede celler med hensyn til ekspression af TK1 mRNA og TK1 enzym. Der er et lavt niveau af TK1 mRNA og TK1 enzym i hvilende celler og et højt niveau af TK1 mRNA og TK1 enzym i delende celler. I kronisk lymfatisk leukemiske celler (CLL celler) viste resultaterne overraskende at TK1 mRNA niveauet var højt, som i delende celler. Det høje niveau af TK1 mRNA blev ikke translateret til aktivt enzym, idét TK1 enzym aktivitet var lige så lavt som i ikke delende celler. Dette indikerer, at CLL celler har en defekt i reguleringen af TK1. For at undersøge dette fænomen nærmere, besluttede jeg ved anvendelse af recombinant teknikker at

undersøge sammenhængen mellem TK1 genet og TK1 proteinet.

I anden del af dette arbejde er et direkte ekspressions system for human TK1 i *E.coli* blevet udviklet for at få en kilde for store mængder TK1 og derved blive i stand til at undersøge strukturen af TK1 proteinet.

Rekombinant TK1 ligner det naturlige TK1 (oprenset fra humane lymfocytter) med hensyn til sub-unit molekylvægt, specifik aktivitet, K_m for thymidin, K_m for ATP, substrat specifitet med AZT og ara-T og molekylvægt af det aktive enzym, som er en tetramer.

Dette indikerer at enzymerne er identiske, men videre undersøgelser viste nogle interessante forskelle. Rekombinant TK1 er ca 10 gange mere følsom overfor TTP som hæmmer. Der ud over findes de effekter, som fjernelse af ATP har på enzym kinetikken og molekylvægten af det naturlige TK1, ikke ved rekombinant TK1. Det naturlige TK1 skifter reversibelt mellem en lav affinitets form - som dimer, når ATP er fjernet fra enzymet til en høj affinitets form - som tetramer, når ATP er til stede. Rekombinant TK1 findes kun som høj affinitets form og kun som tetramer, også selvom ATP er fjernet fra enzymet.

Dette indikerer, at TK1 bliver post-translationelt modificeret i humane celler og at disse modifications mekanismer bliver udført i *E.coli*.

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Paper I

QUANTITATION OF TK1 mRNA IN PATIENTS WITH CHRONIC LYMPHATIC LEUKEMIA

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INTRODUCTION

Thymidine kinase is an enzyme in the pyrimidine salvage pathway that, with ATP as co-substrate, catalyzes the phosphorylation of deoxythymidine to deoxythymidine monophosphate (dTMP) which is subsequently converted to dTTP and utilized for DNA synthesis.

In mammalian cells there are two thymidine kinases (TK), the constitutively expressed TK2, and the S-phase specific TK1 which is only present in dividing cells[1].

Lymphocytes from patients with chronic lymphatic leukemia (CLL) are non-dividing and it is therefore plausible that the low TK activity in these cells almost exclusively is due to TK2. However, a thymidine kinase with similar enzyme kinetic pattern as that observed with TK1 from lymphocytes stimulated to growth by the mitogene phytohemagglutinin has been reported[2]. Since TK1 expression is tightly regulated thoughout the cell cycle with transcriptional, translational as well as post-translational regulatory mechanism[3], the occurrence of TK1 in non-dividing CLL cells may be due to a change in the control of the cell cycle regulated expression of the TK1 gene.

To investigate the transcriptional expression of TK1 mRNA in CLL cells, we have measured the level of TK1 mRNA with the competitive polymerase chain reaction (competitive PCR), and compared this mRNA level with the TK enzyme activity. Surprisingly, we have found that the ratio of TK1 mRNA/TK activity in lymphocytes from CLL patients was about 60-400 fold higher than in lymphocytes from healthy persons.

METHODS

Lymphocytes from peripheral blood from 6 healthy persons and from 5 patients with untreated CLL were isolated by the Ficoll-Isopaque technique.
Lymphocytes from healthy persons were stimulated to growth by PHA in RPMI 1640 medium supplemented with 10% fetal calf serum, 20 μg/ml PHA and 20 μg/ml penicillin/streptomycin at a concentration of 10^6 cells pr ml in 5% CO_2 at 37°C. The lymphocytes were divided in portions of 5 x 10^6 cells, and in each portion, TK activity was determined by the DE-81 paper method as described[4] and total protein was determined by the Bradford assay[5]. Total RNA was isolated with the guanidine thiocyanate method[6], transcribed to cDNA and quantitated by the competitive PCR method[7]. TK1 cDNA, taken as representative for TK1 mRNA, was co-amplified with a dilution series of competitor DNA. Exon 1 and 2 with intron from the TK1 gene served as competitor DNA and exon 1 and 2 of the TK1 gene as the cDNA fragment to be quantitated. The fragments were amplified using a pair of primers identical to those reported by Lipson and Baserga[8]. The sizes of the resulting fragments was 138 bp with cDNA as template and 248 bp with competitor DNA as template. The relative amounts of cDNA versus competitor DNA were measured by scanning of ethidium-bromide stained gels. Because the starting concentration of the competitor DNA was known, the amount of cDNA (in grams) in the sample could be estimated as that amount of competitor DNA where equal intensities of the two amplification products were obtained. The number of TK1 cDNA copies was calculated from the amount of cDNA, by dividing the amount of cDNA with the molecular weight of 1 copy of the 138 bp cDNA fragment.

The amplification was performed in a Perkin-Elmer/Cetus Thermal Cycler according to the following program: denaturation for 1 min at 95°C, annealing for 1 min at 60°C and polymerization for 1 min at 72°C, for 35 cycles.

RESULTS AND DISCUSSION

Table 1 shows the ratio of TK1 mRNA copies and TK activity in non-dividing lymphocytes from 6 healthy persons and in lymphocytes from 5 patients with CLL. As seen, the ratio TK1 mRNA copies/TK activity in CLL cells is 60 to 400 fold higher than in non-dividing lymphocytes. The TK activity in CLL cells is of a magnitude as expected for non-dividing cells, while the expression of TK1 mRNA is very high and in the range of the TK1 mRNA level in PHA stimulated healthy donor lymphocytes. In these experiments the TK1 mRNA level is 3-98 x 10^6 copies/mg protein (results are not shown).

The detection limit in the assay is around 6 x 10^4 copies of TK1 mRNA/mg protein or 0.006 copies/cell. Below this level, a 248 bp amplification product, interferes with the competitive PCR. This is probably a result of traces of DNA or non-spliced RNA in the RNA preparation. The results indicate that there, as expected, is no TK1 mRNA in non-dividing lymphocytes from healthy persons.

Due to the high TK1 mRNA level in non-dividing CLL cells it was of importance to clarify whether the dominating TK in CLL cells was TK1 or TK2, using the characteristic differences in phosphate donor specificity towards ATP and CTP. Both enzymes can utilize ATP, but only TK2 is capable of utilizing CTP[9]. The relative TK activity with CTP as phosphate donor was expressed as % of activity with ATP as phosphate donor. PHA-stimulated lymphocytes showed a 85-90% decrease in relative

activity, while non-dividing lymphocytes from healthy persons and lymphocytes from CLL patients showed a 7-30% decrease. The conclusion is that the enzyme in CLL cells is the same as in non-dividing lymphocytes from healthy persons, namely TK2.

Table 1. Ratio of TK1 mRNA copies and TK activity.

	TK1 mRNA copies x 10^6/mg protein	TK activity Units/mg protein	TK1 mRNA copies x 10^6/TK activity
Non-dividing lymphocytes			
1	< 0.06	0.009	< 6.7
2	< 0.06	0.013	< 4.6
3	0.21	0.013	16.2
4	0.06	0.008	7.5
5	< 0.06	0.009	< 6.7
6	< 0.06	0.016	< 3.8
Lymphocytes from CLL patients			
1	10.3	0.008	1287
2	7.4	0.006	1233
3	22.7	0.013	1746
4	15.2	0.005	3040
5	6.1	0.006	1016

The ratio between TK1 mRNA and TK activity as estimated in non-dividing lymphocytes from 6 donors and in lymphocytes from 5 patients with CLL. The numbers refer to the individual donors and patients.
1 unit is the amount of enzyme that phosphorylate 1 nmol substrate per minute.

The occurence of a high level of TK1 mRNA without concomittant expression of TK1 enzyme activity may indicate that CLL cells have an abnormal regulation of the cell-cyclus regulated TK1. The regulations mechanism are not fully understood, but several investigations have shown that the changes in TK1 mRNA during cell cycle can not fully account for the rise in TK activity. Translational and post-translational modifications may contribute to the regulation of TK1. Chang and Huang[10] have demonstrated that seryl residues of the TK1 polypeptide are phosphorylated in cycling HL-60 cells. An increasing phosphorylation of the polypeptide was followed by an increase in enzyme activity, during the cell cycle. Another post-translational mechanism has been reported by Kauffman and Kelly[11]. They have shown that amino acid residues near the C-terminal end are

responsible for degradation of thymidine kinase protein in the G_2 and M phase, and that mutations in this part of the gene allow expression in G_0 cells.
It is possible that a post-translational mechanism serve as a secondary back-up system for the regulation of TK. This may explain why we can measure a high TK1 mRNA level but no TK1 activity.

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Paper II

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OVEREXPRESSION OF HUMAN THYMIDINE KINASE mRNA WITHOUT CORRESPONDING ENZYMATIC ACTIVITY IN PATIENTS WITH CHRONIC LYMPHATIC LEUKEMIA

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Abstract—The level of cytosolic thymidine kinase (TK1) mRNA in lymphocytes from six healthy people and in lymphocytes from five patients with untreated chronic lymphatic leukemia (CLL) was determined with competitive polymerase chain reaction (competitive PCR). Using this procedure we have shown that in patients with CLL, there is an overexpression of TK1 mRNA without corresponding enzymatic activity. The TK1 mRNA level is approximately 100-fold higher in lymphocytes from CLL patients than in lymphocytes from healthy persons. A high level of TK1 mRNA without corresponding enzyme activity may indicate a defect in the processing of the enzyme. This may disturb the cells' normal feedback system and thereby influence the development of malignant conditions.

Key words: Thymidine kinase, mRNA, chronic lymphatic leukemia, competitive PCR, quantification, CLL.

Introduction

Thymidine kinase (ATP: thymidine 5'phosphotransferase E.C. 2.7.1.21) is a pyrimidine nucleoside salvage pathway enzyme with two isoenzymes: TK1 and TK2, also called cytosolic and mitochondrial TK, respectively. Both enzymes catalyse the phosphorylation of thymidine to TMP which is subsequently converted to TTP and utilized in DNA synthesis. TK1 is the dominating form in dividing lymphocytes and TK2 is the only form present in non-dividing lymphocytes, but in low amounts [1]. The two isoenzymes have characteristic differences in their enzyme kinetic pattern and subunit molecular weights [2, 3]. TK1 is cell-cycle regulated and the enzyme level is low or undetectable in quiescent (G_0) cells, but increases dramatically when the cells enter S-phase [4–7]. It is generally accepted that there is a close correlation between TK1 activity and the proliferative state of the cell [1, 6, 8–10].

Lymphocytes from patients with chronic lymphatic leukemia (CLL) are non-dividing and it is, therefore, plausible that the low TK activity in these cells almost exclusively is due to TK2. However, Munch-Petersen and Tyrsted found that the dominating TK activity isolated from lymphocytes from a CLL patient displayed an enzyme kinetic pattern similar to that observed with TK1 [11].

The occurrence of TK1 in quiescent CLL cells may be due to a change in the control of the cell-cycle regulated expression of the TK1 gene. The cell-cycle regulation of TK1 is a very complex system involving transcriptional, post-transcriptional [4, 12, 13], translational and post-translational regulation mechanisms [14, 15]. To investigate the expression of TK1 mRNA in CLL cells at the transcriptional level we have measured the level of TK1 mRNA by the very sensitive competitive polymerase chain reaction (competitive PCR), and compared this with the TK enzyme activity. Surprisingly, we have found that the level of TK1 mRNA in lymphocytes from CLL patients was about 100-fold higher than in lymphocytes from healthy persons.

Materials and Methods

Materials

^{3}H-thymidine (2 Ci/mmol), [α^{32}P]dCTP (3000 Ci/mmol), Megaprime DNA labeling systems (RPN 1604) and

Abbreviations: CLL, chronic lymphatic leukemia; *TK*, thymidine kinase; *TK1*, thymidine kinase characteristic for dividing cells; *TK2*, thymidine kinase characteristic for non-dividing cells; *PCR*, polymerase chain reaction; *PHA*, phytohemagglutinin; *FCS*, fetal calf serum; *ATP*, adenosine triphosphate; *CTP*, cytidine triphosphate.

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Hybond N^+ membrane were from Amersham Denmark ApS. Isopaque–Ficoll was from Nycomed. RPMI-1640, fetal calf serum (FCS), phytohemagglutinin (PHA) and bovine serum albumin (BSA) were from Gibco. All nucleosides were from Boehringer and Mannheim, RNasin, random hexamer and M-MLV reverse transcriptase RNase H minus were from Promega. *Thermus aquaticus* DNA polymerase (AmpliTaq) was from Perkin Elmer/Cetus. The primers used were synthesized at the Department of Microbiology at the Technical University of Denmark. SpinBind DNA extraction Unit was from FMC Bioproducts Europe. DEAE and 3MM filters were from Whatman. All other reagents were of the highest quality generally available.

Cells

Peripheral blood from six healthy persons was collected in heparin vacuum tubes. Peripheral blood, similarly collected from five patients with untreated CLL was kindly provided by Sven Erik Nielsen (M.D.), Roskilde Hospital. The lymphocytes were isolated by Isopaque–Ficoll gradient centrifugation [16] and washed in RPMI-1640 containing 10% heat-inactivated FCS. The cell number was estimated in a Coulter counter and cell pellets were stored at −80°C.

Phytohemagglutinin (PHA) stimulation of lymphocytes from healthy persons

The lymphocytes were stimulated by PHA in RPMI-1640 medium supplemented with 10% FCS, 20 μg/ml PHA and 20 μg/ml penicillin/streptomycin, at a concentration of 10^6 cells per ml in 5% CO_2 at 37°C. For flow cytometry analysis aliquots of 10^6 cells were centrifuged at 50 *g* for 10 min and fixed in 250 μl buffer (6.1 mM glucose, 140 mM NaCl, 5 mM KCl, 2.7 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 0.5 mM EDTA) and 750 μl EtOH. The DNA was stained with 20 μg/ml EtBr and 10 μg/ml mithramycin and monitored by flow cytometry. In quiescent lymphocytes the S-fraction was about 8%. In lymphocytes cultured for 48 h with PHA the S-fraction was in the range of 30%. These data were in agreement with previous observations [17].

Extraction of lymphocytes for TK activity measurement

The cells were suspended in Loebs buffer (20 mM K-phosphate buffer (pH 7.4), 15% glycerol, 1 mM K-EDTA, 10 mM dithiotreitol (DTT)), lysed by sonication (40 W, 3 × 1–2 s) and centrifuged at 20,000 *g* for 30 min. The supernatant (enzyme extract) was used for TK activity measurement and total protein measurement.

TK activity assay

TK activities were determined as initial velocities using the DEAE-cellulose 81 paper square method as previously described [2, 3]. The standard assay mixture contained: 50 mM Tris–HCl (pH 7.5), 10 mM DTT, 2.5 mM ATP, 2.5 mM $MgCl_2$, 3 mM NaF, 0.5 mM CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulphonate), 3 mg/ml bovine serum albumin (BSA) and 10 μM ^{3}H-thymidine (2 Ci/mmol). In assays with CTP as phosphate donor ATP was substituted with equimolar CTP. Samples of 13 μl were applied on DEAE filters 5, 10 and 15 min after starting the reaction by addition of enzyme extract to the assay mixture to a total volume of 50 μl. The reaction temperature was 37°C. The filters were washed, eluted and the radioactivity determined by scintillation counting as described [3].

One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 nmol dTMP per min.

Protein determination

The protein content was measured by Coomassie brilliant blue as described [18].

RNA isolation

Total RNA was isolated using the guanidine thiocyanate method described by Chomczynski and Sacchi [19]. To improve purification an extra phenol extraction and alcohol precipitation was applied. RNA concentration was estimated from the optical density at 260 nm, and the RNA quality was examined by agarose gel electrophoresis.

Estimation of RNA recovery

^{3}H-uridine was added to the growth medium (5 μCi/ml, 5 Ci/mmol) during PHA-stimulation in two experiments. A total of 5×10^6 labeled cells were harvested on 3 MM filters and non-incorporated ^{3}H-uridine was washed away. From an equal number of cells, RNA was isolated and applied to 3 MM filters. The radioactivity on the filters was determined by scintillation counting. The amount of isotope in RNA was compared with the amount of isotope in the cells. The RNA recovery estimated from these comparisons was in the range of 70–90%.

Reverse transcription

RNA (2.5 μl) was transcribed to cDNA in a 50 μl volume of PCR buffer (10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.015% gelatine, 0.1% Tween 20), 7.5 mM $MgCl_2$, 1 mM of each of the dNTPs, 40 U RNasin, 7 μM random hexamers and 250 units of M-MLV reverse transcriptase RNase H minus. The reaction was terminated after 2 h at 37°C. The extent of reverse transcription was controlled by a parallel reaction where ^{3}H-TTP, instead of TTP was added. Aliquots of the reaction mixture were applied on 3 MM filters. The non-incorporated ^{3}H-TTP was removed from the filter by washing 3 × 10 min in 1 M HCl containing 0.6 mM $Na_5P_3O_{10}$, 10 min in 0.26 M NaAc/EtOH and finally in EtOH. The radioactivity was measured by scintillation counting (results not shown).

Competitive PCR

The principle in the competitive PCR method, as reported by Gilliand *et al.* [20], is a co-amplification of target cDNA concurrently with the corresponding genomic DNA. Thus, the two templates compete for the same substrates and primers ensuring equal efficiency of amplification. The genomic DNA serves as internal standard. In our experiments we chose exons 1 and 2 with intron 1 from the TK gene as internal standard and exons 1 and 2 as the target cDNA fragment. The fragments were amplified using a pair of primers identical to those reported by Lipson and Baserga [21]. The sizes of the resulting fragments were 138 bp with cDNA as template and 248 bp with genomic DNA as template. The internal standard of 248 bp genomic DNA was prepared by PCR with DNA as a template and using the primers mentioned above. The product was quantified by agarose gel electrophoresis together with different known amounts of DNA. The unknown amount of cDNA was estimated from a set of PCR reactions performed in a dilution series with known amounts of the

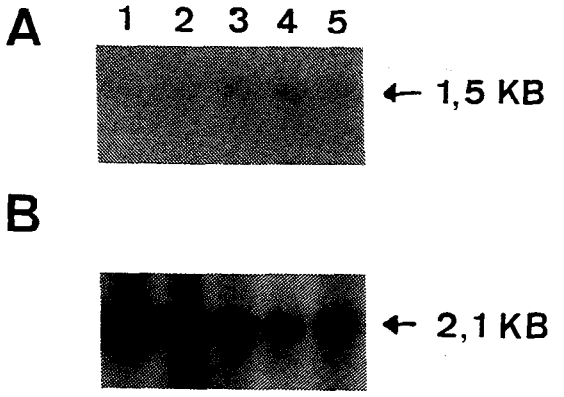

Fig. 1. Northern blot analysis of TK1 mRNA. Total RNA was isolated from lymphocytes from donor 5 with 24 μg loaded in each lane. The lymphocytes were stimulated to grow with PHA. Non-stimulated (lane 1); 48 h (lane 2); 72 h (lane 3); 96 h (lane 4); 168 h (lane 5). (A) Hybridization with a TK probe detecting a single mRNA species of 1.5 kilobases. (B) After stripping the filter in (A) for TK probe, the filter was rehybridized to a probe for the constitutively expressed β-actin, detecting a mRNA species of 2.1 kb.

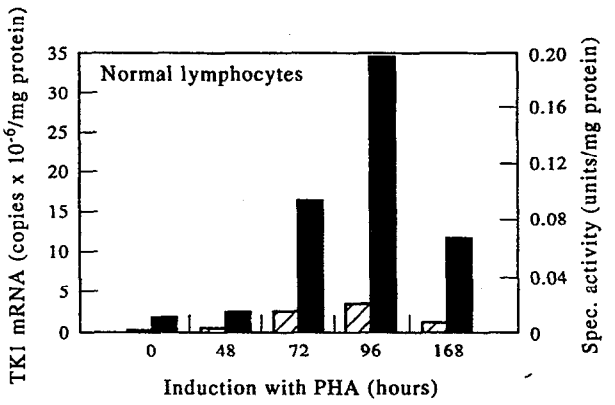

Fig. 2. Cell-cycle-specific variation of TK1 mRNA (copies/mg protein) ▨ and TK activity (units/mg protein) ■ are illustrated for donor 1. The lymphocytes were stimulated with PHA for the indicated time periods. TK1 mRNA was estimated by competitive PCR and TK activity was measured at standard conditions as described in 'Methods'.

genomic DNA. The PCR products were separated by agarose gel electrophoresis. The amount of cDNA (in grams) in the sample was estimated as that amount (in grams) of genomic DNA giving equal intensity of the two amplification products. The number of TK1 cDNA copies was calculated from the amount of cDNA, by division with the molecular weight of the 138 bp cDNA fragment (average molecular weight/base = 308). The number of copies of TK1 cDNA was taken as being representative for the number of copies of TK1 mRNA.

The competitive PCR analyses were performed in a 25 μl vol. of PCR buffer, 7 pmol of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 unit of *Thermus aquaticus* DNA polymerase. Heat-denatured cDNA (100°C, 2 min) and internal standard were added to the reaction mixture with a layer of mineral oil to avoid evaporation. The amplification was performed in a Perkin-Elmer/Cetus Thermal Cycler according to the following program: denaturation for 1 min at 95°C, annealing for 1 min at 60°C and polymerization for 1 min at 72°C, for 35 cycles.

Northern blot

Total RNA was prepared as described above. RNA preparations were denatured and electrophoresed through a 1.8% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond N^+ membrane according to published procedures [22]. The probes used were human TK1 cDNA from plasmid pTK11 [23] and human β-actin cDNA labeled with ^{32}P-dCTP using Megaprime DNA labeling systems. The TK1 probe was 720 bp and the actin probe was 400 bp. Non-incorporated nucleotides were removed with a SpinBind DNA extraction unit. In a 20 ml hybridization reaction a 100 ng (3.4 μCi/μg) probe was used. Hybridization was performed according to the guidelines from Amersham Denmark ApS with high stringency washing: 0.1 × SSPE-buffer (3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA pH 7.7) and 0.1% SDS at 68°C.

Results

Determination of TK1 mRNA by Northern blotting

Expression of TK1 mRNA during the cell cycle of PHA stimulated lymphocytes was analysed for two donors by Northern blot analysis. As seen in Fig. 1(A) it is clear that TK1 mRNA is not expressed in quiescent lymphocytes, whereas a 1.5 kb band, corresponding to TK1 mRNA, is seen in lymphocytes cultured with PHA. The level of TK1 mRNA increases reaching a maximum after 96 h of culture with PHA, whereafter the level decreases. Hybridization with a probe for the constitutive expressed β-actin shows that equal amounts of RNA were applied to each lane.

TK1 mRNA and TK activity in lymphocytes from healthy donors

With competitive PCR it is possible to measure TK1 mRNA in quiescent lymphocytes. Figure 2 shows the level of TK1 mRNA (copies/mg protein) and the TK activity (units/mg protein) in lymphocytes from donor 1. It is clearly demonstrated that the amounts of TK1 mRNA and TK activity increase concomitantly during incubation of the lymphocytes with PHA, reaching a peak level at 96 h. The same cell-cycle regulated pattern was observed in lymphocytes from the five other donors. Table 1 shows the actual amounts of TK1 mRNA and TK activity in the six donors. In quiescent lymphocytes, the level of TK1 mRNA is very low and in four out of six donors below the limit of detection which in our reactions correspond to 0.006 copies of TK1 mRNA/cell. After PHA stimulation, the level of TK1 mRNA transcripts increases to a maximum level, between

Table 1. The amount of TK1 mRNA (copies/mg protein) and TK activity (units/mg protein) in non-stimulated lymphocytes and lymphocytes incubated with PHA for 96 h

Donor No.	TK1 mRNA (copies × 10^6/mg protein)		TK activity (units/mg protein)	
	Non-stimulated lymphocytes	PHA-stimulated lymphocytes	Non-stimulated lymphocytes	PHA-stimulated lymphocytes
1	N.D	3.2	0.009	0.195
2	N.D	4.9	0.013	0.120
3	0.215	20.0	0.013	0.292
4	0.061	5.4	0.008	0.503
5	N.D	85.7	0.009	0.285
6	N.D	98.8	0.016	0.760

N.D = Not detectable.

Table 2. The amount of TK1 mRNA (copies/mg protein) and TK activity (units/mg protein) in lymphocytes from five patients with CLL

CLL patient No.	TK1 mRNA (copies × 10^6/mg protein)	TK activity (units/mg protein)
1	10.3	0.008
2	7.4	0.006
3	22.7	0.013
4	15.2	0.005
5	6.1	0.006

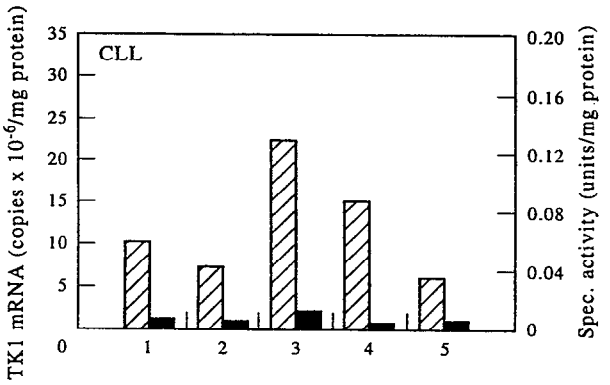

Fig. 3. The amounts of TK1 mRNA (copies/mg protein) ▨ and TK activity (units/mg protein) ■ in lymphocytes isolated from CLL cells. The numbers on the *x*-axis refer to the five CLL patients.

50 and 5000-fold higher than in quiescent cells. The broad range in TK1 mRNA level may reflect individual variation between the different donors.

TK1 mRNA and TK activity in CLL cells

Our results show that the TK activity in CLL cells is low and in the same range as in quiescent lymphocytes, between 0.005–0.013 units/mg protein (Table 2). Surprisingly, despite the low TK activity, lymphocytes from CLL patients express very high levels of TK1 mRNA (Fig. 3). The level of TK1 mRNA/mg protein was between 30 and 300-fold higher than the level in quiescent cells from healthy donors.

Characterization of the TK isoenzyme in CLL cells

Owing to the high TK1 mRNA level it was important to establish to which degree the TK activity was due to TK1 or TK2. The low amounts of TK activity in the crude extracts, however, did not allow any further separation of TK1 and TK2. Therefore, we have distinguished between TK1 and TK2 using the pronounced differences in substrate specificity.

Both isoenzymes utilize adenosine triphosphate (ATP) efficiently as phosphate donor, but TK2 can also use cytidine triphosphate (CTP) efficiently, whereas it is a poor phosphate donor for TK1 [24].

To clarify whether the isoenzyme in CLL cells is similar to TK2 in quiescent cells or to TK1 in dividing cells, we have compared the phosphate donor efficiency of ATP and CTP in quiescent lymphocytes, PHA stimulated lymphocytes and CLL cells. TK activity with CTP as donor is given as a percent of TK activity with ATP as donor.

As seen in Fig. 4, CTP was a poor phosphate donor for the TK enzyme in PHA stimulated lymphocytes, but an efficient phosphate donor for the TK enzyme in quiescent lymphocytes and CLL cells. These results indicate that the TK in CLL cells is the same as in quiescent lymphocytes, namely TK2.

Discussion

The development of the PCR reaction has revolutionized the area of molecular biology. With its high sensitivity, competitive PCR makes it possible to measure gene expression of even a low copy gene,

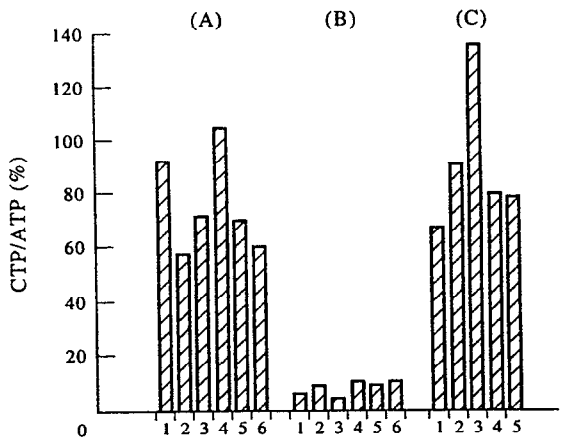

Fig. 4. The ratio between phosphate donor capacity of CTP and ATP. In each experiment the enzyme activity is normalized to 100% with ATP as phosphate donor. (A) Non-stimulated lymphocytes; (B) lymphocytes 96 h after PHA stimulation; (C) CLL cells. The numbers on the *x*-axis refer to the individual donors and patients.

such as thymidine kinase. We wanted to quantitate TK1 gene expression in lymphocytes from untreated CLL patients, but were restricted by the limited availability of material. The most frequently used method for determination of gene expression is Northern blot analysis, but the results were very weak when carried out on PHA stimulated lymphocytes. To be able to detect TK1 mRNA in the very limited CLL samples, a more sensitive method was required. Using competitive PCR it was possible to quantify the level of TK1 mRNA, even in quiescent lymphocytes. We have estimated a very low, but detectable level, in two donors. The amount of TK1 mRNA in quiescent lymphocytes is very close to the detection limit, which is about 0.006 copies of TK1 mRNA/cell. Below this level, a 248 bp amplification product interferes with the competitive PCR. This is probably as a result of traces of DNA or non-spliced RNA in our RNA preparation. As there is a minor loss during the RNA purification and the cDNA synthesis (see Methods), the actual level of TK1 mRNA is slightly underestimated. However, we presume that the underestimation is in the same range in all samples, since we have used the same protocols for all donors.

As a model system we have used human lymphocytes which are truly G_0 cells. Culturing the quiescent lymphocytes in the presence of PHA stimulates the cells to enter the cell cycle, allowing events in the G_1 and S-phases to be investigated. A clear advantage of using lymphocytes as representatives for normal cells instead of immortal cell lines is the ability of cell lines to grow continuously. This is due to the occurrence of at least one feature required to turn normal cells into cancer cells.

The expression of TK has been shown to be regulated on multiple levels and the regulation mechanisms differ, depending on the cell system. For example, in cycling HeLa cells, the S-phase increase in TK activity is largely accounted for by an increase in the rate of TK protein translation [7]. In serum-starved cells stimulated to re-enter the cell cycle, the increase in TK activity is accompanied by a corresponding increase in TK1 mRNA. In this system both transcription and post-transcriptional mechanisms account for the induction of TK1 mRNA [4, 25, 26]. In PHA stimulated lymphocytes it is well known that TK activity increases dramatically when the cells enter the cell cycle [1, 27, 28]. However, for this cell system, we have not been able to find any reports regarding the fluctuations in TK1 mRNA level as a characteristic of regulatory mechanisms.

Our results show that both TK1 mRNA and TK activity in PHA stimulated lymphocytes display the same cell-cycle regulated pattern as stimulated serum-starved cells. An increase in TK1 mRNA in lymphocytes at the entry to S-phase is followed by an increase in TK enzymatic activity. The TK1 mRNA level increases about 100-fold when the cells are stimulated. When cells leave S-phase, the TK1 mRNA level and TK enzyme activity decrease.

An important difference between the two cell systems is that lymphocytes are truly G_0 cells, they do not enter S-phase before 48 h after addition of PHA. This is in contrast to stimulated serum-starved cells which reach S-phase after 12 h [25].

In CLL cells we found, surprisingly, that TK1 mRNA expressed per mg protein is 30–300 fold higher than in quiescent lymphocytes. The TK activity level is very low and in the same range as in quiescent lymphocytes. Exploiting the different substrate specificities of TK1 and TK2 using CTP instead of ATP as phosphate donor, it was shown that the TK activity in the CLL cells is due to TK2. This phenomenon of a high TK1 mRNA level with no TK1 enzyme activity has to our knowledge not been described elsewhere.

Our experiments indicate that CLL cells have an abnormal regulation of the S-phase-regulated TK1. TK1 mRNA level is high and is prevented from translation into active enzyme. Owing to its association with the proliferative state of cells, TK activity is regarded as a useful tumor marker with prognostic value for a number of malignant diseases such as human breast cancer [29–31], non-Hodgkins lymphoma [32] and acute lymphatic leukemia [33]. In CLL cells the TK activity is very low and can, therefore, not be used as a tumor marker. On the other

hand, the level of TK1 mRNA is very high and may, therefore, serve as an alternative tumor marker in these cells to predict the diagnosis at an earlier stage.

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Paper III

ALTERED KINETIC PROPERTIES OF RECOMBINANT HUMAN CYTOSOLIC THYMIDINE KINASE (TK1) AS COMPARED WITH THE NATIVE FORM.

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INTRODUCTION

Human cytosolic thymidine kinase (TK1) is a cell cycle regulated key enzyme in the salvage pathway of the nucleoside metabolism catalyzing the first phosphorylation step in dTTP synthesis. Thymidine kinase (TK) is an enzyme of high interest, primarily because of the specific cell cycle regulated expression. In addition, TK phosphorylates a number of nucleoside analogs which in the phosphorylated form interfere with DNA replication and repair and hence are useful in the chemotherapeutic treatment of cancer and viral infections.

A more detailed knowledge of TK will be valuable for constructing more TK specific and efficient nucleoside analogs. A main problem in the structural an physical investigation of TK is the extremely low level of TK protein in mammalian cells. In this work we describe an expression vector for human TK1. Surprisingly, the enzymatic properties of the recombinant TK1 differ from those of the native TK1 with respect to the recently described specific regulatory effect of ATP[1]. This may indicate that native TK1 is subject to posttranslational modifications, such as phosphorylation, modifications that are not operable in *E.coli*.

METHODS.

Constuction of TK1-pet3a vector.

The TK1-pet3a vector was constructed by standard methods[2] by inserting the amino-acid coding region of the human TK1 into the polylinker of the pet3a vector[3] which is under control of a T7 RNA polymerase promotor. The aminoacid coding region was PCR amplified from the pTKII[4] containing the complete human TK1 cDNA. The two primers in the PCR flank the aminoacid coding sequence and each has a restriction site in the 5' end (NdeI and BamHI, respectively). The PCR product was blunt-end ligated into the EcoRV site of

pBR322. This cloning was nessesary because it was not possible to cut the amplified product directly by NdeI and BamHI. The pBR322 with the insert was cleaved by BamHI and NdeI and the insert was purified by agarose gel electrophoresis and band purification by standard methods[2]. The insert and the pet3a vector were ligated and used to transform a host E.coli (HB101). The TK1-pet3a vector was isolated and sequenced by T7 DNA polymerase to control that no mutations had occurred during the PCR.

Expression of TK1.

E.coli strain BL21(DE3)lysS containing T7 RNA polymerase under control of the IPTG inducible UV5 promotor was transformed by the TK1-pet3a vector. The expression was carried out in LB medium (10 g tryptone, 5 g yeast, 10 g NaCl/liter) at 25°C. When OD_{600} was 0,2, IPTG was added to a final concentration of 0,4 mM to induce the UV5 promotor. The cells were harvested by centrifugation (14,000 g, 3 min), resuspended in 1/4 vol of lysis buffer (50 mM Tris-HCl (pH 8), 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, 5 mM DTT, 0.5 mM PMSF, 5 mM Benzamidine, 10% glycerol, 50 mM NaF) and sonicated 3 times 15 sec (40 Watt) on ice. Cell debris was removed by centrifugation 20,000 g at 4°C for 30 min.

Purification of TK1.

All steps in the purification were performed essentially as decsribed[5]. Enzyme extract from 1 liter culture was filtered on a G-25 column (5.5 x 20 cm) and the enzyme chromatographed on a 3'-dTMP-Sepharose column (1 x 4 cm).

For enzyme kinetics studies, thymidine was removed by CM-sepharose chromatography as described[1]. *E.coli* TK is removed together with thymidine in this step, because in contrast to the human TK, it does not bind to the negative carboxy groups[6].

Enzyme kinetics.

The thymidine kinase activity was assayed by the DEAE-cellulose 81 paper method[7]. Standard assay conditions were: 50 mM Tris-HCl, pH 8, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 3 mg/ml BSA, 2.5 mM ATP, 10 μM radiolabelled thymidine. One unit of enzyme activity is defined as the amount of enzyme that can phosphorylate 1 μmol of nucleoside per min at 37°C under standard assay conditions. K_m values and Hill coeficients were determined from Hill plots (log v/(V-v) versus log s).

Sephadex G-200 chromatography.

Sephadex G-200 superfine was swelled and packed according to standard procedures (600 x 20 mm). V_e values were determined for five marker proteins (beta-amylase (200 KD), alcohol dehydrogenase (150 KD), bovine serum albumin (66 KD), carbonic anhydrase (29 KD), cytochrome C (12,4 KD)) and for TK1 in absence and presence of ATP. Before determinating the V_e value in the presence of ATP the enzymes were preincubated with 2.5 mM ATP and the column was pre-equilibrated with buffer containing 2.5 mM ATP.

RESULTS AND DISCUSSION.

An expression-vector was constructed to ensure unmodified ends of the TK1. When BL21(DE3)lysS is transformed by this vector the yield is around 1 mg TK1 per liter culture (25°C, 24 hours).

The recombinant TK was purified by affinity chromatography on a 3'-dTMP-Sepharose column to more than 95% homogeneity as estimated from a SDS-PAGE (figure 1). The sub-unit molecular weight was as expected, similar to the native TK1 - around 24 KD.

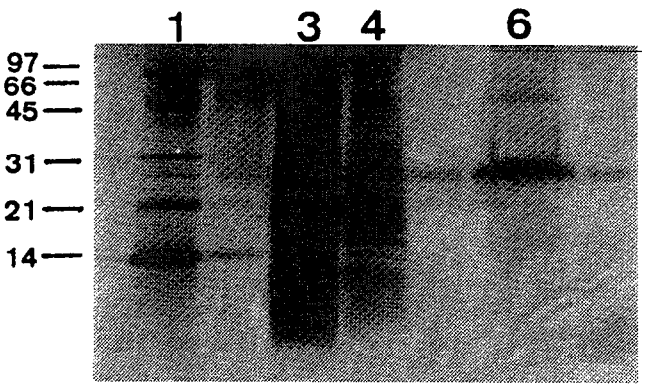

Figure 1.SDS-PAGE of purified recombinant TK1. Lane 1: Proteinmarker (97 KD, 66 KD, 45 KD, 31 KD, 21 KD, 14 KD), lane 3: Bacteria lysate, lane 4: Wash fractions of affinity column, lane 6: Eluate from affinity column.

Table 1. Comparison of the properties of lymphocyte TK1 and recombinant TK1.
* Hill coefficient = n

		Lymphocyte TK1	Recombinant TK1
Spec. act. nmol/min/mg		9.500	10.000
K_m (μM)	+ATP	0.5	0.4
	-ATP	12	0.4
Hill coefficient	+ATP	1.25	1.5
	-ATP	0.7	1.4
Subunit molecular mass (KD)		24	24
Native molecular mass	+ATP	150	150
	-ATP	70	150

The kinetic data for the native TK1 (Table 1) are in agreement with our previously reported data, and show that incubation or storage with ATP induces a kinetically slow transition from a low affinity form (Km=12μM, n=0.7) to a high affinity form of TK1 (Km=0.5 μM, n=1.25)[1]. For the recombinant enzyme, the Km and n values were 0.4 μM and 1.4 - 1.5, respectively, for both the -ATP and +ATP form. This indicates that the recombinant enzyme only occurs as the high affinity form with the low Km value. In addition the Hill coefficients of 1.4 - 1.5 indicate a slightly higher degree of positive homotropic cooperativity than found for the +ATP form of the native TK1.

The different kinetics of the +ATP and -ATP forms were related to the ability of the native TK1 to appear as a dimer in the absence of ATP and a tetramer in the presence of ATP[1]. When the apparant molecular weights of the native and recombinant TK1 were determined in absence and presence of ATP (figure 2), we found that recombinant TK1 was a tetramer also in absence of ATP.

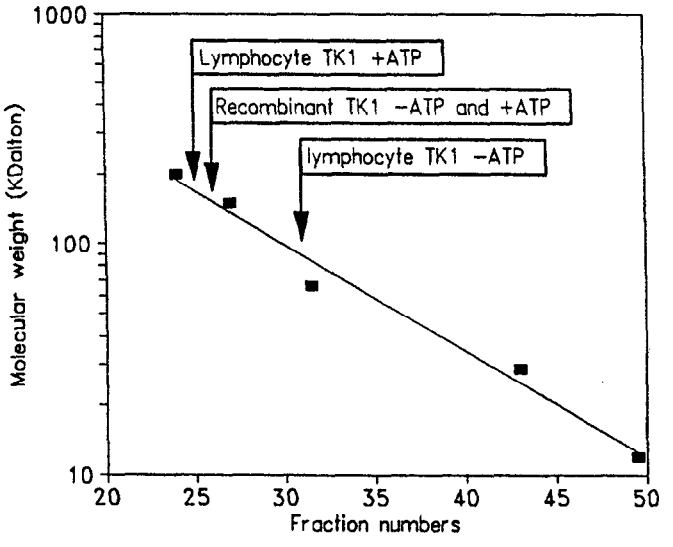

Figure 2. The molecular weight determined by G-200 superfine Sephadex. The column is calibrated by protein standards (12 - 200 KD) marked as black squares. The recombinant and lymphocyte TK1 preincubated with and without ATP are marked with black arrows.

Two conclusions are implicated in these results: Firstly the ATP induced kinetic transition of the native TK1 is associated with the dimer-tetramer transition. Secondly, the missing ability of recombinant TK1 to undergo the ATP induced transition between two distinct molecular forms, may be due to the lack of post-translational modifications when expressed in E.*coli*. Such modifications may enable native TK1 to undergo the reversible transition, and we find it likely that this mechanism is involved in the cell cycle regulated expression of TK1 as previously suggested[1,8].

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Paper IV

HUMAN CYTOSOLIC THYMIDINE KINASE EXPRESSED IN *E.coli* OCCURS SOLELY AS THE HIGH AFFINITY TETRAMER FORM WITH THE LOW K_m VALUE.

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Running title: Recombinant and native human cytosolic thymidine kinase differ in enzymatic properties.

Keywords: Thymidine kinase, recombinant enzymes, enzyme kinetics, regulation, expression-vector, pET-3a.

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Abbreviations:

TK, thymidine kinase; TK1, cytosolic thymidine kinase; PCR, polymerase chain reaction; DTT, dithiothreitol; IPTG, isopropyl-ß-D-thiogalactopyranoside; BSA, bovine serum albumine: PMSF, phenylmethylsulfonyl fluoride; AZT, 3'-azidothymidine; ara-T, 2'-arabinosol-thymidine.

ABSTRACT

An expression system for human "cytosolic" thymidine kinase (TK1) in E.coli was developed. cDNA encoding the entire polypeptide of TK1 was inserted in the polylinker of the pet3a vector and expressed in BL21(DE3)lysS. The yield was about 1 mg/liter culture after 24 hours of induction at 25°C. The TK1 enzyme was purified to homogeneity by thymidine affinity chromatography. The recombinant TK1 has the samme sub molecular weight and specific activity as the native enzyme (purified from PHA stimulated lymphocytes). Also, when stored in ATP, recombinant and native thymidine kinase were identical with respect to thymidine kinetics as a high affinity form (K_m = 0.5 - 0.7 μM) and molecular size as a tetramer. Surprisingly, this was not the case when storing the enzymes without ATP. Thus, as shown recently, the native enzyme is a dimer with low affinity for thymidine (K_m = 15 μM)(Munch-Petersen,B., Tyrsted,G. and Cloos,L. (1993) *The Journal of Biological Chemistry*. **268**, 15621-15625). Recombinant TK1 were a tetramer both in presence and absence of ATP and solely showed the kinetics of a high affinity form. The lack in ability to appear as the low affinity dimer may be due to different posttranslational mechanisms in human cells and *E.coli*.

INTRODUCTION

Human cytosolic thymidine kinase (TK1)(ATP: thymidine 5'phophotransferase E.C. 2.7.1.21) is a cell cycle regulated key enzyme in the salvage pathway of the nucleoside metabolism, catalyzing the first phosphorylation step in TTP synthesis. TK1 is an enzyme of high interest, primarily because of the specific cell cycle regulated expression, involving transcriptional, post-transcriptional (Coppock & Pardee, 1987)(Gudas et al, 1988)(Groudine & Casimir, 1984), translational and post-translational regulation mechanisms (Ito & Conrad, 1990)(Sherley & Kelly,1988)(Kaufman & Kelly,1991). In addition, TK phosphorylates a number of nucleoside analogs used in chemotherapeutic treatment of cancer and viral infections. In the phosphorylated form the analogs interfere with DNA replication and repair and abolish the cellular proliferation.

The amount of TK1 is extremely low in human tissues. Furthermore, the availability of human tissue is limited and it is very timeconsuming and expensive to purify TK1. In addition the enzyme is very unstable especially in the pure form. These factors encouraged us to construct an expressionvector in *E.coli*. In this report we describe the expression of TK1, the purification and characterization of the recombinant enzyme as compared to the native form. The specific activity and sub molecular weight were the same as found for the native TK1 purified from human lymphocytes.

Recently Munch-Petersen et al (1993) found a specific reversible effect of ATP on TK1, purified from human lymphocytes.

After preincubation or storage with ATP (+ATP form) the native TK1 appear as a high affinity form (K_m = 0.5 μM) and changes reversible to a low affinity form (K_m = 15 μM) by removal of ATP (-ATP form). Furthermore, the -ATP form of native TK1 is a dimer, and the +ATP form is a tetramer of 24 kDa. We investigated recombinant TK1 both incubated with ATP and without ATP. Recombinant TK1 has the molecular size as a tetramer and thymidine substrate kinetics identical to the +ATP form of native TK1. Surprisingly, the recombinant TK1 was not able to change to the dimer conformation and low affinity kinetics when ATP was removed from the enzyme. Recombinant TK1 is a tetramer and appear as the high affinity form both in absence and presence of ATP. This may indicate that native TK1 is subject to posttranslational modifications, such as phosphorylation, modifications that are not operable in *E.coli*.

MATERIALS AND METHODS

Materials.

E.coli strains: BL21(DE3)lysS, BL21(DE3)lysE, KY895 (Igarashi et al, 1967) and HB101.

Plasmids: pBR322, pET3a (Studier et al,1990) and pTK11 (Bradshaw & Deininger,1984).

^{3}H-thymidine (25 Ci/mmol), ^{3}H-3′-azidothymidine (^{3}H-AZT) (14 Ci/mmol), ^{3}H-2′-arabinosol-thymidine (^{3}H-AraT) (6 Ci/mmol) and [α-^{35}S] dATP(1000 Ci/mmol) were from Amersham ApS Denmark.

Enzymes: EcoRV, NdeI and BamHI were from New England Biolab,

ligase and Klenow polymerase from Promega and Taq polymerase from Perkin Elmer.

Oligonucleotides: pOliIII was kindly provided by Dr. Reinhold Hofbauer. pP4, pP5, pFB, pF2, pBaf2 and pBaf1 were from DNA Technology ApS, DK.

pOliIII: 5′ GCACTGGATGGGACCTTCCAGAGGAAG 3′ (sense)
pP4: 5′ ACACATATGAGCTGCATTAACCTGCCCACTGTG 3′ (sense)
pP5: 5′ CACGGATCCTCAGTTGGCAGGGCTGCATTGCAG 3′ (antisense)
pFB: 5′ ACACATGACCGGAACACCATGGAGGC 3′ (sense)
pF2: 5′ GTCATAGGCATCGACGAGGGGCAGTTT 3′ (sense)
pBaf2: 5′ GTCAGCTTCACCACGCTCTC 3′ (antisense)
pBaf1: 5′ CGGTCATGTGTGCAGAAGCT 3′ (antisense)

Columns: G-25 sepharose, 3′-dTMP-sepharose, Carboxymethyl sepharose and G-200 sephadex superfine were from Pharmacia.
Nucleotides were from Boeringer Manheim.
DNA sequencing kit was sequenase version 2.0 from United States Biochemical Corporation. DEAE filters were from Whatman.
Protein markers were from Sigma.
All other reagents were of the higest quality available.

Buffers.

Lysisbuffer: 50 mM Tris-HCl (pH 8), 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, 5 mM DTT, 0.5 mM PMSF, 5 mM Benzamidine, 10% glycerol, 50 mM NaF.

Buffer A: 20 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 5 mM NaF, 10% Glycerol, 5 mM Dithiothreitol (DTT), 0.5 mM CHAPS, 5 mM Benzamidine, 0.5 mM PMSF.

Buffer B: 10 mM Tris-HCl (pH 7), 5 mM $MgCl_2$, 5 mM NaF, 10% Glycerol, 5 mM DTT, 0.5 mM CHAPS, 5 mM Benzamidine, 0.5 mM PMSF.

Buffer C: Buffer B + 1 mM thymidine

Buffer D: 10 mM Tris-HCl (pH 7), 5 mM $MgCl_2$, 10 % glycerol, 5 mM DTT.

Buffer E: 100 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 10% glycerol, 5 mM DTT, 0.5 mM CHAPS, 100 mM KCl.

Buffer F: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM KCl, 5 mM DTT.

Construction of the TK1-pet3a vector.

The TK1-pet3a vector (figure 1) was constructed by standard methods (Sambrook et al,1989) by inserting the amino-acid coding region of the human TK1 into the polylinker of the pet3a vector (Studier et al, 1990) which is under control of a T7 RNA polymerase promotor. The aminoacid coding region was PCR amplified from the pTK11 (Bradshaw & Deininger, 1984) containing the complete human TK1 cDNA. The two primers in the PCR flank the aminoacid coding sequence and each has a restriction site in the 5′ end (NdeI and BamHI, respectively). After blunt-ending the PCR product with Klenow polymerase the PCR product was blunt-end ligated into the EcoRV site of pBR322. This cloning was nessesary since it was impossible to cut the amplified product directly by NdeI and BamHI. The pBR322 with the insert was cleaved by BamHI and NdeI and the insert was purified by agarose gel electrophoresis and band purification by standard methods (Sambrook et al, 1989). The insert and the pet3a vector were ligated and used to transform a host E.coli (HB101). The TK1-

pet3a vector was isolated and sequenced by the ddNTP chain terminating method (Sanger et al, 1977) to ensure that no mutations had occurred during the PCR and that the base sequence was identical the published sequence for human TK1 (Bradshaw & Deininger, 1984). The sequencing strategy is illustrated in figure 2.

Expression of TK1.

E.coli strain BL21(DE3)lysS containing T7 RNA polymerase under control of the IPTG inducible UV5 promotor was transformed by the TK1-pet3a vector. The expression was carried out in LB medium (10 g tryptone, 5 g yeast, 10 g NaCl/liter) at 25°C. When OD_{600} was 0,2, IPTG was added to a final concentration of 0,4 mM to induce the UV5 promotor. The cells were harvested by centrifugation (14,000 g, 3 min), resuspended in 1/4 vol of lysis buffer and sonicated 3 times 15 sec (40 Watt) on ice. Cell debris was removed by centrifugation 20,000 g at 4°C for 30 min.

Purification of TK1.

All steps in the purification procedure were performed essentially as described (Munch-Petersen et al,1991). The enzyme extract from one liter culture was filtered on a G-25 column (55 mm x 200 mm) with buffer A and the desalted enzyme fractions were chromatographed on a 3'-dTMP-sepharose column (10 mm x 40 mm)

pre-equilibrated with buffer A. After washing the affinity column with buffer A and B, TK1 was eluted with buffer C. Fractions of 3 ml were collected.

For enzyme kinetics studies, thymidine was removed from the enzyme by CM-sepharose chromatography (Munch-Petersen et al, 1993). The column (10 mm x 20 mm) was washed with buffer D, and TK1 eluted with buffer E. *E.coli* TK was removed together with thymidine in this step, since this enzyme does not bind to the negative carboxy groups (Okazaki & Kornberg,1964). The enzyme fractions from the CM column were divided in two and 2.5 mM ATP was added to one of these. The ATP containing enzyme fraction was referred to as the +ATP form, and the ATP free fraction as the -ATP form.

TK activity assay.

TK activity was determined as initial velocities using the DEAE-cellulose 81 paper square method as described (Munch-Petersen,1984)(Munch-Petersen et al,1991). The standard assay mixture contained: 50 mM Tris-HCl (pH 7.5), 10 mM DTT, 2.5 mM ATP, 2.5 mM $MgCl_2$, 3 mM NaF, 0.5 mM CHAPS, 3 mg/ml bovine serum albumin (BSA) and 10 μM ^{3}H-thymidine (2 Ci/mmol). After addition of enzyme extract to the assay mixture to a total volume of 50 μl, samples of 13 μl were applied on DEAE filters after 5, 10 and 15 min of reaction at 37°C. The filters were washed with 5 mM ammoniunformiate, eluted by 0.2 M KCl/0.1 M HCl and the radioactivity determined by scintillation counting as described (Munch-

Petersen et al,1991).

The thymidine and ATP data were analysed by Hofstee plots (v versus v/s). Thymidine kinetics by Hill plots (log $v/(V_{max}-v)$ versus log s). The TTP inhibition were determined as I_{50}, which is the inhibitor concentration at 50% inhibition.

Sephadex G-200 chromatography.

Sephadex G-200 superfine was swelled and packed according to standard procedures (20 mm x 600 mm) and the chromatography was performed with buffer F. The elution fraction number (V_e values) were determined for five marker proteins (beta-amylase (200 KD), alcohol dehydrogenase (150 KD), BSA (66 KD), carbonic anhydrase (29 KD), cytochrome C (12,4 KD)) and for the enzymes in absence and presence of ATP. When determinating the enzymes value in the presence of ATP, the enzymes were preincubated with 2.5 mM ATP, the column was pre-equilibrated and the elution performed with buffer F containing 2.5 mM ATP.

Other methods.

Protein concentrations were determined by coomassie brilliant blue as described (Bradford, 1976) with BSA as the standard protein.

The sub-unit molecular weight of TK1 was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4.5%

stacking gel and a 15% separation gel prepared by standard methods (Sambrook et al, 1989). The samples were denatured at 95°C for 3 min in 125 mM Tris-HCl, 10 mM DTT, 1% SDS, 0.01% Bromphenol Blue and 25% glycerol before loading on the gel. The protein bands were visualized by Coomassie brilliant Blue or silver staining and dried on a slab gel drier for 2 hours at 80°C.

The TK1 vector was sequenced by the Sequenase DNA sequencing kit using ^{35}S-ATP and the primers listed in MATERIALS. The sequencing polyacrylamide gels were prepared by standard methods (Sambrook et al, 1989).

RESULTS

An expression-vector for direct expression of TK1 was constructed as outlined in figure 1 and sequenced by the sequencing-strategy shown in figure 2. As the sequence was identical to the published sequence for TK1 (Bradshaw & Deininger,1984), we conclude that no mutations had occurred during the initial PCR.

When BL21(DE3)lysS is transformed by this vector and the expression of TK1 is induced by IPTG, the yield is around 1 mg per liter culture. Initially we attempted to transform an *E.coli* TK minus strain, KY895, but it was not possible to transform this strain by the vector. We also tried to transform a strain suitable for the expression of toxic genes, BL21(DE3)lysE. But induction of this transformant resulted in a yield 20 fold less

the yield obtained with BL21(DE3)lysS.

The recombinant TK1 was purified by affinity chromatography to more than 95% homogeneity as estimated from an SDS-PAGE (figure 3) and the sub-unit molecular mass was about 26 kDa as predicted from the cDNA sequence and slightly above that of the native TK1 - which is about 24 kDa. The specific activity of the recombinant TK1 was similar to the native TK1 about 10 μmol/min/mg.

In a recent investigation of the enzymatic properties of purified TK1 from human lymphocytes we uncovered a hitherto unrecognized property (Munch-Petersen et al, 1993). When ATP was removed from the pure enzyme, the kinetics with thymidine changed remarkably from a high affinity kinetics with a low K_m value of 0.5 - 0.7 μM to a low affinity kinetics with a high K_m of 15 μM. The low affinity kinetics was non-Michaelis-Menten, with biphasic curves in Hofstee plots (v versus v/s). The high affinity form was regained after incubation or storage in ATP of the low affinity form. A second removal of ATP restored the low affinity form. Thus, the ATP effect was reversible. Since the native TK1 eluted as a dimer in the absence and a tetramer in the presence of ATP, it was concluded that the low affinity kinetics was associated with the dimer and the high affinity kinetics to the tetramer. The biphasic kinetics was explained as the simultaneous presence of the high and low affinity enzyme forms in a ratio dependent on the thymidine and enzyme concentrations.

Previous investigations of the kinetics of mammalian TK1 have normally been performed on the ATP form, since ATP has routinely been used to stabilize the otherwise extremely unstable

enzyme (Sherley and Kelly, 1988; Ellims et al, 1982; Gan et al, 1983; Munch-Petersen, 1984; Munch-Petersen et al, 1991). For this reason, the properties of the -ATP form and the reversible effect of ATP have not been described before.

In the present work we wanted to investigate if the recombinant TK1 had the same reversible properties with ATP as the native TK1. In addition, we wanted to compare the properties of the native and recombinant TK1 regarding the ATP and TTP kinetics and the substrate specificity towards the thymidine analogs, AZT and ara-T. The data for the comparison of the properties of recombinant and native TK1 are shown in table 1.

The acitivities of the recombinant TK1 with the thymidine analogs, AZT and ara-T were identical to those obtained with the native TK1. With 5 μM of AZT the activity of the +ATP and -ATP form of recombinant TK1 was 46% and 38%, respectively, of the activity obtained with 5 μM thymidine. Ara-T was not phosphorylated by recombinant TK1. These data was in complete agreement with previous data for the native enzyme (Eriksson et al.1991; Munch-Petersen et al.1991).

When investigating the ATP substrate kinetics at 10 μM thymidine, both the +ATP and -ATP form of recombinant enzyme showed a positive cooperative kinetics with a K_m of about 30 μM. This is quite similar to the +ATP form of native TK1 where K_m is about 14 μM but very diverging to the -ATP form where K_m is about 140 μM.

Examining the inhibitory effect of TTP at 10 μM thymidine showed surprisingly that the recombinant TK1 is around 10 times more sensitive to TTP than the native TK1. This was investigated

by varying the TTP concentration. As the inhibition was cooperative for both recombinant and native TK1, the sensitivity to TTP was expressed by I_{50}, where I_{50} is the TTP concentration necessary for 50% inhibition. Recombinant TK1 has a I_{50} value at about 1,5 μM TTP whereas the native TK1 has a I_{50} value at about 10 μM. In these experiments both the +ATP and -ATP forms of recombinant and native TK1 showed identical results.

The thymidine substrate kinetics of the +ATP form of recombinant TK1 was Michaelis-Menten type with a straight line in the Hofstee plot of v versus v/s (figure 4), and a K_m value of 0.4 μM. This pattern was identical to that found with the native TK1. However, as indicated from figure 4, the -ATP form of recombinant TK1 showed exactly the same kinetics as the +ATP form, with Michaelis-Menten kinetics and K_m=0.4 μM. The Hill coefficents for the +ATP and -ATP form of recombinant TK1 were 1.5 and 1.4 respectively, and thus slightly higher than the Hill coefficient of 1.2 found for the +ATP form of native TK1.

This indicated that the recombinant TK1 solely occurred as the high affinity form both in the absence and in the presence of ATP.

The different thymidine substrate kinetics of the -ATP and +ATP forms of the native TK1 were related to the different molecular mass of the two forms. Thus the molecular mass of the native TK1 as determined by elution on Superose 200 was a dimer (50 kDa) in the absence of ATP and a tetramer (100 kDa) in the presence of ATP. This indicates that ATP induces a tetramerization of the 24 kDa TK1 from human lymphocytes (Munch-Petersen et al,1993).

In the present work we have determined the apparant molecular mass of the native and recombinant TK1 by elution on G-200 sephadex. The results are shown in figure 5. In absence and presence of ATP the apparent molecular mass of the native TK1 were 75 kDa and 175 kDa respectively. The recombinant TK1 however, exclusively appeared with the high molecular mass. The apparent molecular mass in absence and presence of ATP were identical and about 155 kDa. This indicates that the ability to dissociate from a tetramer structure to a dimer structure was lost in the recombinant TK1.

DISCUSSION.

As outlined in RESULTS we tried to express the TK1-pET3a vector in a *E.coli* TK minus strain (KY895) to obtain an expression system without any interfering bacterial TK, but we were not able to transform this strain. As there were no problems with transforming the strain with other vectors, we assume that the TK1-pET3a vector were toxic for the strain. The next attempt to express the vector was in a strain permissive for toxic genes (BL21(DE3)lysE). The lysE plasmid has a high transcription of lysozyme inhibiting T7 RNA polymerase. This gave an expression of 50 μg/liter culture. Finally we transformed BL21(DE3)lysS containing the lysS plasmid which have a low expression of lysozyme with the TK1-pET3a vector and achieved a yield of 1 mg/liter culture. These results indicate that only a moderate direct expression of human TK1 is tolerated by *E.coli* otherwise

it is toxic to the cell.

The recombinant TK1 was very unstable during purification. As the yield was improved by addition of proteinase inhibitors, we assume that the increased instability as compared to the native TK1 is owned to proteinases from *E.coli*. The binding properties of recombinant TK1 to TMP sepharose and CM sepharose were similar to the native TK1 as recombinant TK1 was purified with exactly the same procedures as used for the native TK1 and with a yield of 25 - 50%.

By examining both the +ATP and -ATP form of recombinant TK1 we found identical specific activity and substrate specificity with two nucleoside analogs compared to the results with native TK1. There were no differences between the +ATP and -ATP form. This was also the case when the ATP and TTP kinetics were investigated. However, recombinant TK1 was 10 fold more sensitive towards TTP as compared to native TK1.

Regarding the thymidine substrate kinetics, the +ATP form of the recombinant TK1 had similar properties as the native TK1. The K_m values were low, 0.5 μM, and the kinetics was Michaelis-Menten. Unexpectedly, the kinetic properties of the -ATP form of the recombinant TK1 were identical to those of the +ATP form, and the biphasic kinetics with the high K_m value was not observed, in contrast to what was obtained with the -ATP form of the native TK1 (Munch-Petersen et al, 1993) (figure 4).

The similarity of the +ATP and -ATP form of the recombinant TK1 was also reflected in the gel-filtration studies. Here, like the native TK1, the recombinant TK1 eluted as a tetramer in the presence of ATP. But when eluted in the absence of ATP, the

recombinant TK1 maintained the tetramer form, whereas native TK1 dissociated to a dimer (figure 5).

As mentioned in results, we achieved higher apparant molecular weights of the native TK1 by elution on G-200 sephadex than in the previous elution experiments with the same enzyme preparation on superose 12 (Munch-Petersen et al.1991; Munch-Petersen et al.1993). This may be due to a non-globular shape of the proteins resulting in different filtration on the two column matrices. Similarly, the slightly lower molecular mass of recombinant TK1 (155 kDa) as compared to the native TK1 (175 kDa) may be explained by an altered conformation of the tetramer. As the recombinant TK1 can not dissociate to a dimer, a tighter association of the four sub-units is probable.

In the previous investigation of the native TK1, the biphasic kinetics obtained with the ATP free enzyme form was explained as the result of the simultaneous presence of two enzyme forms, a dimer with low affinity and a tetramer with high affinity (Munch-Petersen et al.1993). The observation in the present study that the ATP free form of recombinant TK1 is a tetramer with a Michaelis-Menten type of thymidine kinetics, supports the previous connection of the kinetic pattern with the molecular mass.

Recently Chang et al (1994) has showed that TK1 from HeLa cells is differentially phosphorylated during the cell cycle and that this phosphorylation affects the TK1 activity. They document that mitotic arrested HeLa cells had a hyperphosphorylated low affinity form of TK1 (K_m = 15.5 μM) and dividing HeLa cells had a hypophosphorylated high affinity form of TK1 (K_m = 1.5 μM).

They show that in HeLa cells a Staurosporine sensitive mitotic kinase was responsible for the mitotic phosphorylation.

The K_m values for the +ATP and -ATP form of the pure native TK1 were 0.5 - 0.7 and 15 μM, respectively (Munch-Petersen et al,1993). Therefore it is very likely that these two forms are similar to the differentially phosphorylated forms found by Chang et al (1994) in G_1/S phase cells and in mitotic arrested cells, respectively. As the K_m of the +ATP and -ATP form of recombinant TK1 are identical, this further implies that the recombinant TK1 have lost the ability to shift form.

One question remains to be answered. Chang et al (1994) suggested that a kinase catalyzed phosphorylation is needed for the shift from the high affinity hypophosphorylated form to the low affinity hyperphosphorylated form. On the other hand Munch-Petersen et al (1993) showed that purified TK1 can shift reversibly between the high and low affinity forms by adding or removing ATP at 4°C. It is therefore unlikely that a protein kinase is involved, unless it can operate a 4°C and is a function of the TK1 protein. However, a reversible autophosphorylation mechanism would also require a phosphatase activity and this is impossible within a 24 kDa protein.

To explain the present results we propose the following model: Newly synthesized TK1 protein in mammalian cells is modified by post-translational mechanisms in a way that prevents immidiate association to a tetramer. After binding of ATP a conformational change is induced that enable a tetramerization accompanied with a shift to higher affinity. The conformational change is promoted by increased thymidine and enzyme

concentration. Removal of ATP from the enzyme results in a change to a dimer form with low thymidine affinity. *E.coli* can not perform the post-translational modification and therefore the newly synthesized protein sub-units associates into a tight tetramer immidiately after translation. This tetramer can not dissociate into dimers at physiological conditions.

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Figure legends.

Figure 1:

The construction of the TK1-pET3a vector. The aminoacid-coding region of human TK1 cDNA is PCR amplified by primers including restriction sites in the 5'end and inserted into the pET3a expression-vector using the restriction sites NdeI and BamHI.

Figure 2:

The sequencing-strategy to verify that the aminoacid-coding region in TK1-pET3a was identical to the published sequence of human TK1 (Bradshaw & Deininger, 1984). The primers used are listed in MATERIALS. The small arrows indicate the primers and the long arrows show the bases sequenced by the primer.

Figure 3:

SDS-PAGE of purified recombinant TK1. Lane 1: Proteinmarker (97 kDa, 66 kDa, 45 kDa, 31 kDa, 21 kDa, 14 kDa), lane 3; bacteria lysate, lane 4; wash from affinity chromatography, lane 6; purified recombinant TK1 from affinity chromatography.

Figure 4:

The effect of ATP incubation on the thymidine substrate kinetics of purified recombinant TK1 and TK1 purified from lymphocytes. The open circles are the +ATP form of the enzyme and the filled circles are the -ATP form of the enzyme. (a) Hofstee plot of lymphocyte TK1 and (b) Hill plots of the same data. (c) Hofstee plots of recombinant TK1 and (d) Hill plots.

Figure 5:

The molecular mass determined by G-200 superfine Sephadex. The column was calibrated by protein standards (12-20 KD). The +ATP forms are open circles and the -ATP form are filled cicles. The x-asis show fraction numbers collected from the column. (a) Elution of the +ATP and -ATP form of lymphocyte TK1. (b) Elution of the +ATP and -ATP form og recombinant TK1. (c) Protein standards are marked as filled squares with respect to molecular mass and elution fraction number. The molecular mass of the TK1 enzymes are determined from elution fraction number.

Table 1. Comparison of the properties of lymphocyte TK1 and recombinant TK1.

		Lymphocyte TK1	Recombinant TK1
Sub-unit molecular mass (kDa)		24	26
Molecular mass (kDa)	+ATP	175	155
	-ATP	75	155
Thymidine Spec.act. nmol/min/mg		9,500	10,000
[a]AZT as substrate	+ATP	40%	46%
	-ATP		38%
[a]ara-T as substrate	+ATP	0%	0%
	-ATP		0%
[b]ATP K_m (μM)	+ATP	14*	31
	-ATP	140*	27
[b]Thymidine K_m (μM)	+ATP	0.5-0.7	0.4
	-ATP	12-15	0.4
[c]thy. Hill coefficient	+ATP	1.2	1.5
	-ATP	0.7	1.4
[d]TTP inhib. I_{50} (μM)	+ATP	9-10*	1.5
	-ATP	9-10*	1.5

[a]The substrate analogs AZT and ara-T were determined in % of the activity with thymidine as substrate.

[b]K_m values for thymidine and ATP were calculated from Hofstee plots (v versus v/s).

[c]The thymidine Hill coefficient were determined from Hill plots (log v/V_{max}-v versus log s).

[d]I_{50} for TTP is the inhibitor concentration at 50% inhibition.

* Munch-Petersen et al (1995b)

Figure 1

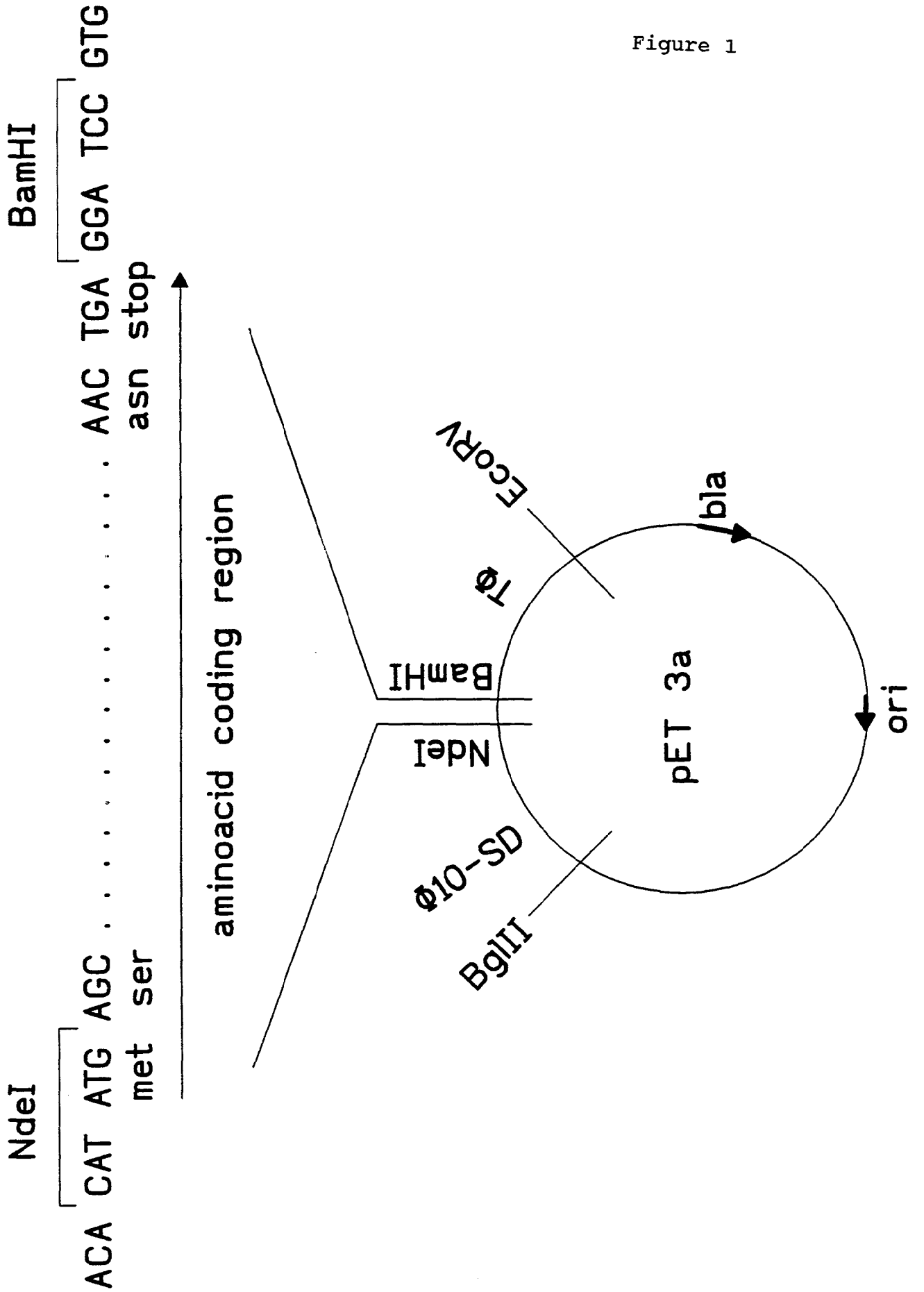

BamHI
GGA TCC GTG
AAC TGA
asn stop
NdeI
ACA CAT ATG AGC
met ser
aminoacid coding region
EcoRV
Tø
BamHI
NdeI
Ø10-SD
BglII
bla
pET 3a
ori

Figure 2

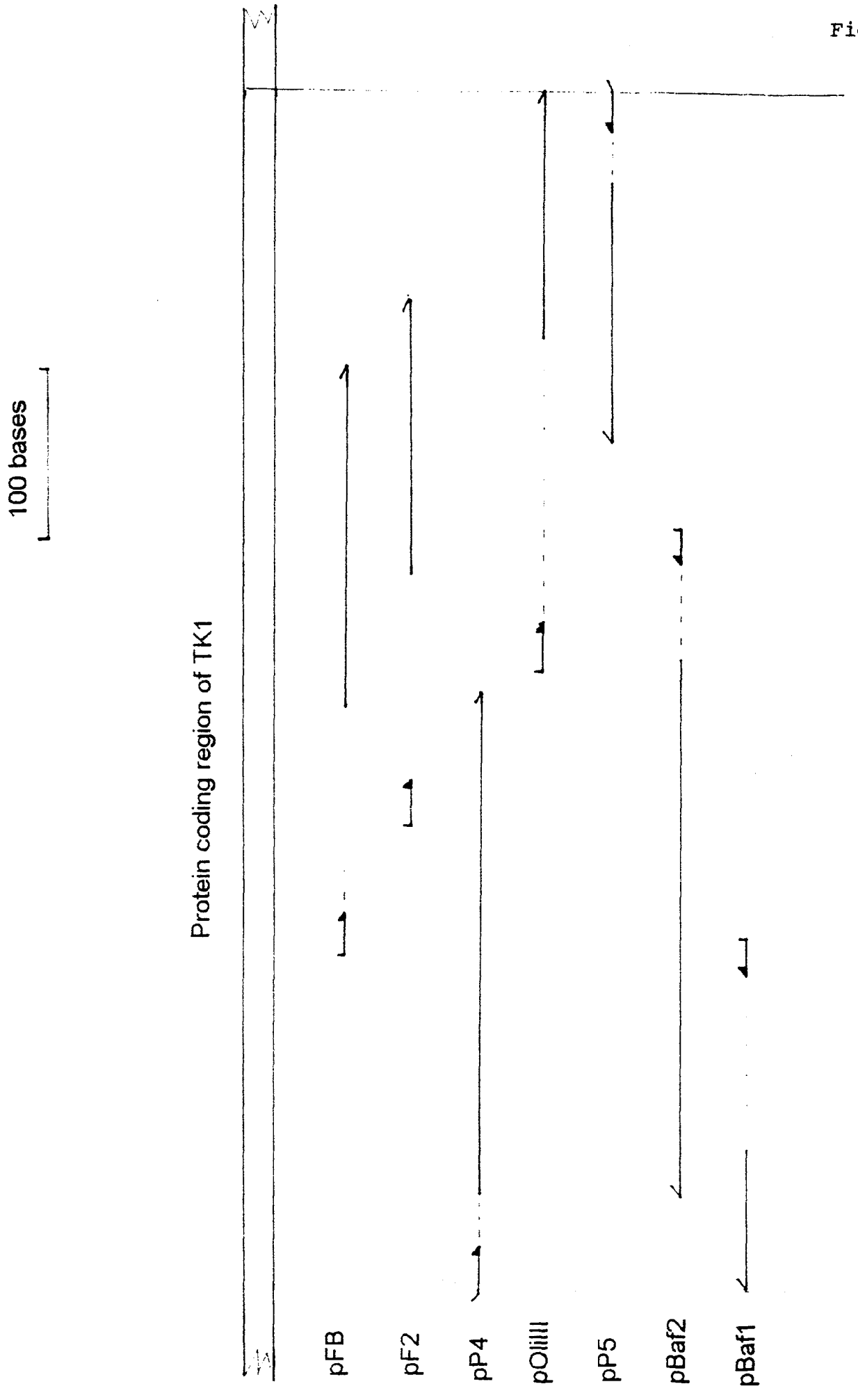

100 bases
Protein coding region of TK1
pFB
pF2
pP4
pOIIIII
pP5
pBaf2
pBaf1

Figure 3

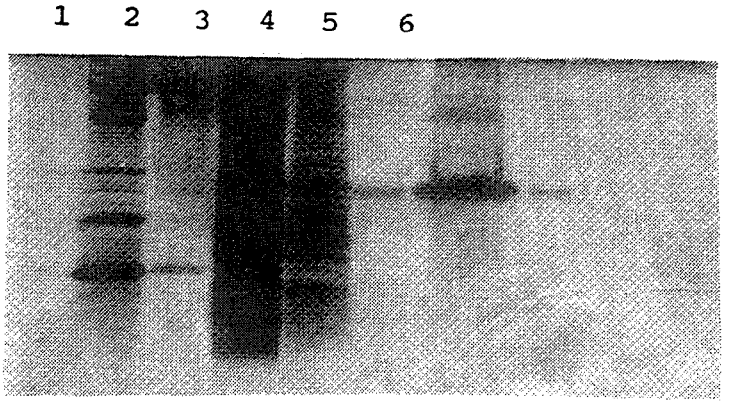


Figure 4

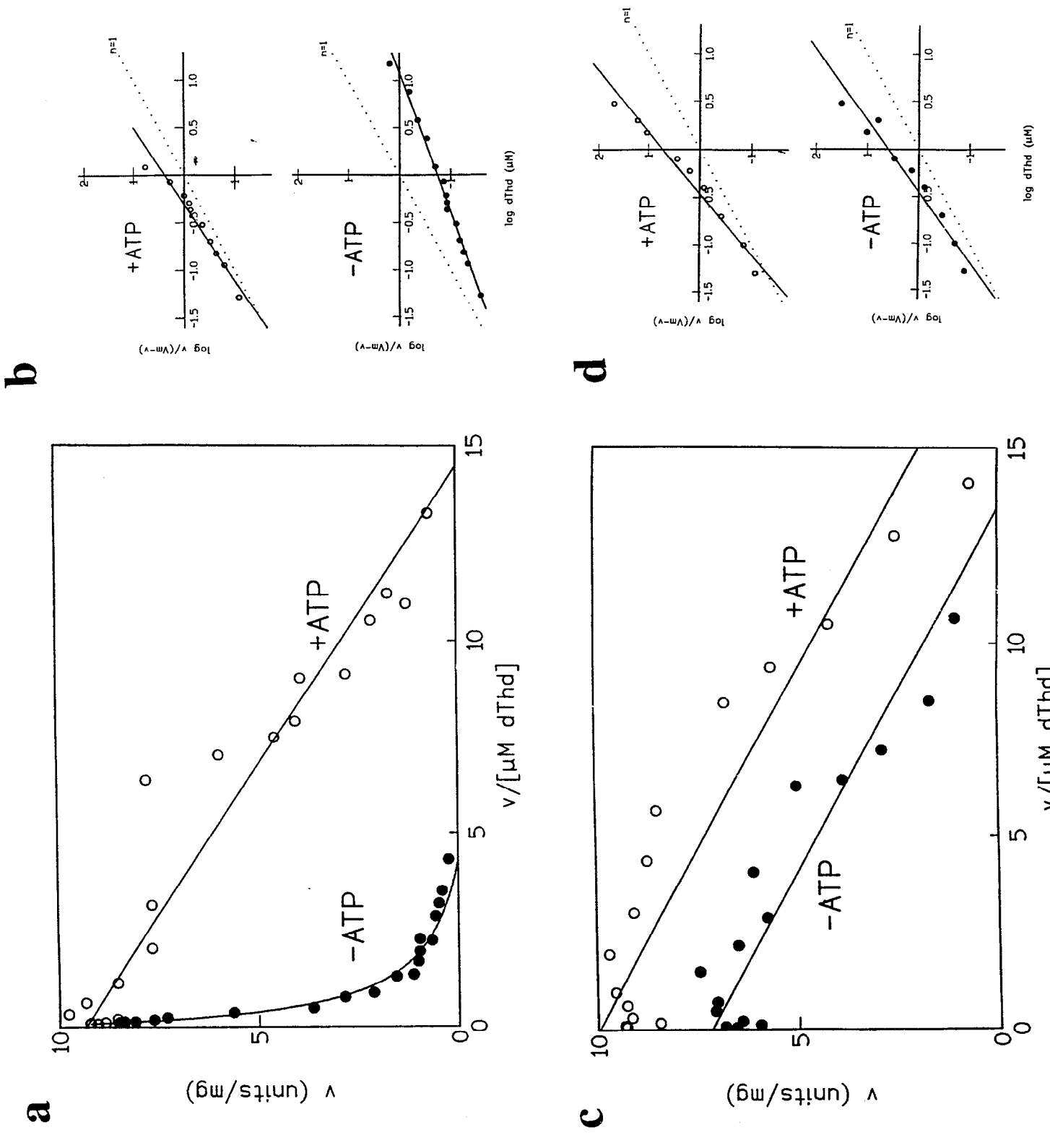
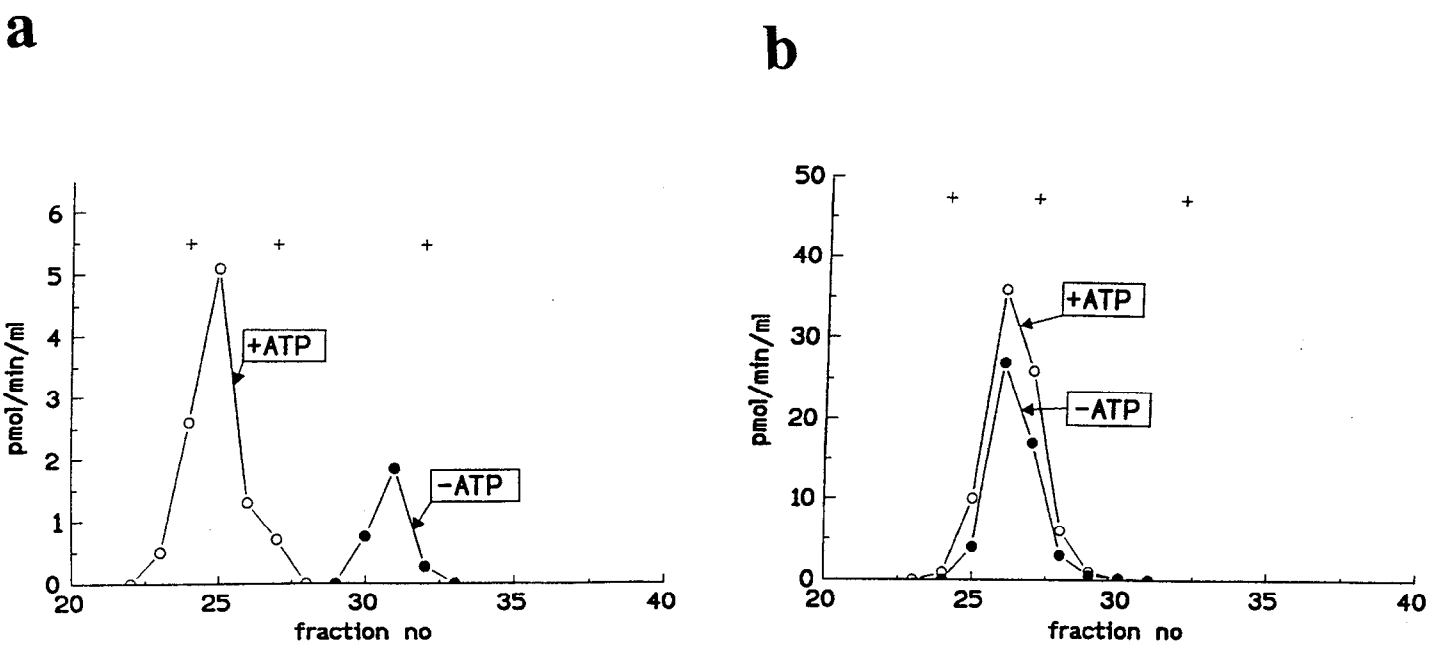
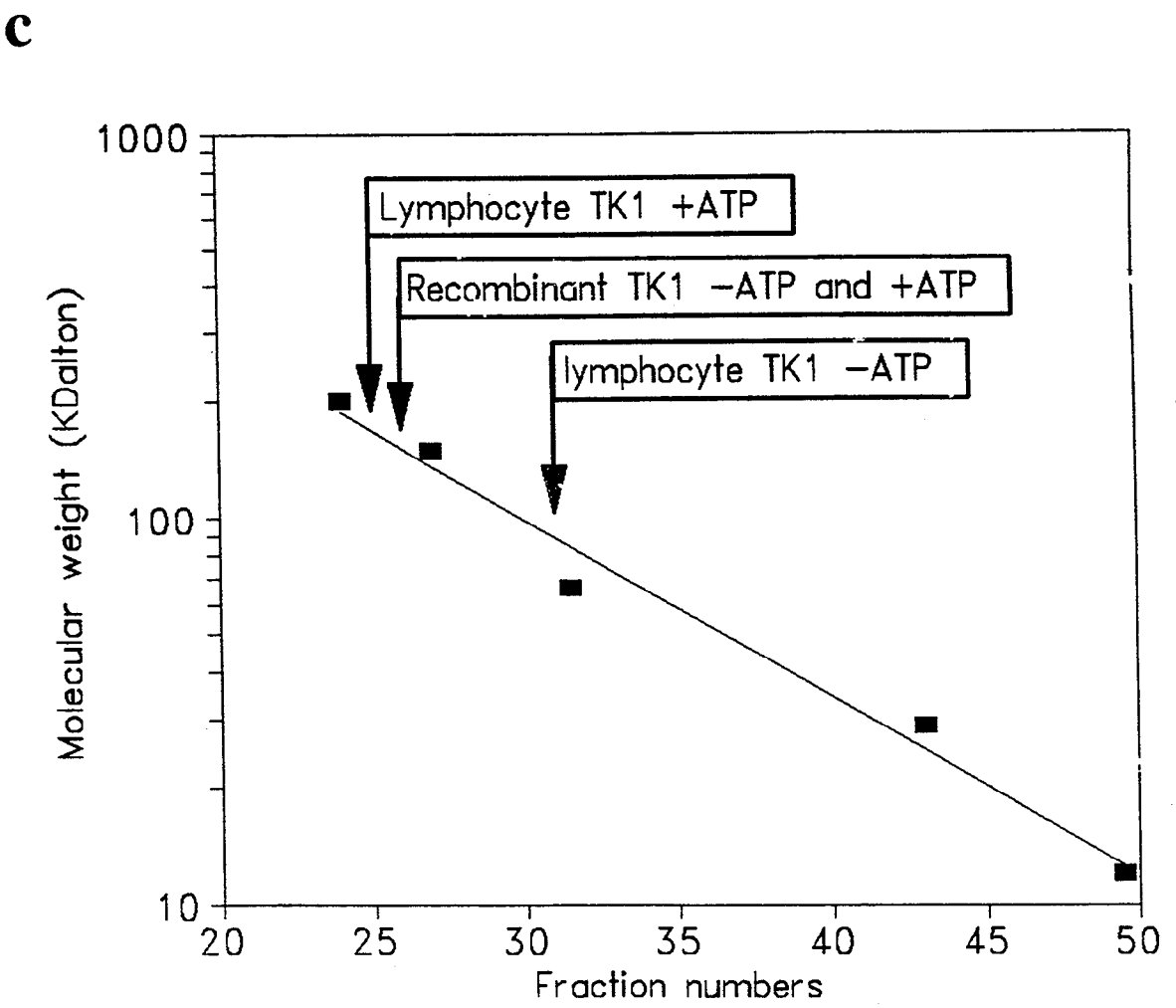


Figure 5


a
b
pmol/min/ml
+ATP
-ATP
fraction no
0
1
2
3
4
5
6
10
20
30
40
20
25
30
35
40


c
Lymphocyte TK1 +ATP
Recombinant TK1 −ATP and +ATP
lymphocyte TK1 −ATP
Molecular weight (KDalton)
1000
100
10
20
25
30
35
40
45
50
Fraction numbers