Valine, Not Methionine, Is Amino Acid 106 in Human Cytosolic Thymidine Kinase (TK1)

IMPACT ON OLIGOMERIZATION, STABILITY, AND KINETIC PROPERTIES

Cytosolic thymidine kinase (TK1) cDNA from human lymphocytes was cloned, expressed in *Escherichia coli*, purified, and characterized with respect to the ATP effect on thymidine affinity and oligomerization. Sequence analysis of this lymphocyte TK1 cDNA and 21 other cDNAs or genomic TK1 DNAs from healthy cells or leukemic or transformed cell lines revealed a valine at amino acid position 106. The TK1 sequence in NCBI GenBank has methionine at this position. The recombinant lymphocyte TK1<sup>Val-106</sup> (rLy-TK1<sup>Val-106</sup>) has the same enzymatic and oligomerization properties as endogenous human lymphocyte TK1 (Ly-TK1); ATP exposure induces an enzyme concentration-dependent reversible transition from a dimer to a tetramer with 20–30-fold higher thymidine affinity ($K_m$ about 15 and 0.5 μM, respectively). Substitution of Val-106 with methionine to give rLy-TK1<sup>Met-106</sup> results in a permanent tetramer with the high thymidine affinity ($K_m$ about 0.5 μM), even without ATP exposure. Furthermore, rLy-TK1<sup>Met-106</sup> is considerably less stable than rLy-TK1<sup>Val-106</sup> ($t_{1/2}$ at 15 °C is 41 and 392 min, respectively). Because valine with high probability is the naturally occurring amino acid at position 106 in human TK1 and because this position has high impact on the enzyme properties, the Val-106 form should be used in future investigations of recombinant human TK1.

The human cytosolic thymidine kinase, TK1 (EC 2.7.1.21), is a key enzyme in the salvage synthesis of TMP from thymidine. Intracellular TMP is quickly phosphorylated to TDP and TTP. In a key enzyme in the salvage synthesis of TMP from thymidine. Intracellular TMP is quickly phosphorylated to TDP and TTP. Because TTP is an allosteric effector of ribonucleotide reductase, imbalances in the TTP pool disturb the supply of both purines and pyrimidines for DNA synthesis and repair. In turn, imbalanced deoxynucleoside triphosphate (dNTP) pools increase the mutation rate and probability of carcinogenesis (1–3).

TK1 is a cell cycle-regulated enzyme. Its activity fluctuates with DNA synthesis, being high in dividing and malignant cells and low in quiescent cells (4, 5). The expression of TK1 is meticulously controlled on the transcriptional and post-transcriptional level (6, 7). At the enzymatic level, ATP, besides being a cosubstrate, has been shown to be a regulator of the catalytic activity of TK1 (8). Thus, exposure to ATP induces a reversible enzyme concentration-dependent transition from a low thymidine affinity dimer of about 50 kDa ($K_m = 15 \mu M$) to a high affinity tetramer ($K_m = 0.7 \mu M$). To further investigate the effect of ATP, we constructed a pET3a-TK1 plasmid (9) containing the amino acid coding region of TK1 cDNA from the pTK11 plasmid of Bradshaw and Deininger (10), who had used SV40 transformed human fibroblasts as the mRNA source. We expressed the resulting recombinant TK1 (rTFi-TK1) in *Escherichia coli*, and purified and characterized the enzyme. To our surprise we found that the enzymatic properties of rTFi-TK1 differed markedly from those of the endogenous Ly-TK1 with respect to the regulatory effect of ATP (9). Irrespective of pre-exposure to ATP, the recombinant rTFi-TK1 was a permanent tetramer of about 100 kDa with high affinity to thymidine with a $K_m$ value about 0.4 μM (9). At that time, we assumed that the amino acid sequences of rTFi-TK1 and Ly-TK1 were identical and explained the divergent properties of rTFi-TK1 by the absence of post-translational modification of TK1 when expressed in *E. coli* (9). Because the pET3a-TK1 expression system was not satisfactory in terms of amount of TK1 protein produced, we constructed another expression plasmid, pGEX-2T-LyTK1. Here, the amino acid coding region of TK1 from normal human lymphocytes was cloned into the pGEX-2T vector, and the recombinant TK1 was expressed as an isopropyl-β-D-galactopyranoside-inducible glutathione S-transferase fusion protein. In contrast to the findings with rTFi-TK1, our preliminary kinetic experiments showed that the recombinant lymphocyte TK1 (rLy-TK1)<sup>1</sup> behaved essentially as the endogenous lymphocyte enzyme, Ly-TK1, regarding kinetic and oligomerization properties. Therefore, absence of post-translational modification of rTFi-TK1 in *E. coli* cannot explain the divergent properties of recombinant TK1 expressed from the pET3a-TK1 plasmid. By comparison of the sequence of lymphocyte TK1 cDNA with that of TK1 cDNA in our pET3a-TK1 plasmid, as well as in the clone of Bradshaw and Deininger (10), we discovered that lymphocyte TK1 cDNA had a

<sup>1</sup>The abbreviations used are: Ly-TK1, TK1 purified from human lymphocytes; rLy-TK1<sup>Val-106</sup>, recombinant TK1 expressed from cDNA derived from human lymphocytes; rLy-TK1<sup>Met-106</sup>, rLy-TK1<sup>Val-106</sup> with valine<sup>106</sup> mutated to a methionine; rTFi-TK1, recombinant TK1 expressed from cDNA derived from SV40 transformed human fibroblasts (10); TK1-ATP, TK1 incubated and stored with 2.5 mM ATP/MgCl₂; CHAPS, 3-[3-cholamidopropyl]-dimethylammonionio-1-propusulfonate; PCR, polymerase chain reaction; DTT, dithiothreitol; BSA, bovine serum albumin.
GTG codon for valine at amino acid position 106, whereas TK1 cDNA cloned by Bradshaw and Deininger (10) had an ATG codon for methionine at this position. Amino acid 106 is located in a highly conserved area and is valine in TK1 from chicken, Chinese hamster, mouse, and vaccinia virus. The present investigation was started to clarify the naturally occurring amino acid at site 106 in human TK1 and to examine the significance of valine or methionine at this site for the oligomerization and enzymatic properties.

**EXPERIMENTAL PROCEDURES**

**Recombinant TK1 Enzymes**

**Bacterial Strains and Growth Conditions**—KY895, a thymidine kinase-deficient strain of *E. coli* (11), and *E. coli* strain BL21 (Amersham Pharmacia Biotech) were used for propagation of recombinant plasmids. BL21 was used for expression of recombinant TK1. *E. coli* XLI-blue supercompetent cells (Stratagene) were used for transformation of DNA after site-directed mutagenesis. Unless otherwise indicated, the strains were grown in LB medium at 37 °C and with 50 μg ml−1 ampicillin for plasmid-containing strains.

**Construction of pGEX-2T-LyTK1**—Total cytoplasmic RNA was isolated from normal human lymphocytes (donor 1) according to the procedure of Chomczynski and Sacchi (12) and reverse transcribed by avian myeloblastosis virus-reverse transcriptase (Promega). The resulting cDNA was PCR amplified under the following conditions: 40 cycles, 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min, and with a sense primer 5′-TGGATCCATGAGCGTTGTA-3′ and an antisense primer 5′-CCGCGTACGATCTGACTG-3′ (the position numbers are according to Bradshaw and Deininger in Ref. 10). Bases shown in bold type were altered in comparison to the original sequence to introduce BamHI and SpH1 restriction sites into the cDNA. The PCR fragment was cloned into BamHI-Sph1 restriction sites of pGEM-3zf(+) vector. The KpnI-HindIII fragment from pGEM-3zf-TK1 plasmid, containing the entire TK1 coding sequence, was subcloned into KpnI-HindIII sites of pBluescript II KS(−). This plasmid was cleaved with BamHI and EcoRI, and the TK1 coding fragment was inserted into BamHI and EcoRI cleaved pGEX-2T vector (Amersham Pharmacia Biotech). The resulting plasmid was called pGEX-2T-LyTK1. Because sequencing showed a GTG codon for valine instead of an ATG codon for methionine at TK1 amino acid position 106. After propagation in *E. coli* KY895, pGEX-2T-LyTK1 (the position 106 was purified by the Wizard kit (Promega) and stored at 4 °C in 10 mM Tris, 0.5 mM EDTA, pH 8.0. For insertion of the restriction sites and for introduction of thrombin cleavage site, it was necessary to modify the N-terminal to start with GSSC instead of MCS and the C-terminal to end with ILQCMSPA.

**Construction of pGEX-2T-LyTK1 Val-106**—The KpnI-HindIII fragment of pGEM-3zf-TK1 plasmid, containing the entire TK1 coding sequence, was subcloned into KpnI-HindIII sites of pBluescript II KS(−). This plasmid was cleaved with BamHI and EcoRI, and the TK1 coding fragment was inserted into BamHI and EcoRI cleaved pGEX-2T vector (Amersham Pharmacia Biotech). The resulting plasmid was called pGEX-2T-LyTK1Val-106, because sequencing showed a GTG codon for valine instead of an ATG codon for methionine at TK1 amino acid position 106. After propagation in *E. coli* KY895, pGEX-2T-LyTK1Val-106 was purified by the Wizard kit (Promega) and stored at 4 °C in 10 mM Tris, 0.5 mM EDTA, pH 8.0. For insertion of the restriction sites and for introduction of thrombin cleavage site, it was necessary to modify the N-terminal to start with GSSC instead of MCS and the C-terminal to end with ILQCMSPA. Neither of the 10 first N-terminal amino acids nor the C-terminal amino acids of thymidine kinases are evolutionarily conserved (13), and therefore these amino acid changes were regarded to be of no importance. This expectation was confirmed by our experimental results.

**Construction of pGEX-2T-LyTK1 Met-106**—The codon GTG at TK1 amino acid position 106 was replaced by ATG using the QuickChange™ site-directed mutagenesis kit from Stratagene according to the protocol supplied. The mutagenic primers were: sense primer 5′-5′TTTCTTGCTACATGGAAATCTCTGGAGGC-3′ and antisense primer 5′-5′GGCTGCAGATCTGCTGACTG-3′. The ATG codon replacing GTG is underlined, and the altered bases are shown in bold type (the other base change G to A in the sense primer and C to T in the antisense primer was introduced to get a control EcoRI restriction site introduced in the cDNA without changing the amino acid; the numbering is according to Bradshaw and Deininger in Ref. 10). The potentially correct plasmids were transformed into *E. coli* BL21 and sequenced.

**Sequencing of pGEX-2T-LyTK1 Plasmids**—After propagation in *E. coli* BL21, plasmid DNA was isolated by the Wizard kit (Promega). The cDNA inserts in pGEX-2T for rLy-TK1 Val-106 and rLy-TK1 Met-106 were sequenced on both strands using the Sequenase version 2.0 DNA sequencing kit (Amersham Pharmacia Biotech).

**Expression and Purification of rLy-TK1 Enzymes**—Overnight bacterial cultures were diluted to an A590 = 0.6 in LB medium with 50 μg ml−1 ampicillin, and expression of the glutathione S-transferase-TK1 fusion protein was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 6 h at 25 °C. The bacterial pellet was resuspended in 1/10 of the original culture volume in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 50 mM e-aminoacproic acid) and homogenized by the French Press. After centrifugation and filtration as described (14), the bacterial lysate was applied to a Glutathione Sepharose 4B column (Amersham Pharmacia Biotech) pre-equilibrated with buffer B (Buffer A without EDTA and with Tris-HCl replaced by phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3). After washing with 20 column volumes of buffer B, the column was equilibrated with phosphate-buffered saline with 0.1% Triton X-100, and TK1 was cleaved from the bound glutathione S-transferase by recirculation for 2 h at room temperature with one column volume of phosphate-buffered saline with 0.1% Triton X-100 and 50 units/ml thrombin (Amersham Pharmacia Biotech). The eluate containing TK1 was collected on ice. For storage at −80 °C, glycerol, Triton X-100, DTT, and MgCl2 were added to 10%, 1%, 5 mM, and 5 mM, respectively. For kinetic and stability experiments, the thrombin cleavage fractions were pooled, desalted by Sephadex G-25 (Amersham Pharmacia Biotech) in buffer C (10 mM potassium-phosphate buffer, pH 6.0, 10% glycerol, 2 mM DTT, 0.5 mM CHAPS, and 5 mM MgCl2), and further purified by CM-Sepharose CL6B (Amersham Pharmacia Biotech) equilibrated with buffer C. After washing with 20 column volumes of buffer C, the enzyme was eluted with buffer D (50 mM potassium phosphate buffer, pH 8.0, 10% glycerol, 2 mM DTT, 0.5 mM CHAPS, and 5 mM MgCl2). Protein concentration was determined according to Bradford (15) using BSA as standard. The yield of recombinant protein in the cleavage fractions obtained from 1 liter of bacterial culture was 4–8 mg.

**Storage and ATP Incubation of the Purified Enzymes**—The enzymes were diluted in buffer E or F to 5 μg/ml without or with 2.5 mM ATP/MgCl2 and incubated at 4 °C for 2 h before storage at −80 °C. The cleavage fractions were diluted in buffer E (50 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM CHAPS, 5 mM MgCl2, and 0.1 mM KCl) and the CM fractions were diluted in buffer F (50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM CHAPS, and 3 mg/ml BSA). The enzymes stored without and with ATP are referred to as TK1−ATP and TK1+ATP, respectively.

**Subunit Molecular Size and Protein Purity**—Discontinuous SDS-polyacrylamide gel electrophoresis was performed in Tris-HCl 4.5% stacking gel, pH 6.8, and 15% separation gel, pH 8.8. As shown in Fig. 1, CM fractions of both rLy-TK1 Val-106 and rLy-TK1 Met-106 gave single bands corresponding to 25 kDa, the same size as the subunit size of Ly-TK1 (8).

**Native Molecular Size**—The apparent molecular size of nondenatured recombinant enzymes was determined by gel filtration on a Superose 12 column (10 × 300 mm) connected to a Gradient automatic sampler (Amersham Pharmacia Biotech) as described earlier (8). The column was equilibrated and eluted with 50 mM imidazole-HCl buffer, pH 7.5, containing 5 mM MgCl2, 5 mM DTT, 2 mM CHAPS, and 0.1 mM

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**FIG. 1. SDS gel electrophoresis of fractions from CM-Sepharose chromatography. Lanes 1, marker proteins; from bottom to top: a-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase B (94 kDa). Lanes 2, rLy-TK1 Val-106 (A) and rLy-TK1 Met-106 (B).**
KCl. The fractions were assayed for thymidine kinase activity at standard assay conditions and 10 μM thymidine.

**Thymidine Kinase Assay—**The TK1 activity was assayed by the DE-81 filter paper method as described previously (8). Standard assay conditions were: 50 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 10 mM DTT, 0.5 mM CHAPS, 3 mg/ml BSA, 2.5 μM TP, and the indicated concentrations of [methyl-³²]H thymidine (925 GBq/nmol; Amersham Pharmacia Biotech) in a total volume of 50 μl. The enzyme was diluted immediately before start of the reaction with ice-cold buffer F but without glycerol. For dilution of the TK1+ATP form, 2.5 mM ATP/MgCl₂ were included. The enzyme concentration in the kinetic experiments was 5 ng/ml in the assay mixture.

**Enzyme Kinetics—**The kinetic parameters and reaction mechanism were determined by fitting the data to the following equation.

\[ v = \frac{V \cdot s}{K_m + s} \]  (Eq. 1)

using the nonlinear regression software from Graphpad Prism®. \( V \) is the maximal velocity, and \( s \) is the Hill constant.

**Stability—**The stored enzymes (5 μg/ml) were diluted 200-fold into 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM CHAPS and 5 mM DTT and incubated at 15 °C. At various times, 10-μl samples were assayed for thymidine kinase activity at standard assay conditions and 100 μM thymidine.

**Genomic and cDNA Sequencing**

**Cell Lines—**The leukemic cell lines: Raji, CEM-C, Molt-3, Reh, K-562, KG-1, RS4;11, AML-193; the colon cancer cell lines: DLD-1, Hct-116, LoVo, SW408; and the HeLa cells were purchased through American Type Culture Collection. The human fibroblast cell line WTs3 and the SV40 transformed human fibroblast cell line W126 VA4 were purchased from European Collection of Cell Cultures.

**Lymphocytes—**Lymphocytes were isolated from peripheral blood from healthy volunteers by the Isopaque-Ficoll technique. For isolation of mRNA, the lymphocytes were cultured for 72 h with PHA as described (16). The harvested cells were stored at −80 °C.

**Cell Culture Conditions—**The cell lines were cultured in RPMI 1640 (Life Technologies, Inc.) with L-glutamine, 15% fetal bovine serum (Life Technologies, Inc.), and 1% penicillin-streptomycin in atmospheric air and 5% CO₂ at 37 °C. Subculturing was performed routinely at a cell concentration of about 10⁶ cells/ml.

**Isolation and PCR Amplification of a Genomic TK1 DNA Fragment Containing the Codon for Amino Acid 106—**Genomic DNA was isolated by the same extraction standard method after SDS lysis and proteinase K treatment (17). DNA concentration was measured at 280 nm. A 166-base pair genomic DNA fragment containing the codon for amino acid 106 was amplified with the sense primer 5'-11824AGCGTCT-TCGCTGGGGCTCC11843-3' and the antisense primer 5'-11699TGTC-CTTCGTGAA42-3' (the numbers are according to Bradshaw et al., Ref. 18). The PCR reaction conditions were optimized with the PCR Optimizer² kit, version C (Invitrogen), and performed at the following parameters: 25–30 cycles, denaturation 94 °C for 1 min, annealing 55 °C for 2 min, and polymerization 72 °C for 3 min.

**Amplification of TK1 cDNA—**RNA was isolated by the RNAqueous™ phenol-free total RNA isolation kit (Ambion, TX). cDNA was amplified with Titan™ one-tube reverse transcription-PCR kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions but with 400 μM of each primer. The sense primer was 5'-22GAGAGTACTCGGGT-TCGCTGGGCTCC11843-3' and the antisense primer was 5'-11699TGTC-CTTCGTGAA42-3' (the numbers are according to Bradshaw and Deininger in Ref. 10). This gave an 804-base pair fragment containing the entire coding region of TK1 cDNA.

**Amplification and Sequencing of the Genomic DNA Fragments and of TK1 cDNAs—**Both strands were sequenced using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech) with the dITP nucleotide master mix. [α-³²P]Deoxynucleotides were from Amersham Pharmacia Biotech.

**RESULTS**

**cDNA Sequence of Lymphocyte TK1—**Two differences were uncovered when the nucleotide sequence of the lymphocyte TK1 cDNA insert in the pGEX-2T-LyTK1Val106 plasmid was compared with the published human TK1 sequences, which are the coding sequence of TK1 derived from SV40 transformed human fibroblasts (the pTK11 clone) (10) and the entire TK1 genomic sequence derived from HeLa cells (the Atk46 clone) (18). The differences were: Base 373, A, was changed to G in pGEX-2T-LyTK1, and base 689, A, was changed to G in pGEX-2T-LyTK1 (numbers refer to TK1 cDNA insert in the pTK11 clone). The first change resulted in change of codon ATG for methionine to GTG for valine. The second change was from codon AAG for lysine to codon AGG for arginine. Thus, at the amino acid level we observed two differences in our recombinant TK1 (rLy-TK1) when compared with the published human TK1 sequences: valine in place of methionine at position 106 and arginine in place of lysine at position 211 (A in the ATG codon for the first methionine is nucleotide number 58, according to the numbering of Bradshaw and Deininger in Ref. 10).

**The Significance of the Amino Acid Changes—Alignment of TK1 amino acid sequences from mammals and vaccinia virus with iso-functional enzymes (adenylate kinase and protein elongation factor EF-Tu) has predicted the presence of several conserved regions essential for substrate binding and transfer of the phosphate group (13). Amino acid 106 of human TK1 is in a region of 44 highly conserved amino acids starting with amino acid 93, and each amino acid in this region is expected to be functionally important (13). All the published mammalian TK1 sequences, as well as the vaccinia virus TK sequence, have valine at the site corresponding to site 106 in human TK1, except for the published human TK1 sequence with methionine at position 106 (10, 18). However, the amino acid 106 derived from our nucleotide sequence of the lymphocyte cDNA clone is not a methionine but a valine in agreement with the known sequences from different mammalian enzymes. The second difference we found was arginine instead of lysine at position 211, which does not belong to any conserved region in the C-terminal of thymidine kinases. According to Bordo and Argos (19), exchange of lysine for arginine belongs to so-called “safe” substitutions, resulting in very small conformational changes, if any. Further, the 40 C-terminal amino acids are not required for TK1 activity but are involved in cell cycle regulation of the enzyme (7). Therefore, the amino acid change at position 211 is not expected to be important for the enzymatic properties of TK1.

We have investigated the significance of amino acid 106 by comparison of the properties of the recombinant lymphocyte TK1 enzymes, rLy-TK1Val106 and rLy-TK1Met106, with TK1 isolated from human lymphocytes, Ly-TK1. Because amino acid 211 is an arginine in both recombinant enzymes, eventual differences in enzymatic properties will solely be due to the different amino acids at position 106.

**Kinetic Properties—**Fig. 2 shows the relationship between the initial velocity of dTMP formation and the thymidine concentration at saturating concentration of ATP for both the TK1−ATP (TK1 incubated and stored without ATP) and TK1+ATP (TK1 incubated and stored with ATP) form of rLy-TK1Val106 and rLy-TK1Met106. The thymidine substrate kinetics of rLy-TK1Val106 (Fig. 2A) is essentially the same as that previously observed for Ly-TK1 (8): rLy-TK1Val106 ATP displays a nonhyperbolic, “creeping” binding curve and gives a clear biphasic kinetic pattern in the Hofstee plot (Fig. 2A, inset). From the nonlinear regression analysis, the \( K_m \) value was determined to be 15 μM, and the Hill coefficient was determined to be 0.4, indicating a negative cooperative reaction mechanism. The rLy-TK1Val106+ATP form displays rectangular hyperbolic kinetics and a straight line in the Hofstee plot, with a \( K_m \) value of 0.6 μM and a Hill coefficient of about 1. The activating effect of ATP is dependent on the concentration of TK1 (8) and does not occur at assay enzyme concentration.

Similar experiments with rLy-TK1Met106 (Fig. 2B) indicate that the kinetics of this enzyme was independent of preceding
exposure to ATP; the same rectangular hyperbolic reaction mechanism and straight lines in the Hofstee plots were observed for both the + ATP and the − ATP form. Furthermore, \( K_m \) for thymidine of the two forms is the same, about 0.5 \( \mu M \), and the same as the \( K_m \) of rLy-TK1Val-106 + ATP and thus about 30-fold lower than the thymidine \( K_m \) of rLy-TK1Val-106 − ATP, which is 15 \( \mu M \). Also, the Hill coefficients for the + ATP and − ATP forms of rLy-TK1Met-106 are the same, about 1, indicating hyperbolic reaction mechanism. In addition, rLy-TK1Met-106 + ATP show the same relationship of velocity toward substrate concentration throughout the applied concentrations of thymidine (Fig. 2B), whereas at thymidine concentrations below 15 \( \mu M \), the velocity of rLy-TK1Val-106 + ATP was 3–5-fold higher than the velocity of rLy-TK1Val-106 − ATP (Fig. 2A). These results demonstrate two important issues. Firstly, rLy-TK1Val-106 behaves like the endogenous Ly-TK1 purified from human lymphocytes, whereas rLy-TK1Met-106 differs by having a permanently high thymidine affinity irrespective of preceding ATP exposure. Secondly, the kinetic properties of rLy-TK1Val-106 are the same as those previously described by Munch-Petersen et al. (9) for rTF-TK1 derived from the cDNA clone of Bradshaw and Deininger (10).

Polymorphisms in Human DNA—To clarify the naturally occurring bases and the resulting amino acids at positions 106 and 211, we have sequenced genomic DNA and cDNA from a number of cell lines and from stimulated lymphocytes from several healthy donors. Genomic DNA fragments of 166 base pairs containing the codon for amino acid 106 from four colon cancer cell lines, HeLa cells, and lymphocytes from four healthy donors, all had a GTG codon for valine at position 106. The complete coding region of TK1 cDNAs from leukemic cell lines, fibroblasts, and lymphocytes from five healthy donors, all had a GTG codon for valine at position 106.

**Effect of Amino Acid 106 on Human TK1**

TK1Val-106 is a permanent tetramer. This behavior of rLy-TK1Val-106 is in agreement with our earlier results (9) with rTF-TK1, derived from the cDNA clone of Bradshaw and Deininger (10), with methionine as the amino acid at position 106.

Enzyme Stability—Fig. 4 demonstrates that the stability of rLy-TK1Met-106 is considerably lower than that of the rLy-TK1Val-106. At 25 °C, rLy-TK1Met-106 was very unstable, and more than 50% of the activity was lost within 4 min. Therefore, the stability experiments were performed at 15 °C. Also here, rLy-TK1Met-106 appeared unstable because the \( t_{1/2} \) value was 41 min. In contrast, when incubated at 15 °C, more than 50% of the activity of rLy-TK1Val-106 was preserved after incubation for 6 h (\( t_{1/2} \) was 392 min). At assay conditions, where ATP, BSA, and CHAPS are present, both enzymes are stable. The different stabilities stress the impact of amino acid 106 for the properties of TK1.
### Effect of Amino Acid 106 on Human TK1

#### TABLE 1

| Source of cDNA | Nucleotide position |
|---------------|---------------------|
|               | 90      | 115     | 279     | 282     | 373     | 651     | 689     |
| pTK11         | CCG     | CAG     | GAG     | GGG     | ATG     | GCC     | AAG     |
| Lymphocytes from healthy donors |         |         |         |         |         |         |         |
| Donor 1       | Pro     | Gln     | Gln     | Ala     | Met     | Ala     | Lys     |
| Donor 2       | Pro/Pro | Gln     | Gln/Glu | Ala/Ala | Val     | Ala     | Arg     |
| Donor 3       | Pro/Pro | Gln/Glu | Ala/Glu | Val     | Ala     | Lys     |         |
| Donor 4       | Pro/Pro | Gln/Glu | Ala/Glu | Val     | Ala     | Lys     |         |
| Donor 5       | Pro/Pro | Gln     | Gln     | Ala/Ala | Val     | Ala     | Lys     |
| Leukemic cell lines |       |         |         |         |         |         |         |
| RS4;11        | CCT     | CAG     | GAG     | GCA     | GTG     | GCC     | AAG     |
| Molt-3        | Pro     | Gln     | Gln     | Ala     | Val     | Ala     | Lys     |
| Reh           | Pro/Pro | Gln/Glu | Ala/Glu | Val     | Ala     | Lys     |         |
| KG-1          | Pro     | Gln     | Glu     | Ala     | Val     | Ala     | Lys     |
| AML-193       | Pro/Pro | Gln/Glu | Ala/Glu | Val     | Ala     | Lys     |         |
| K562          | Pro     | Gln     | Glu     | Ala     | Val     | Ala     | Lys     |
| CEM-C         | Pro     | Gln     | Glu     | Ala     | Val     | Ala     | Lys     |
| Fibroblasts   | CCT     | CAG     | GAG     | GCA     | GTG     | GCC     | AAG     |
| WI38          | Pro     | Gln     | Glu     | Ala/Ala | Val     | Ala     | Lys     |
| WI26          | Pro     | Gln     | Glu     | Ala     | Val     | Ala     | Lys     |

The numbers are according to the published sequence of Bradshaw and Deininger (10) and correspond to the underlined bases, where the polymorphisms are located. Bases and amino acids that differ from (10) are shown in bold type. Sequence differences occurring in only one allele are in parentheses. The translation initiation is at position 58, wherefore the codon for amino acid 106 starts at 373.

**DISCUSSION**

We have cloned and sequenced TK1 cDNA derived from human lymphocytes, expressed and purified the corresponding enzyme, rLy-TK1, and examined its catalytic and oligomerization properties. When the cDNA sequence of rLy-TK1 was compared with the published TK1 cDNA (10), we observed two differences. In rLy-TK1 cDNA, the codon for amino acid 106 was GTG for valine instead of ATG for methionine, and the codon for amino acid 211 was AGG for arginine instead of AAG for lysine. Our characterization of rLy-TK1 showed that the enzymatic properties with respect to the effect of ATP on oligomerization and thymidine affinity were the same as found for the native TK1 previously purified from human lymphocytes, i.e. exposure to ATP induces a reversible transition from a low affinity dimer ($K_m$, 15–17 μM, 50 kDa) to a high affinity tetramer ($K_m$, 0.5–0.7 μM, 100 kDa) (8).

Furthermore, we have shown that mutation of Val-106 to methionine changed rLy-TK1 to a permanent tetramer with high thymidine affinity ($K_m$, 0.4–0.6 μM, 100 kDa) irrespective of pre-exposure to ATP. Additionally, our results confirm our previous supposition that high thymidine affinity is associated with a tetrameric state of the enzyme (9).

The behavior of rLy-TK1Met-106 is the same as we have observed previously with recombinant human TK1 (rTFl-TK1) (9) expressed from the cDNA clone of Bradshaw and Deininger (10), also with a codon for methionine at position 106. cDNA of this clone is deposited in the NCBI GenBank™. Thus, our results demonstrate that the enzymatic properties of TK1 expressed from the published cDNA sequence (10, 18) differ noticeably from the properties of the native TK1 and recombinant TK1 from human lymphocytes, because of a single amino acid at position 106. The discrepancy regarding the different amino acid codons in the published TK1 cDNA and genomic DNA, and in the cDNA we have isolated from human lymphocytes may be due to the different origins of the mRNAs. To clarify this question, we have sequenced cDNA and genomic DNA from various human cells and cell lines. The mRNA source in the cDNA we have isolated from human lymphocytes may be noticeably from the properties of the native TK1 and recombinant TK1 from human lymphocytes, because of a single amino acid at position 106. The discrepancy regarding the different amino acid codons in the published TK1 cDNA and genomic DNA, and in the cDNA we have isolated from human lymphocytes may be due to the different origins of the mRNAs. To clarify this question, we have sequenced cDNA and genomic DNA from various human cells and cell lines. The mRNA source in the investigations of Bradshaw and Deininger (10) and Flemington et al. (18) were SV40 transformed fibroblasts and HeLa cells, respectively. Therefore, these two cell lines and a nontransformed fibroblast cell line were purchased at American Type Culture Collection and European Collection of Cell Cultures (see "Experimental Procedures") and included in our sequence analysis. Altogether, we have examined the codon for amino acid 106.
acid 106 in genomic and cDNA from seven healthy volunteers and 15 cell lines. In each of these, a GTG codon corresponding to valine at position 106 was found in both alleles. Valine at this position has also been found in the human lymphoblastoid cell line, TK6 (20). Furthermore, amino acid 106 is positioned in a highly conserved region, where a valine is found in TK1 from several other vertebrate organisms as well as in TK from several vira of the pox family.

Other polymorphisms at the DNA level were observed as well, but only two of these resulted in amino acid changes. However, such “silent” mutations may in fact influence translation if a “high usage” codon is changed into a “low usage” codon (21). In one cell line, Reh, there was a C to T transition at nucleotide position 115 changing the codon CAG for amino acid 20 to a stop codon, TAG. If the resulting truncated polypeptide is expressed in the Reh cells, this may result in the formation of nonfunctional dimers and tetramers, which very likely would impair the enzymatic activity. The same C to T transition at nucleotide position 115 resulting in a stop codon at amino acid position 20 was observed in the TK6 human lymphoblastoid cell line after X-irradiation (22). The presence of a stop codon in one or both alleles at the tk locus may be the underlying mechanism that could explain our previous observations of a high level of TK1 mRNA but no TK1 activity in lymphocytes from patients with chronic lymphatic leukemia (5).

Our data presented here demonstrate that amino acid 106 is of paramount importance for the thymidine affinity and oligomerization of human TK1. The molecular mechanism behind this effect is, however, not clear at the present and must await the three-dimensional structure of TK1 to be solved. Until now, all ribo- and deoxyribonucleoside kinases, only the structure of Herpes virus type 1 thymidine kinase has been solved (23, 24). Although the sequence homology between the Herpes TK and human TK1 is limited, it is interesting that one of the dimer interface α-helices of the Herpes TK, α-helix number 4 (24), aligns with the section of human TK1 that contains amino acid 106 (Cluster X 1.8). Furthermore, prediction of the secondary structure places Val-106 in the middle of an α-helix (25–27). Replacement of the nonpolar valine with the partially charged methionine that allows larger solvent accessibility can be imagined to destabilise the hydrophobic interactions in the interface between the two subunits of a dimer. The looser monomer-monomer interaction may on the other hand energetically favor interaction between two pairs of dimer and result in a tetramer irrespective of exposure to ATP.

It remains to be explained why a methionine at position 106 was found in previous investigations of TK1 derived from SV40 transformed fibroblasts (10) and from HeLa cells (18). It may be that both the Val-106 and Met-106 forms of human TK1 are present in nature. However, our results obtained from 22 independent isolations of genomic DNA and cDNA indicate that valine with high probability is the naturally occurring amino acid at position 106 in human TK1. At the present, all research with recombinant TK1 is performed with enzyme expressed from the pTK11 clone with methionine at position 106. In our present work, we have shown that this position has a pronounced impact on the enzymatic properties and regulation of human TK1. To obtain representative data in future investigations, TK1 with valine at position 106 should be used instead of TK1 expressed from the pTK11 cDNA.

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