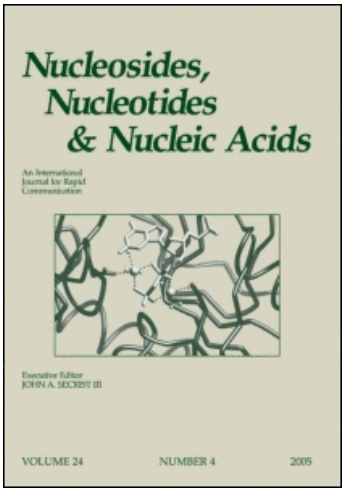

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PURIFICATION AND CHARACTERIZATION OF WILD-TYPE AND MUTANT TK1 TYPE KINASES FROM *CAENORHABDITIS ELEGANS*

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□ Caenorhabditis elegans *has a single deoxynucleoside kinase-like gene. The sequence is similar to that of human TK1, but besides accepting thymidine as a substrate, the C. elegans TK1 (*Ce*TK1) also phosphorylates deoxyguanosine. In contrast to human TK1, the* Ce*TK1 exclusively exists as a dimer with a molecular mass of ~60 kDa, even if incubated with ATP. Incubation with ATP induces a transition into a more active enzyme with a higher k_{cat} but unchanged K_m. This activation only occurs at an enzyme concentration in the incubation buffer of 0.5 μg/ml (8.42 nM) or higher. C-terminal deletion of the enzyme results in lower catalytic efficiency and stability.*

Keywords Thymidine kinase; TK1; *Caenorhabditis elegans*; ATP activation; Characterization; Enzyme kinetics

INTRODUCTION

Caenorhabditis elegans thymidine kinase (*Ce*TK1) is a TK1 type deoxynucleoside kinase that catalyzes the transfer of γ-phosphate from ATP to the 5′-OH of thymidine and deoxyguanosine to form dTMP and dGMP, respectively. In humans and other mammalians TK1 is a key enzyme in the salvage of dTTP for DNA synthesis.

Deoxynucleoside kinases are also important for activation of antiviral and anticancer drugs such as the nucleoside analog AZT used for HIV treatment. A thymidine kinase from Herpes Simplex virus type 1, HSV1-TK, has been used in suicide gene therapy in an attempt to cure cancer.[1,2] In tumor cells expressing the HSV1-TK, the analog is phosphorylated to the monophosphate level. Subsequently, cellular kinases add 2 more phosphate groups to form the triphosphate analog, which after incorporation into

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DNA prevents DNA elongation and causes cell cycle arrest or apoptosis. In order to find a better deoxynucleoside kinase with higher analog specificities for this purpose, new enzymes are continuously characterized and new analogs are created.

In the present characterization of *Ce*TK1, the enzyme is compared with human TK1 (HuTK1) with regard to native size, activation by ATP, stability and kinetic parameters for thymidine. *Ce*TK1 is truncated in the C-terminal in order to see how the truncation affects stability and activity.

MATERIALS AND METHODS

The *Ce*TK1 gene was provided from ZGene (Hoersholm, Denmark) and cloned into the expression vector pGEX-2T by PCR amplifcation (Primers: Forward: 5′-CGG CGC CCG GGC ATG GAC ATT GAA GCG GCC AAG AAT GAG ATG ACT TGC-3′; Reverse: 5′ CCG GAA TTC TTA AGT CCG AGC TGT GGC CTC CAG AGC 3′) followed by cleavage and ligation at XmaI and EcoRI restriction sites. *Ce*TK1 was expressed in *E. coli* BL21 as a GSH fusion protein and purified by GSH affinity chromatography as described.[3]

The C-terminally truncated mutant was constructed using the QuickChange site-directed mutagenesis kit from Stratagene (AH Diagnostics, Denmark) (Primers: Forward: 5′-GC AGAGAA TGT TAT GTT CAA AAG AGC TAA GAA AAA GAT GC-3′; Reverse: 5′ GC ATC TTT TTC TTA GCT CTT TTG AAC ATA ACA TTC TCT GC-3′. Mutation marked with grey). The truncated enzyme was expressed and purified as the wild type.

Effect of ATP on the enzymes was examined by incubating 5 μg/ml (84.2 nM) enzyme for 2 hours on ice in buffer A (50 mM Tris HCL pH 7.5, 5 mM $MgCl_2$, 0.1 M KCl, 2 mM 3-[(3-Cholamidopropyl)-dimethyl-ammonium]-1-propanesulfonate (CHAPS), 10% Glycerol, 5 mM dithiothreitol (DTT)) with 2.5 mM ATP. For comparison, an aliquot of enzyme was incubated in the same buffer without ATP.

Enzyme activities were measured as initial velocities determined by 3 time samples (5, 10, and 15 minutes) with the DE-81 filter paper assay using ^{3}H labelled substrates.[3] Reaction mixture of 50 μl contained 50 mM TRIS-HCl, pH 7.5, 2.5 mM $MgCl_2$, 10 mM DTT, 0.5 mM CHAPS, 3 mM NaF, 3 mg/ml BSA, 2.5 mM ATP, and [*methyl*-^{3}H]-Thymidine (1.8 Ci mmol^{-1} Amersham Pharmacia Biotech, now GE Healthcare Bio-Sciences, Hilleroed, Denmark) and 0.074 nM enzyme.

The native size was determined by gel filtration on a superdex 200 column as described.[3]

*Ce*TK1 stability was determined by incubating the enzyme at room temperature (5 μg/ml, 84.2 nM) in buffer A with or without ATP.

RESULTS AND DISCUSSION

The entire genome of *C. elegans* is sequenced and appears to contain only a single deoxynucleoside kinase-like sequence. Humans have 4 different deoxynucleoside kinases whereas insects, such as the fruit fly *Drosophila melanogaster*, only have a single one. However, in contrast to the human deoxynucleoside kinases, the insect deoxynucleoside kinase can phosphorylate all 4 naturally occurring deoxynucleosides and differ greatly in structure from the TK1-type kinases.[4–6] A BLAST search on the *Ce*TK1 protein sequence reveals the closest similarity to HuTK1, with 46% identical and 63% positive matches. A sequence alignment of HuTK1 and *Ce*TK1 is presented in Figure 1, and it is evident from the alignment that the two enzymes have high sequence similarity with exception of the N- and C-terminal regions.

In spite of the high similarity, HuTK1 and *Ce*TK1 differ in respect to both substrate specificity and ATP-activation pattern: When HuTK1 is incubated with ATP it changes from a dimer form with a mass of ~50 kDa and a high K_m of ~15 μM into a tetramer of ~100 kDa with an approximately 20– to 30- fold lower K_m (Table 1).[7,8] k_{cat} for HuTK1 is in the same range for both the dimer and tetramer. In contrast to this, *Ce*TK1 is a dimer of ~60 kDa regardless of ATP incubation, but even so ATP has an effect on enzyme activity. The ATP incubated *Ce*TK1 has a higher k_{cat} than the enzyme incubated without ATP, but K_m is unchanged. Hence, ATP activates both enzymes, but HuTK1 is activated with regard to K_m and *Ce*TK1 with regard to k_{cat}. In general, the k_{cat} values for HuTK1 and *Ce*TK1 are in the same range, but for the enzymes not incubated with ATP, the HuTK1 k_{cat} is higher than


```
HuTK1   ---------MSCINLPT------VLPGSPSKTRGQIQVILGPMFSGKSTELMRRVRRFQI 45
CeTK1   MDIEAAKNEMTCCSSNSSLSDFNTLPRCPNR-VGSITVILGPMFSGKTTELLRLHDRQII 59
                 *:* .  :      .** .*.:  *.* **********:***:*   *  *

HuTK1   AQYKCLVIKYAKDTRYSSS-FCTHDRNTMEA--LPACLLRDVAQEALG--VAVIGIDEGQ 100
CeTK1   AKRTCVLVKYAGDTRYDADLVATHSKMTGQGRTVKAHRLSEVQSQIFNDEVQVVSIDEGQ 119
        *: .*:::*** ****.:. ..**.: * :.  : *  * :* .: :.  * *:.*****

HuTK1   FFPDIVEFCEAMANAGKTVIVAALDGTFQRKPFGAILNLVPLAESVVKLTAVCMECFREA 160
CeTK1   FFEDLAETCEELAQRGKVVCVAALNGTFERKPFPQISLLLPYANEIKQVTAVCVECGSQA 179
        ** *:.* ** :*: **.* ****:***:****  *  *:* *:.: ::****:**  :*

HuTK1   AYTKRLGTEKEVEVIGGADKYHSVCRLCYFKKASGQPAGPDNKENCP----------VPG 210
CeTK1   NFSFRSTLDKKVEVIGGSDTYTALCRECYVQKSEEKDAEEQMKTGCDKNENDITGIFLAK 239
         :: *   :*:******:*.* ::** **.:*:. : *  : * .*           :.

HuTK1   KP----GEAVAARKLFAPQQILQCSPAN-- 234
CeTK1   KEQRSDGSVSPPRKKIGLSKSMALEATART 269
        *     *.. ..** :. .: :  ..:
```

FIGURE 1 ClustalX 1.8 alignment of sequences of HuTK1 (GeneBank nr. AAH06484) and *Ce*TK1 (GeneBank nr. NM_069485). (* Indicates a conserved amino acid.) The gray boxes indicate the position for C-terminal truncation of the 2 enzymes.

TABLE 1 Kinetic Values with the Substrate Thymidine

HuTK1[a]	WT (25.5 kDa)		CΔ40 (21.1 kDa)	
Storage conditions	−ATP	+ATP	−ATP	+ATP
k_{cat} (sec^{-1})	6.3 ± 0.9	6.9 ± 0.2	9.5 ± 1.1	9.7 ± 0.2
K_m (μM)	16 ± 3.4	0.7 ± 0.2	1.4 ± 0.6	0.6 ± 0.1
k_{cat}/K_m	0.4×10^6	9.9×10^6	6.8×10^6	16.2×10^6
CeTK1	WT (29.7 kDa)		CΔ56 (23.6 kDa)	
Storage conditions	−ATP	+ATP	−ATP	+ATP
k_{cat} (sec^{-1})[b]	5.0 ± 0.1	8.8 ± 0.2	1.1 ± 0.0	3.5 ± 0.2
K_m (μM)	2.3 ± 0.3	2.6 ± 0.3	7.4 ± 0.8	5.8 ± 0.9
k_{cat}/K_m	2.2×10^6	3.4×10^6	0.1×10^6	0.6×10^6

[a]Kinetic values for HuTK1 are from.[8]

[b]k_{cat} values were calculated presuming one binding site per subunit using the theoretical MW for the enzyme. +ATP and −ATP denotes whether the enzyme has been pre-incubated in a buffer with or without 2.5 mM ATP/$MgCl_2$.

the *Ce*TK1 k_{cat}, and for the ATP incubated enzymes, *Ce*TK1 k_{cat} is slightly higher than the HuTK1 k_{cat}.

Due to a high K_m value and a low solubility of deoxyguanosine accurate kinetic values for phosphorylation of this substrate could not be achieved.

The ATP-activation of *Ce*TK1 depends on enzyme concentration in the incubation buffer. At concentrations of 0.1 μg/ml (1.68 nM) and lower there is no transition to the more active +ATP form of the enzyme, whereas at 0.5 μg/ml (8.42 nM) and higher concentrations the transition to the higher k_{cat} form takes place. The low enzyme concentration in the assay (0.074 nM) explains our linear progression curves and absence of transition during the kinase assay although there is 2.5 mM ATP in the assay mixture.

*Ce*TK1 is remarkably stable compared to the human TK1. When incubated in TRIS buffer, the half-life of *Ce*TK1 is 222 hours whereas the half-life of HuTK1 incubated in phosphate buffer (50 mM K-phosphate with 0.5 mM CHAPS) is only 58 minutes.[8] The half-lifes are not directly comparable due to the different incubation buffers, but since HuTK1 has a lower stability in TRIS buffer than in phosphate buffer (B. Munch-Petersen, personal communication) the stability of *Ce*TK1 is more than 540 fold higher than the stability of HuTK1. However, where deletion of the C-terminal of the HuTK1 increases the stability,[8] similar deletion of the C-terminal of *Ce*TK1 has a destabilizing effect. Thus, the half-life for HuTK1 deleted by 40 amino acids in the C-terminal is 256 minutes,[8] which is 32 = fold higher than the undeleted enzyme. Deletion of the corresponding amino acids (the position of deletion is marked on Figure 1) in *Ce*TK1 (*Ce*TK1-CΔ56) results in a 1.4-fold decrease in half-time from 222 hours to 156 hours. Further, the C-terminal deletion in the human kinase increases the catalytic activity,

whereas the C-terminal deletion of *Ce*TK1 decreases the catalytic activity. In general, *Ce*TK1 is a remarkably stable enzyme both with and without the C-terminal. During more than two weeks of storage at 4°C it maintains full activity.

In summary, the results presented here show that in contrast to the C-terminal of HuTK1 that decreases the stability and enzymatic activity, the C-terminal of *Ce*TK1 maintains stability and enzymatic activity.

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