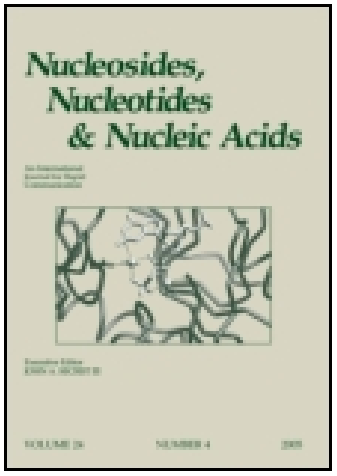

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EFFECT OF C-TERMINAL OF HUMAN CYTOSOLIC THYMIDINE KINASE (TK1) ON IN VITRO STABILITY AND ENZYMATIC PROPERTIES

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□ *Thymidine kinase (TK1) is a key enzyme in the salvage pathway of nucleotide metabolism and catalyzes the first rate-limiting step in the synthesis of dTTP, transfer of a gamma-phosphate group from a nucleoside triphosphate to the 5′-hydroxyl group of thymidine, thus forming dTMP. TK1 is cytosolic and its activity fluctuates during cell cycle coinciding with the DNA synthesis rate and disappears during mitosis. This fluctuation is important for providing a balanced supply of dTTP for DNA replication.*

The cell cycle specific activity of TK1 is regulated at the transcriptional level, but posttranslational mechanisms seem to play an important role for the level of functional TK1 protein as well. Thus, the C-terminal of TK1 is known to be essential for the specific degradation of the enzyme at the G2/M phase.

In this work, we have studied the effect of deletion of the C-terminal 20, 40, and 44 amino acids of TK1 on in vitro stability, oligomerization, and enzyme kinetics. We found that deletion of the C-terminal fold markedly increased the stability as well as the catalytic activity.

Keywords Thymidine kinase 1; TK1; Cell cycle regulation; C-terminal; Stability

INTRODUCTION

Cytosolic thymidine kinase 1, TK1 (E.C.2.7.1.21) is a key enzyme in the salvage of thymine nucleotides, catalyzing the first phosphorylation step of thymidine to TMP which thereafter is quickly phosphorylated to TDP and TTP. TK1 activity is cell cycle regulated, being low or absent in nondividing cells, increases during G1/S phase in close correlation to the increase in DNA synthesis and disappears rapidly in G2/M phase due to specific

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proteolytic degradation by the ubiquitin-dependent proteasomal pathway which recognizes a specific KEN box motif in the C-terminal end of TK1.[1] Deletion of the 40 C-terminal amino acids of TK1 delayed the G2/M phase specific degradation.[2]

We previously have found another regulatory mechanism operating at the enzymatic level, where TK1 can occur in two reversibly interchangeable forms.[3,4] At low TK1 concentrations, the enzyme is a low activity dimer (K_m about 15 μM). At higher concentrations (>0.2 μg ml^{-1}) and in the presence of ATP, TK1 occurs as a high activity tetramer (K_m about 0.5 μM). Both enzyme forms have a V_{max} about 18–20 μmol min^{-1} mg^{-1}. We believe it is very likely that this enzymatic regulation confers a fine-tuning mechanism of the regulation of TK1 activity during the cell cycle.

The present work was undertaken to investigate the C-terminal amino acids effect on the enzymatic regulation of TK1, if any.

MATERIALS AND METHODS

TK1 mutants. C-terminal truncated TK1 mutants lacking 20, 40, and 44 C-terminal amino acids (TK1 CΔ20, TK1 CΔ40, TK1 CΔ44) were constructed from the plasmid pGEX-2T-LyTK1^{val106} by inserting stop codons at the desired sites using the QuickChange site-directed mutagenesis kit (Stratagene, AH Diagnostics, Denmark).

Recombinant enzyme expression and purification. TK1 enzymes were expressed in *E.coli* from using the glutathione S-transferase (GST) fusion system (Amersham pharmacia Biothech) and purified in two steps on glutathione-sepharose 4B (Amersham Pharmacia Biotech) and CM-sepharose, as previously described.[4] According to SDS PAGE, the purity was higher than 98%.

Enzyme assay. TK1 activity was assayed as initial velocities by the DE-81 filter paper method as described.[3] The standard reaction mixture contained 50 mM TRIS-HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM DTT, 0.5 mM CHAPS, 3 mg/ml BSA, 2.5 mM ATP and [*methyl*-^{3}H]-Thymidine (66.6 GBq/mmol calculate GBq) Amersham Pharmacia Biotech) in a total volume of 50 μL.

TK1 stability. TK1 (25 ng mL^{-1}) was incubated in 50 mM K-phosphate buffer with 0.5 mM CHAPS at 20°C for 5 hours. Time samples of 10 μL were taken after 0, 10, 20, 45, 60, 90, 120, 180, 240, and 300 minutes of incubation and added to the standard TK assay reaction mixture with 10 μM [*methyl*-^{3}H]-Thymidine (66.6 GBq mmol^{-1}).

Molecular size. Subunit molecular size is determined by SDS PAGE. Native molecular size is determined by gelfiltration on a Superdex 200 column (10 × 300 mm) as described.[5] The elution buffer is 50 mM imidazole/HCl pH 7.5, 5 mM $MgCl_2$, 0.1 M KCl, 2 mM CHAPS, 5 mM dithiothreitol. 0.7 μg pure

TABLE 1 Enzymatic Properties of Untruncated and C-Terminal Truncated TK1

	TK1 WT		TK1 CΔ40	
Storage conditions	−ATP	+ATP	−ATP	+ATP
k_{cat} (sec^{-1})[a]	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
Molecular size (kD)[b]	60	100	60	90

[a]kcat values (with SDs based on 2 experiments) were calculated presuming one binding site per subunit using the theoretical MW of TK1. [b]The molecular size was determined as described in MATERIALS AND METHODS.

TK1 was applied. The +ATP samples contained 2.5 mM ATP and 2.5 mM ATP was added to the elution buffer.

RESULTS AND DISCUSSION

We have examined the effect of deletion of the C-terminal amino acids on the enzymatic properties and stability of human recombinant TK1. Deletion of the 40 C-terminal amino-acids of TK1 had minor impact on the enzymatic properties (Table 1), since the difference between the + and −ATP forms is considerably less pronounced compared to the WT TK1. However, when studying the ability to occur as dimer in absence and tetramer in presence of ATP, the truncated form behaved essentially as the untruncated (Table 1).

In contrast to the minor effect of truncation on kinetics and native molecular size, the impact on the stability was pronounced. As seen from Table 2, the half-life of TK1 increases with increasing truncation. Deletion of 44 amino acids increased the half life 6-fold. However, deletion of these additional four amino acids had a marked impact on k_{cat} that decreased almost 4-fold compared to the TK1CΔ40 which may be due to the close vicinity to the highly conserved Zn-binding domain.[6]

Clearly, the C-terminus confers instability to TK1 and may have the function to introduce unfolding of the enzyme to expose the KEN box for cell-cycle specific degradation.

TABLE 2 Stability and k_{cat} Values for TK1 WT and C-Terminal Deletion Mutants

	TK1 WT	TK1 CΔ20	TK1 CΔ40	TK1 CΔ44
$t^{1/2}$ (min)[a]	83 ± 7	78 ± 6	335 ± 24	500 ± 70
k_{cat} (sec^{-1})	6.3 ± 0.9	3.7 ± 0.7	9.5 ± 1.19	2.5 ± 0.3

[a]The time of incubation at 20°C, where 50% activity is lost. The incubation buffer was 50 mM potassium phosphate pH 7.5 with 0.5 mM CHAPS.

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