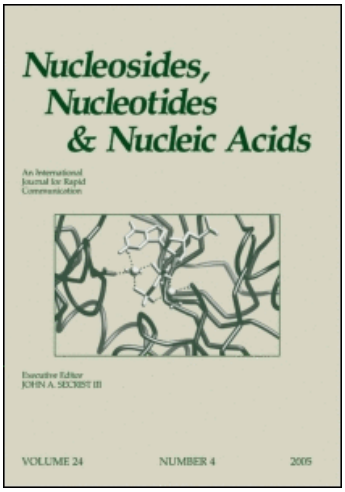

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ENZYMATIC REGULATION OF CYTOSOLIC THYMIDINE KINASE 1 AND MITOCHONDRIAL THYMIDINE KINASE 2: A MINI REVIEW

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□ *The central enzyme on the de novo pathway for synthesis of DNA precursors, the deoxyribonucleoside triphosphates, is ribonucleotide reductase (RNR). Deoxythymidine triphosphate (dTTP) has a key role in control of RNR activity shifting the specificity from pyrimidine to purine nucleotide reduction. Apart from the complex de novo synthesis of dTTP through UDP reduction, dTTP is provided through salvage of thymidine catalyzed by the thymidine kinases, the cytosolic and cell cycle regulated TK1 and the mitochondrial and constitutively expressed TK2. The complex enzymatic regulation of TK1 and TK2 and the possible physiological significance of this regulation will be discussed.*

Keywords Thymidine kinase; TK1; TK2; mitochondria; enzyme regulation

It has long been known that the immediate precursors for DNA synthesis, the deoxyribonucleoside triphosphates (dNTPs), closely relate to the DNA synthesis rate: they are low in nonproliferating cells, increase through G1/S phase and decline at G2/mitosis.[1] Deoxythymidine triphosphate (dTTP) appears to have the most pronounced variation, as it is the lowest pool in nonproliferating cells and the highest pool in dividing cells.[2–5] Further, dTTP is a key regulator of ribonucleotide reductase activity and shifts the specificity from pyrimidine to purine nucleotide reduction.[5] Thus, dTTP plays a central role in the balanced supply of dNTPs for DNA replication and repair. The significance of dTTP regulation is underlined by recent studies showing that a disbalanced dTTP pool confers genetic and chromosomal instability.[6,7] The de novo biosynthesis of dTTP is performed through many energy demanding steps: UDP reduction, phosphorylation to dUTP, dephosphorylation to dUMP, and methylation by thymidylate synthase to dTMP, and finally two phosphorylation steps to dTTP. Alternatively, dTMP is derived from the salvage pathway by phosphorylation of thymidine, easily taken up by the cell from external sources.

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The two key enzymes in salvage of thymidine to dTMP are cytosolic thymidine kinase 1 (TK1) and mitochondrial thymidine kinase 2 (TK2). Both enzymes have the same EC number (EC 2.7.1.21), although they are encoded by different nuclear genes and belong to two fundamentally different structural families that indicates different evolutionary origin.[8–10] TK1 is located at chromosome 17q25 and TK2 at chromosome 16q22. Besides thymidine phosphorylation, they both phosphorylate deoxyuridine and TK2 also phosphorylates deoxycytidine. Both enzymes have complex enzymatic and cooperative regulation mechanisms. These mechanisms and their physiological impact will be briefly discussed.

CYTOSOLIC THYMIDINE KINASE–TK1

TK1 is a comprehensively regulated enzyme. Its activity increases during G1/S phase, declines through G2/M phase and is absent in nonproliferating cells. Transcriptional and translational mechanisms control the increased expression in early S-phase[11] and the G2/M specific disappearance is due to specific degradation of TK1 protein by the ubiquitin-proteasome pathway.[12] In addition, TK1 has been shown to be regulated at the enzymatic level in a complex manner. In 1991,[13] TK1 was purified to homogeneity and the subunit size of 25 kDa agreed with the mass of 25,505 Da predicted from TK1 cDNA encoding a protein of 234 amino acids.[14] The native size determined by gel filtration showed a dimer about 60 kDa, whereas gel filtration in the presence of ATP gave a tetramer of about 120 kDa.[13,15] Gel filtration of the 120 kDa peak without ATP restored the 60 kDa form which indicated that the ATP oligomerization effect was reversible and that TK1 switched between a dimer and a tetramer.[15] Analysis of the thymidine substrate kinetics at saturating assay concentrations of ATP (2–3 mM) showed the same turnover (k_{cat}) for both enzyme forms but different K_m values. The dimer form had a K_m about 15–17 μM thymidine and the tetramer form about 0.5 μM thymidine. This implies that the tetramer form has a catalytic efficiency (k_{cat}/K_m) about 30-fold higher than the dimer.[15,16] The transition from dimer to tetramer depended also on the enzyme concentration. Irrespective of pre-assay exposure to ATP, TK1 remained a dimer below a critical enzyme concentration of 10 ng·ml^{-1} that corresponds to 0.4 nM.[15] Standard assay concentrations of TK1 were below this critical limit which indicated that there was no transition between the two forms at assay conditions and explained the linear progress curves of product versus time, consistently obtained with TK1.

The concentration effect was seen when gel filtration was performed with higher concentrations of TK1. Without ATP exposure at enzyme concentrations above 0.2 mg·ml^{-1} (8 μM) TK1 eluted exclusively as a tetramer.[16] This was also shown by Birringer et al who used concentrations in the range of 0.4 to 20 mg·ml^{-1}.[17]

The tetramer form of TK1 followed Michaelis Menten kinetics, but the dimer form of TK1 exhibited a complex kinetics that deviated from Michaelis-Menten kinetics.[15,16] The *v* versus *s* curve had a "creeping" form and the upward concave form of the Hofstee plot of *v* versus *v/s* indicated a negative cooperative behavior. This apparent cooperation may be the result of the simultaneous presence in the assay of two forms of TK1, where the ratio between these two forms depends on the thymidine concentration in the assay as previously proposed.[18] The physiological concentration of ATP in the cell is about 1–2 mM,[7] but that of thymidine is not known. However, the plasma thymidine concentration was determined to be below 0.05 μM.[19] Presuming equal distribution of thymidine between plasma and cytosol, at 0.05 μM thymidine the cellular kinase activity of the dimer TK1 with the high K_m would be about 30-fold lower than the kinase activity of the tetramer TK1.

The cellular concentration of TK1 is zero in beginning of G1, and is estimated to increase to about 0.16–0.25 μM in peak S-phase cells.[15,16] This means that in early G1, TK1 cannot oligomerize to the tetramer form but will be a dimer with the high Km. As the TK1 concentration increases during the S-phase, more and more TK1 enzyme will go to the tetramer with the 30-fold lower K_m. In Figure 1 is proposed a model for the variation of TK1 activity during cell cycle presuming constant ATP concentration of about 1–2 mM and a thymidine concentration of 0.05 μM or lower. In the beginning of a new cell cycle, TK1 protein is absent due to the specific degradation by the ubiquitin-proteasome pathway in the previous G2/M phase. Then, during early G1, TK1 concentration increases due to the transcriptional regulation. Initially, TK1 will be on the dimer form with the high K_m due to the low

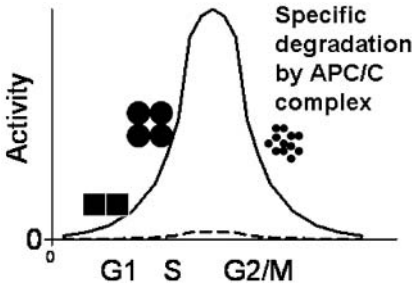

FIGURE 1 A model for variation of TK1 activity during cell cycle. A constant ATP concentration of about 1–2 mM and a thymidine concentration of 0.05 μM or lower are presumed. In the beginning of a new cell cycle TK1 protein is absent due to specific degradation by the ubiquitin-proteasome pathway in the previous G2/M phase. Then, during early G1, TK1 concentration increases due to the transcriptional regulation. Initially, TK1 will be on the dimer form with the high K_m due to the low TK1 concentration. As TK1 concentration increases, more and more of TK1 protein will be on the tetramer form with the 30-fold lower Km. Eventually, when TK1 concentration peaks, all TK1 will be on the tetramer form and the TK1 enzyme molecules will be 30-fold more active than if they were still at the dimer form. Unbroken line: TK1 activity with dimer-tetramer transition. Broken line: TK1 activity without dimer-tetramer transition.

TK1 concentration. As TK1 concentration increases, more and more of TK1 will be on the tetramer form with the 30-fold lower K_m. Eventually, when TK1 concentration peaks, all TK1 will be on the tetramer form and the TK1 enzyme molecules will be 30-fold more active than if they were still at the dimer form. It is very likely, that the dimer-tetramer shift serves as a fine-tuning regulatory mechanism of TK1.

MITOCHONDRIAL THYMIDINE KINASE–TK2

TK2 is expressed from a nuclear gene and imported into mitochondria very likely signalled by its N-terminal leader sequence.[20,21] The primary role of TK2 apparently is to sustain the pyrimidine deoxynucleotide pool in mitochondria. TK2 is not cell cycle regulated and is present at low levels in all tissues, but actually has a complex regulation at the enzymatic level. The kinetic behavior with deoxycytidine followed Michaelis-Menten kinetics with a hyperbolic velocity versus substrate curve, but with thymidine TK2 showed a biphasic dependency that indicated negative cooperativity.[13,22] This behavior was initially observed already in 1977 with semi-pure preparations of TK2 and interpretated as the result of the presence of to thymidine phosphorylating enzymes with different K_m and V_{max}.[23] K_m values estimated from the biphasic kinetic plots of velocity versus velocity/substrate were about 0.5 μM at thymidine concentrations below 3–6 μM and above this concentration they were 16 μM or higher.[13,23]

Negative cooperativity implies that at low substrate concentrations the substrate affinity is high but decreases when substrate concentration increases. The kinetic parameters can be obtained from a fit to the Hill equation $v = V_{max} \times [S]^h/(K_{0.5}^h + [S]^h)$ where $K_{0.5}$ defines the substrate concentration [S] when $v = \frac{1}{2} V_{max}$ and h is the Hill coefficient and indicate interaction between the substrate binding sites. Noncooperative kinetics is defined by $h = 1$, positive cooperativity by $h > 1$, and negative cooperativity by $h < 1$. With TK2, h was in the range of 0.3 to 0.5. [13,21,24] Compared to TK1, the substrate specificity of TK2 was broader and TK2 was able to phosphorylate a broad variety of nucleoside analogs.[13,25] The apparent negative cooperative behavior of TK2 seemed to be associated with the 5-methyl group as only azidothymidine showed this behavior, whereas dUrd and FdUrd followed Michaelis-Menten kinetics.[13] For a cell to have a TK2 with this apparent negative cooperative regulation, the result will be that the activity at physiological thymidine concentrations below 0.05 μM will be several hundred -fold higher than with a noncooperative TK2 with the same K_m.

The molecular mechanism behind the apparent negative cooperative behavior is not clear. Generally, a precondition for cooperative behavior is the presence of more than one substrate binding site with the consequence that when the first substrate is bound, the affinity for the next substrate

is changed. In the case of negative cooperativity, the first substrate bound decreases the affinity for the next substrate. However, existence of more than one substrate binding site on a TK2 peptide with a size of 30 kDa is not very likely. Whether TK2 can oligomerize to a dimer or higher is still an open question. TK2′s closest relative regarding peptide sequence is the broadly specific ultrafast deoxyribonucleoside kinase from *Drosophila melanogaster* (Dm-dNK) that phosphorylated all four natural substrates with the same high k_{cat} of about 17 sec^{-1}, manyfold higher than the TK2 k_{cat} of 0.3 sec^{-1}.[26] Dm-dNK, however, did not show cooperative behavior with any of its substrates. Gel filtration of Dm-dNK on superpose 12 gave a mass of about 33 kDa.[26] However, in the crystal structure, Dm-dNK was a homodimer.[27] Since interaction between the Superose matrix and Dm-dNK may influence the elution profile, the native size was determined by nondenaturing polyacrylamide gel electrophoresis and by gel filtration on superdex, and found to be 48.5 kDa and 57.7 kDa, respectively, indicating a homodimer.[28] Likewise, the multisubstrate deoxyribonucleoside kinase from *Bombyx mori* and the TK2 like kinase from *Xenopus laevis* behaved as dimers using superdex gel filtration and nondenaturing polyacrylamide gel electrophoresis.[28] In spite of the close sequence relation between these kinases and TK2, several gel-filtration analysis indicated TK2 to be a monomer rather than a dimer. Gel filtration on superose 12 as well as on Superdex 200 showed TK2 to elute later than ovalbumine (45 kDa), but with a slightly higher mass than that of carbonic anhydrase of 29 kDa.[13,21] The elution profile of TK2 thus corresponded to a mass of about 30–40 kDa. Thymidine or ATP have not been found to change the elution profiles.[13] The nature of the TK2 peak in the gel filtration was broad and this may indicate that there is a rapid equilibrium between a monomer and dimer TK2 that will result in a single peak with a mass of about 40 kDa.[21]

An interesting issue for discussion is whether the complex kinetic behavior of TK2 has any physiological consequence for regulation of the mitochondrial dNTP pools. In dividing cells, the mitochondrial dNTP pools have been shown to be in dynamic exchange with the cytoplasmic pools.[29] In non cycling cells where de novo and salvage of thymine nucleotides are minimal due to down-regulation of ribonucleotide reductase and TK1, mitochondrial TK2 is vital for dTTP synthesis. In 2001, it was shown that mutations in TK2 were associated with the severe mitochondrial DNA (mtDNA) depletion syndrome, MDS, resulting in isolated skeletal myopathy, mtDNA depletion and death at early age.[30] Since then, several studies have confirmed this association with further identification of mutated TK2 genes in MDS patients. The connection between mtDNA depletion and a defect TK2 gene was further supported from studies of mouse models with deficient TK2. The TK2 deficient animals showed growth retardation and premature death.[31] In another study, a TK2 mutant knockin mouse was generated with a mutant version of TK2 homologous to the H121N mutation found in MDS patients.

The mouse with this TK2 mutation showed significantly reduced TK2 activity and unbalanced dTTP pools.[32] The effect of this mutation on the enzymatic properties of TK2 was previously studied by Wang et al.[21] where WT-TK2 and TK2 with the H121N mutation were expressed and purified. The H121N-TK2 had essentially the same k_{cat}/K_m for thymidine as WT-TK2, as a result of a minor decrease in K_m and $k_{cat,}$ but the negative cooperativity of the mutated TK2 was absent. When the kinetic data from this study are used to calculate the thymidine kinase activity at physiological thymidine concentrations of or below 0.05 μM, the mutant TK2 would have less than 7% kinase activity compared to WT-TK2.

This difference between WT-TK2 and H121N-TK2 may explain at least partly the lower thymidine kinase activity found in the knockin TK2 mice and suggests that the negative cooperative regulation of the mitochondrial TK2 is of physiological significance and very likely plays an important role in the regulation of the mitochondrial dTTP pool.

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