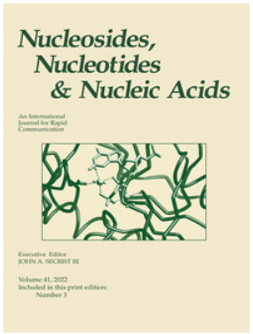

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Mutational analyses of human thymidine kinase 2 reveal key residues in ATP-Mg^{2+} binding and catalysis

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ABSTRACT

Mitochondrial thymidine kinase 2 (TK2) is an essential enzyme for mitochondrial dNTP synthesis in many tissues. Deficiency in TK2 activity causes devastating mitochondrial diseases. Here we investigated several residues involved in substrate binding and catalysis. We showed that mutations of Gln-110 and Glu-133 affected Mg^{2+} and ATP binding, and thus are crucial for TK2 function. Furthermore, mutations of Gln-110 and Tyr-141 altered the kinetic behavior, suggesting their involvement in substrate binding through conformational changes. Since the 3D structure of TK2 is still unknown, and thus, the identification of key amino acids for TK2 function may help to explain how TK2 mutations cause mitochondrial diseases.

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thymidine kinase 2; mutagenesis; substrate binding; ATP/Mg^{2+} binding; enzyme kinetics

1. Introduction

Thymidine kinases catalyze the initial phosphorylation of thymidine (dThd) to thymidine monophosphate in the salvage pathway of deoxythymidine triphosphate (dTTP) synthesis. In mammalian cells there are two thymidine kinases expressed; thymidine kinase 1 (TK1) preferentially expressed in cytosol and thymidine kinase 2 (TK2) mainly expressed in mitochondria. The expression of TK1 is highest during the S-phase of the cell cycle and thus TK1 level is high in proliferating cells. The expression of TK2, on the other hand, is highest in the stationary phase cells and thus TK2 level is very low in rapidly dividing cells. These two enzymes differ also in substrate specificity; TK1 phosphorylates dThd and deoxyuridine (dUrd) while TK2 phosphorylates dThd, dUrd and deoxycytidine (dCyd).[1–3]

Mitochondrial TK2 plays an important role in mitochondrial DNA (mtDNA) maintenance since deficiency in TK2 activity due to genetic alterations causes devastating early onset mtDNA depletion syndrome

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(MDS) or late onset mtDNA depletion/deletion diseases. Since the first report of TK2 deficiency causing MDS a total of 58 TK2 genetic variants have been reported in patients with mtDNA depletion and/or deletion diseases.[4–6] Some of the mutations have been characterized and the results have helped to clarify the role of TK2 mutations in mitochondrial diseases as well as in the development of deoxynucleoside based supplemental therapy.[4,7–11]

In this study, we investigated several amino acid residues in the conserved sequence motifs in human TK2 by site-directed mutagenesis and found that residue Gln-110 and Glu-133 were directly involved in binding of the ATP/Mg^{2+} complex. Alteration of Gln-110 and Tyr-141 resulted in changes in kinetic behavior and Val-55 and Ala-139 were also critical for enzyme activity.

2. Material and methods

Radiolabelled nucleosides: [methyl-^{3}H]-Thymidine (^{3}H-dThd, 25 Ci/mmol) was obtained from PerkinElmer and [5-^{3}H]-deoxycytidine (^{3}H-dCyd, 24 Ci/mmol) was from Moravek Biochemicals, Inc.

PCR-based site-directed mutagenesis: Wild type human TK2 cDNA was used as template in a PCR reaction to generate the desired mutations by using two complementary oligonucleotides essentially as previously descr ibed.[12,13] The final PCR product was cleaved with restriction enzymes and cloned into the pET-14b vector. The mutations were verified by sequencing using Sanger dideoxynucleotide method (Bigdye™ terminator kit and Prisma 300 system, Perkin Elmer).

Expression and purification: The plasmid DNA that contained the desired mutation was transformed into *E. coli* BL21 (DE3) pLysS cells, and the recombinant enzymes were expressed and purified as previous described.[12] Wild type TK2 was expressed and purified in parallel. The purified enzymes were stored at −70 °C in aliquots until further analysis. Protein concentration was determined by Bio-Rad protein assay using bovine serum albumin as standard.

Enzyme assay: TK2 activity was determined by using ^{3}H-dThd and ^{3}H-dCyd as substrates as described previously.[12] The steady-state kinetics was performed by using a broad substrate concentration range in order to identify deviation from Michaelis-Menten kinetics. The data were fitted into either the Michaelis–Menten equation ($V0 = V_{max}\,[S]/(K_M + [S])$) or the Hill equation ($V0 = V_{max}\,[S]^n/(K_{1/2} + [S]^n)$), where n is the Hill coefficient, V_0 is the initial velocity, $V_{max}$is the maximal velocity and S is the substrate concentration. K_M and $K_{1/2}$ are the substrate concentration required to reach ½ V_{max}. All assays were performed

at least 3 times and the data were given as mean ± SD (standard deviation).

3. Results

TK2 belongs to an enzyme family that includes cytosolic deoxycytidine kinase (dCK), mitochondrial deoxyguanosine kinase (dGK), *Drosophila melanogaster* deoxynucleoside kinase (DmdNK) and herpes simplex viral 1 thymidine kinase (HSV1 TK).[14] Among these enzymes only the TK2 structure has not yet been determined.[15–17] At the amino acid sequence level TK2 shows 11%, 31%, 35% and 43% sequence identity to HSV1 TK, dGK, dCK and DmdNK, respectively. All functional important sequence motifs such as the ATP binding p-loop are conserved in this enzyme family, although the degree of sequence identity varies considerably. Since DmdNK shows the highest sequence identity to TK2, we used the DmdNK structure to generate a structural alignment in order to study structural and function relationship in TK2 (Figure 1). We focused on critical residues that may be involved in ATP/Mg^{2+} and substrate binding and catalysis. Several residues in the sequence motif -ERSXXSD/ARY- in TK2 were investigated. All mutant TK2 enzymes were affinity purified and characterized.

3.1. Residue Gln-110

This residue corresponds Gln-81 in DmdNK, which forms hydrogen bond with the substrate base in the DmdNK structure.[16] When Gln-110 was mutated to Thr the mutant enzyme Q110T was inactive, but Q110A mutation yielded an active enzyme. The binding of dThd to the Q110A mutant was virtually unchanged with a K_M value of 8.9 μM, similar to that of the wild type TK2 (wtTK2). However, the V_{max} value of Q110A mutant was much lower than that of the wtTK2, resulting in a 4-fold lower efficiency (Table 1). When the V_0 data was fitted into the Hill equation, the resulting Hill coefficient n equals to 1, indicating that the phosphorylation of dThd followed Michaelis-Menten kinetics. For dCyd, however, the K_M increased and V_{max} decreased (Table 1). These results indicate that Q110 may not directly be involved in dThd binding but play an important role in the conformational changes involved in substrate binding.

We next investigated the binding of ATP/Mg^{2+} to the Q110A mutant. The K_M and V_{max} values for ATP/Mg^{2+} were 19 μM and 176 nmol/min/mg, respectively, and this 2-fold higher K_M value and 4-fold lower V_{max} value for ATP/Mg^{2+} resulted in about 10-fold lower efficiency when compared

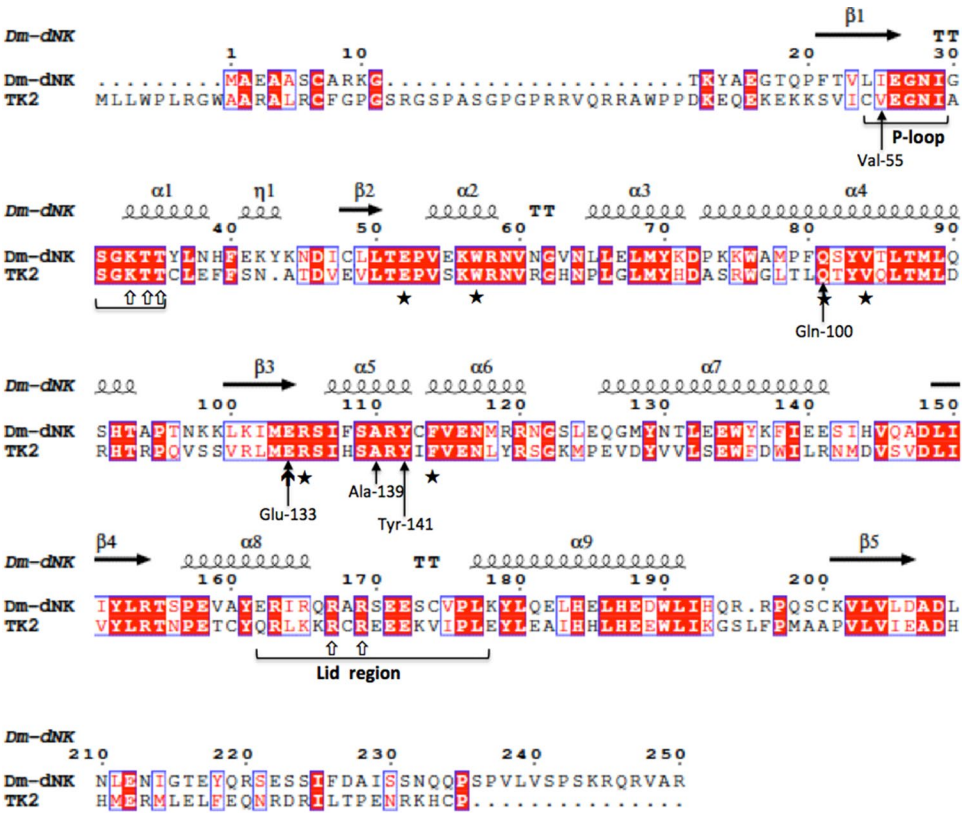

Figure 1. Alignment of human TK2 and DmdNK sequences. The secondary structure and numbering of DmdNK is shown on top of the sequences. Conserved sequence motifs (p-loop and Lid region) and residues in DmdNK that interact with dThd (★), Mg^{2+} (↑) and ATP (⇧) are marked. The mutated TK2 residues are also marked.

Table 1. Kinetic parameters of TK2 mutants with dThd and dCyd as substrate.

	dThd			dCyd	
Enzyme	K_M (μM)	V_{max} (nmol/min/mg)	Hill coefficient	K_M (μM)	V_{max} (nmol/min/mg)
Wild type	6.6 ± 1.6	1396 ± 51	n < 0.5	20 ± 5	714 ± 25
Q110A	8.9 ± 0.8	305 ± 7	n = 1	71 ± 9	331 ± 12
E133D	3.2 ± 0.5	169 ± 21	n < 0.6	16 ± 2	115 ± 8
Y141S	4.8 ± 0.6	1375 ± 81	n = 1	22 ± 2	671 ± 14
Y141F	8.5 ± 0.9	1340 ± 37	n < 0.4	10 ± 3	800 ± 23

Assays were performed in the excess of ATP/$MgCl_2$ and variable ^{3}H-dThd or ^{3}H-dCyd concentrations. Data were from 3 to 5 independent measurements and given as mean ± SD.

to the wtTK2 (Table 2). The Mg^{2+} ion requirement was also tested at fixed ATP concentration (1 mM). As shown in Figure 2, wtTK2 reached maximal activity at 0.2 mM Mg^{2+}, however, >1 mM Mg^{2+} was needed for Q110A mutant to reach maximal activity (Figure 2). These results suggested that Q110 might be directly involved in binding of Mg^{2+} ion and Q110 to A mutation caused inefficient interaction/binding of Mg^{2+} ion, leading to drastically reduced activity.

Table 2. ATP/Mg^{2+} dependence of wild type and mutant TK2.

	K_M (μM)	V_{max} (nmol/min/mg)	V_{max}/K_M
Wild type	8.2 ± 2	693 ± 31	85
Q110A	19 ± 3	176 ± 4	8.8
E133D	26 ± 2	171 ± 2	6.6

*Variable concentration of ATP/$MgCl_2$ and fixed ^{3}H-dThd (11 μM) concentration were used to determine the K_M and V_{max} values. Data were from 3 to 5 independent measurements and given as mean ± SD.

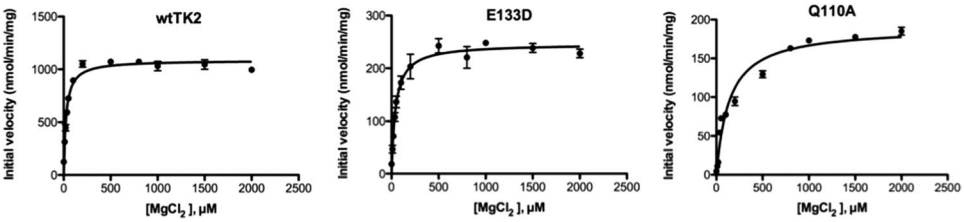

Figure 2. Magnesium requirement for the wtTK2, E133D and Q110A mutant enzymes. The assays were performed with fixed ^{3}H-dThd (11 μM) and ATP (1 mM) concentrations.

3.2. Residue Glu-133

Several residues in the sequence motif -ERSXXSD/ARY- in TK2 were investigated. In DmdNK structure Glu-104 in this motif is involved in Mg^{2+} binding.[16] The corresponding residue in TK2 is Glu-133, which was mutated to Q, D, or A. The E133D mutant showed much lower overall activity as compared to the wtTK2 (Table 1). When assayed in excess of ATP and Mg^{2+}, the K_M value for dThd decreased and the K_M value for dCyd was unchanged, but the V_{max} values were much lower (Table 1). This indicated that the E133D mutation affected the binding of dThd but not dCyd to the enzyme. This is most likely because the adjacent residue in TK2, i.e., Arg-134 corresponds to Arg-105 in DmdNK, which interacts with substrate in the DmdNK structure, and therefore, the E133D mutation could cause changes in microenvironment that favors the binding of the Arg-134 to dThd in TK2 leading to decreased K_M value. Next we examined the ATP/Mg^{2+} kinetics and found that the K_M value was 3-fold higher and the V_{max} value was 4-fold lower as compared with wtTK2 (Table 2). At fixed ATP concentration (1 mM), the Mg^{2+} concentration required to reach maximal activity for wtTK2 was 0.2 mM but for the E133D mutant it was >1 mM, which is 5-fold higher than that of wtTK2 (Figure 2). These data suggested that Glu-133 interacted directly with Mg^{2+} and indirectly with ATP and therefore, functionally involved in the catalysis. E133Q and E133A mutant enzymes showed no detectable activity (data not shown), further demonstrating that the carboxyl group of Glu-133 is critical for TK2 function.

3.3. Residue Ala-139

An important difference between the TK2 enzyme family in the sequence motif -ERSXXS**D**/**A**RY- is Ala-139 in TK2 and Ala-110 in DmdNK but Asp is the corresponding residue in dCK and dGK. Therefore, Ala-139 in TK2 was mutated to either D or E. The A139D and A139E mutant enzymes were inactive. No further analysis could be done. In human patients, mutations of Ala-139 to either Thr or Val caused MDS.[5] Thus, it is likely that Ala-139 is structurally important and any changes may cause local structural perturbation resulting in the loss of activity.

3.4. Residue Tyr-141

Tyr in this motif -ERSXXSD/AR**Y**- is conserved in all of the TK2 family enzymes. When Tyr-141 was mutated to a structurally conserved amino acid Phe, the Y141F mutant did not change either K_M or V_{max} for dThd but the K_M value for dCyd was somewhat lower as compared with wtTK2 (Table 1). This suggests that there are no direct interaction via hydrogen bonds between Tyr-141 and the substrates. When Tyr-141 was mutated to Ala, the mutant enzyme was totally inactive, suggesting that a bulky side chain in this position is important for enzyme function.

However, when Tyr-141 was mutated to a Ser residue, the Y141S mutant showed clearly different properties. The rate of catalysis and the binding affinity of the mutant enzyme were similar to the wtTK2, but the kinetic data for dThd phosphorylation gave a Hill coefficient n = 1 (Table 1). Thus, the Y141 to Ser mutation changed a TK2 with negative cooperative to a non-cooperative enzyme. These results indicated that Tyr-141 is involved in conformational changes that most likely occur during catalysis and substitution of Tyr-141 to Ser most likely alter this conformational flexibility of TK2.

4. Discussion

The nucleotide binding loop among all ATP/GTP-binding proteins has a conserved sequence figure print" -GXXXXGKT/ST-". Earlier studies with HSV1-TK and Vaccinia virus (VVTK) showed that changes of any of the conserved Gly residues resulted in loss of enzyme activity.[18,19] We changed the preceding residue Val-55 in TK2 to a Gly or Arg (Figure 1) and the resulting mutant enzymes were inactive (data not shown), suggesting that any disturbance in the p-loop structure would affect enzyme catalysis.

In a kinase-catalyzed reaction Mg^{2+} ions play a chelating role in catalysis by shielding the charge of the γ-phosphoryl group from the attacking

nucleophile, and enhance the cleavage of the Pγ-O bond by electrophilic effects. The structural role is to orientate the phosphate chains to a proper conformation.[20] We identified two residues in TK2 i.e., Gln-110 and Glu-133 that are apparently directly involved in binding to ATP/Mg^{2+} and thus essential for catalysis. A similar study of Asp-78 in this motif of the heterodimeric deoxyguanosine kinase/deoxyadenosine kinase from *Lactobacillus acidophilus* R26 showed that when Asp-78 was mutated to Ala, Glu or Gln all mutant enzymes were inactive. It was suggested that Asp-78 is essential for enzyme activity and that these mutations may cause disruption in protein folding leading to the loss of activity.[21] This study strongly suggests that in TK2 both Glu-133 and Gln-110 are required for holding the Mg^{2+} ion in a proper position inside the active site.

Human TK2 exhibited negative cooperativity with dThd but the phosphorylation of dCyd followed Michaelis-Menten kinetic.[12,22] An asymmetric conformational change of each subunit induced by binding of the substrate, according to the "induced fit" theory, could explain negative cooperativity.[23] These types of enzymes exist in two conformational states, a low affinity ligand binding state and a high affinity ligand binding state and during catalysis conformational changes occur sequentially for substrate binding and product release. The TK2 Y141S mutant showed no changes of either the K_M or V_{max} values for dThd and dCyd, however, the phosphorylation of dThd was no longer negative cooperative. The Q110A mutant demonstrated altered kinetic mechanism with both dThd so that the phosphorylation of dThd was no longer negative cooperative rather followed Michaelis-Menten kinetics. These results indicated that Tyr-141 and Gln-110 are involved in conformational changes of TK2 during substrate binding and catalysis, probably due to induced fit. Future structure determinations of TK2 will clarify the mechanisms involved in the allosteric behavior of this key enzyme in deoxynucleotide metabolism.

In conclusion structure and function studies of TK2 can provide important information for understanding of how TK2 mutation cause mitochondrial diseases and for future design of new drugs and substitution therapies of great medical value.

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