Kinetic Properties of Mutant Human Thymidine Kinase 2 Suggest a Mechanism for Mitochondrial DNA Depletion Myopathy*

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Thymidine kinase 2 (TK2) is a mitochondrial (mt) pyrimidine deoxynucleoside salvage enzyme involved in mtDNA precursor synthesis. The full-length human TK2 cDNA was cloned and sequenced. A discrepancy at amino acid 37 within the mt leader sequence in the DNA compared with the determined peptide sequence was found. Two mutations in the human TK2 gene, His-121 to Asn and Ile-212 to Asn, were recently described in patients with severe mtDNA depletion myopathy (Saada, A., Shaag, A., Mandel, H., Nevo, Y., Eriksson, S., and Elpeleg, O. (2001) Nat. Genet. 29, 342–344). The same mutations in TK2 were introduced, and the mutant enzymes, prepared in recombinant form, were shown to have similar subunit structure to wild type TK2. The I212N mutant showed less than 1% activity as compared with wild type TK2 with all deoxynucleosides. The H121N mutant enzyme had normal K_m values for thymidine (dThd) and deoxycytidine (dCyd), 6 and 11 µM, respectively, but 2- and 3-fold lower V_max values as compared with wild type TK2 and markedly increased K_m values for ATP, leading to decreased enzyme efficiency. Competition experiments revealed that dCyd and dThd interacted differently with the H121N mutant as compared with the wild type enzyme. The consequences of the two point mutations of TK2 and the role of TK2 in mt disorders are discussed.

Mitochondrial DNA (mtDNA)1 replication is not cell cycle-regulated; therefore, a constant supply of deoxynucleoside triphosphates (dNTPs) is required. Two mitochondrial membranes separate these dNTP pools from the cytosolic/nuclear dNTP pools, and the mt dNTPs are either imported from cytosol or synthesized in situ in mitochondria by salvage enzymes. In proliferating cells the biosynthesis of dNTPs occurs via de novo synthesis, but in resting cells or terminally differentiated cells salvage of pre-existing deoxynucleosides is essential for providing the dNTPs for nuclear DNA repair and mtDNA synthesis (1–3).

In mammalian cells the first step in the salvage of deoxynucleosides is carried out by thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK) in the cytosol and thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) in the mitochondria. Both TK1 and TK2 use thymidine (dThd) and deoxuridine as substrates, while TK2 also phosphorylates deoxycytidine (dCyd). The expression of TK1 is cell cycle-regulated with the highest level in S phase cells and very low or no activity in resting cells. In contrast TK2 is constitutively expressed at low level in all tissues. dCK is able to phosphorylate dCyd, deoxyadenosine (dAdo), and deoxyguanosine (dGuo) and is mainly expressed in lymphoid tissues. Similar to dCK, dGK phosphorylates dGuo, and to some extend dCyd and is constitutively expressed in all tissues (1–3).

Abnormal mt dNTP synthesis has recently been associated with inherited mitochondrial DNA depletion disorders such as mitochondrial neurogastrointestinal encephalomyopathy and external ophthalmoplegia due to deficiencies in thymidine phosphorylase and the mt ADP/ATP translocator protein, respectively (4–6). These enzymes, like all other mtDNA replication factors/encephalomyopathy and external ophthalmoplegia due to deficiencies in thymidine phosphorylase and the mt ADP/ATP translocator protein, respectively (4–6). These enzymes, like all other mtDNA replication factors/zymes, are coded by nuclear genes, and these types of autosomal disorders are defects in the cross-talk between the mitochondrial and nuclear genomes, manifested as multiple deletions and depletion of mtDNA (5).

Very recently individuals lacking dGK activity were described, and they showed severe hepatocerebral symptoms due to mtDNA depletion in the affected tissues (7). The patients had early onset of progressive liver failure, lactic acidosis and neurological abnormalities. The molecular defect was a deletion in the dGK gene leading to truncation of the dGK protein and total lack of the protein in liver. The lack of dGK supposedly led to defective synthesis of dATP and dGTP needed for mtDNA replication (7).

Simultaneously another type of mtDNA depletion disease was described where two single point mutations in the TK2 gene, giving H121N and I122N substitutions, were found. The numbering used here is based on the full-length TK2 sequence, and they were previously numbered as His-90 and Ile-181 (8). This disease was associated with devastating mitochondrial myopathy in infancy of patients from four different families (8). The characterization of the TK2 activity in extracts from muscle mitochondria from the patients was complicated due to the low the levels of TK2 activity found in the extracts. Therefore a more detailed characterization of these two TK2 mutants with purified enzyme was needed.

In the present study we have used site-directed mutagenesis to introduce the same mutations in TK2 as those found in the patients and characterized the recombinant enzymes in kinetic experiments with dThd, dCyd, and ATP. Inhibition studies with the substrates and feedback inhibitors dCTP and dTTP were also performed. A mechanism for the observed mtDNA
depletion is presented, and a characterization of the full-length human TK2 cDNA, including the mt leader sequence, is also presented.

**EXPERIMENTAL PROCEDURES**

**Materials**—Imetaphospho-[3H]thymidine (25 Ci/mmol) and [5-3H]deoxyuridine (24 Ci/mmol) were purchased from Amersham Biosciences. Non-radioactive nucleosides were from Sigma, and all other chemicals were of the highest quality available.

The Cloning and Sequencing of the TK2 Gene—Human TK2 cDNA (10) was used as probe to screen the human genomic BAC library (Genome Systems Inc.), and positive clones were identified and purchased. The TK2 gene was either sequenced directly by using the BAC DNA or subcloned into the BlueScript vector and then sequenced with BigDye™ terminator kit and the Prisma 300 system (PerkinElmer Life Sciences). Sequencing primers were chosen from exon sequences. The first exon was identified and used to search the GenBank™ databases with the BLAST program. Full-length TK2 cDNA clones were identified, and one clone (accession number AL685865) was obtained from ResGen™ (Invitrogen) and sequenced. The full-length human TK2 sequence has been deposited in the GenBank/EMBL/DDBJ databases under the accession number of Y10498.

**Mutagenesis**—An N-terminal-truncated TK2 protein, starting from amino acid number 51 according to the full-length human TK2 sequence presented here, had been characterized earlier, and it showed identical kinetic properties to those of the native enzyme (10). Therefore, this form of the enzyme was used as wild type TK2 in this study. Two complementary oligonucleotides were designed for each mutant, and the forward oligo sequences were for H121N mutant: 5'-TGGGACAGGAATCTCGGTTC-3'; for I212N mutant: 5'-CCTGCGTGCAACTACCTC-3'. Bold letters indicate the desired mutations. The T7 promoter primer was paired with reverse mutant oligos, and forward mutant oligos were paired with the T7 terminator primer, and wild type TK2 cDNA, which has been cloned into the PET-14b vector, was used as template in PCR reactions. The amplified two PCR fragments were purified and fused together due to their sequence complementation and used as templates later to amplify the entire sequence using the T7 promoter primer and the T7 terminator primer. This later PCR product was digested with NotI and BamHI and then subcloned into the PET-14b vector (Novagen, Madison). The mutations were verified by sequencing.

**Expression and Purification**—The plasmids that contained the desired mutations were transformed into BL21 (DE3) pLyS S bacteria. Induction was performed for 2 h in the presence of 0.4 mg isopropyl-1-thio-β-D-galactopyranoside at 37°C. Wild type TK2 expression was performed in parallel as a control. The bacteria were harvested by centrifugation at 3000 × g for 15 min at 4°C, and the pellet was resuspended in lysis buffer (50 mM Tris/HCl, pH 7.6, 0.5 M NaCl, 0.5% Triton X-100, and 140 μM phenylmethylsulfonyl fluoride). Total proteins were extracted by freezing and thawing two times and followed by sonication at 16 A for 30 s. The lysate was centrifuged at 100,000 × g for 90 min at 4°C. Then the enzymes were purified by metal affinity chromatography (TALON, Clontech) essentially as described in the manufacturer's instruction. Both wild type and mutant enzymes were eluted with 0.25 M imidazole in buffer containing 50 mM Tris/HCl, pH 7.9, 0.5 M NaCl, 0.1% Triton X-100, and 10% glycerol. The fractions containing TK2 activity were pooled and concentrated by using a Centricon device (Millipore). Aliquots of purified enzymes were analyzed by 12% SDS-PAGE, and protein concentration was determined by Bio-Rad protein assay using BSA as standard.

**Enzyme Assay**—TK2 activity was determined by using [3H]dThd or [3H]Cyd as substrates as described previously (10). The standard reaction mixture contains 50 mM Tris/HCl, pH 7.6, 2 mM ATP, 2 mM MgCl₂, 0.5 mg/ml BSA, 5 mM DTT, and 11 μM [3H]-labeled nucleoside and purified enzyme. One unit is defined as the formation of 1 nmol product (dTMP or dCMP) per min per mg of protein. For the measurement of the kinetic constants a wide concentration range of each substrate was used. The data were analyzed by the Sigma Plot Enzyme Kinetic Module version 1.1 (SPSS Inc).

**Gel filtration Chromatography**—Purified enzymes were analyzed on a Superdex 200 column using an fast protein liquid chromatography system (Amersham Biosciences) in a buffer containing 20 mM Tris/HCl, pH 7.6, 2 mM MgCl₂, 1 mM DTT, and 0.2 mM KCl. The column was calibrated using following molecular mass markers: blue dextran, β-amylase (200 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa). The active molecular masses of the wt TK2 and the mutant proteins were estimated from the standard curve. Fractions were collected and assayed for dThd kinase activity.

**RESULTS AND DISCUSSION**

The Primary Structure of the TK2 Gene Clarified the mt Location of TK2—Biochemical and cell fractionation studies have shown that TK2 is a mitochondrial enzyme (11, 12). However, controversy exists regarding the existence of a cytosolic form of TK2 (13), and furthermore, two published human TK2 cDNA sequences do not include a mt leader sequence (10, 14). Earlier attempts to clone the 5′-end of human TK2 cDNA were unsuccessful; however, recent methodological development (15) enabled amplification of the TK2 5′-cDNA sequence, which has a very high GC content. The mt leader sequence of human TK2 showed high homology with the mouse TK2 sequence (16) but they differ in the presumed mitochondrial cleavage sites (Fig. 1A). The N-terminal peptide sequence of human TK2 purified from brain (10) showed that amino acid number 37 was a tyrosine. However, direct translation of the cDNA sequence and the genomic DNA sequence give an arginine in this position. At present we can not explain this discrepancy, but one possibility is that the Arg-37 is post-translationally modified and eluted in the position of Tyr in the sequencing procedure. It has been observed earlier with the prion protein that a Tyr and Arg were inverted in the epitope sequence due to post-translational modification of the Arg residue (17, 18).

We have partially sequenced the TK2 genomic DNA fragments, mainly the exons and the intron/exon boundaries, and our results agree with the published human genome sequence (19). The human TK2 gene occupies a 45-kb DNA fragment on chromosome 16 and consists of 10 exons of 32 bp up to 1304 bp and 9 introns of 0.572 kb up to 11.1 kb (Fig. 1B). The mt leader sequence is coded by exon 1, and the entire 3′-untranslated region by exon 10. The 5′ sequences of the TK2 isoform A and B reported earlier (14) are part of the intron sequences, suggesting that these isoforms are alternatively spliced mRNA products that may not be translated. Therefore, we may conclude that TK2 fulfills the criteria for being a mitochondrial enzyme.

Preparation of wt and Mutant TK2 Proteins—A site-directed mutagenesis method was used to produce the two TK2 mutants, H121N and I212N, previously numbered as His-90 and Ile-181, which were shown to be linked to severe mtDNA depletion disorder (8). These mutant enzymes were expressed in Escherichia coli strain BL21 (DE3) pLyS S at high level and purified by one step affinity chromatography to more than 99% purity by the same procedure as the wild type enzyme (10) (Fig. 2). There were no differences in the levels of expression or the yield of purification in case of the wt and the two mutant enzymes. The N termini of these enzymes contain a His tag and a thrombin cleavage site, and they were characterized without removal of the His tag since the presence of the His tag did not affect the kinetic properties of TK2 in previous studies (10).

To rule out possible effects of the mutations of the TK2 on overall structure, purified wt and the two mutant proteins were subjected to gel filtration chromatography on a Superdex 200 column. Wild type TK2 activity eluted as a broad peak, coinciding with the UV absorbance peak at a molecular mass range of 30–40 kDa. Both H121N and I212N mutant TK2 proteins eluted at similar positions during gel filtration chromatography (Fig. 3). Even though the I212N mutant protein had very low activity, a significant dThd phosphorylating activity (and UV) peak could be detected (Fig. 3). In no case were there indications of high molecular forms of TK2. We conclude from this experiment that the mutant enzymes and wt TK2 apparently have similar subunit interaction and are most likely dimers. Thus, there was no evidence for major structural alter-
The unusual elution behavior of TK2 on size exclusion chromatography has been observed earlier (20). Similarly, mouse TK2 and Drosophila melanogaster deoxynucleoside kinase (dNK) also showed different molecular sizes depending on the type of columns used in gel filtration chromatography (16, 21, 22), and this may be related to the interaction between TK2-like enzymes and the gel matrix.

The Kinetic Properties of wt and Mutant TK2—The I212N mutant showed about 100-fold lower $V_{\text{max}}$ values with both dThd and dCyd as substrates compared with wt TK2 when ATP was in excess. The $K_m$ value for dThd was similar to that of wt TK2, while the $K_m$ value for dCyd was very high (Table I). Overall, this mutant enzyme had about 1% efficiency ($V_{\text{max}}/K_m$ value) with dThd and less than 1% with dCyd as compared with wt TK2 (Table I).

The H121N mutant showed a more complex kinetic pattern, and surprisingly, both the $K_m$ and $V_{\text{max}}$ values with dThd as substrate were only somewhat decreased, which resulted in an efficiency similar to wt TK2. However, with dCyd as substrate, the $V_{\text{max}}$ value was only about 30% of that of wt TK2, while the $K_m$ value was similar (Table I). Another significant change with the H121N mutant is the loss of negative cooperativity with dThd as substrate (Hill constant $n = 1$).

TK2 has long been shown to have different kinetics toward its two natural substrates, dThd and dCyd. The phosphorylation of dThd did not follow Michaelis-Menten kinetics but showed negative cooperativity as indicated by biphasic Eadie-Hofstee plot ($v$ versus $v/s$) and a Hill constant $n < 1$. In contrast, the phosphorylation of dCyd followed Michaelis-Menten kinetics and the Eadie-Hofstee plot for dCyd phosphorylation was a straight line, and the Hill constant $n = 1$. To compare the efficiency of the wt and mutant TK2 enzymes, the $K_m$ value with dThd as substrate (Hill constant $n = 1$).

**FIG. 1.** A, N-terminal amino acid sequence alignment of human and mouse TK2. Arrows indicate the mitochondrial cleavage sites and the underlined sequence is the N-terminal sequence of processed mitochondrial TK2 (11, 17). The letter Y above the human TK2 sequence indicates the amino acid found in purified human TK2 (11); B, the human TK2 gene structure.

**FIG. 2.** SDS-PAGE of purified wt and H121N and I212N mutant TK2 enzymes. Lanes 1 and 6, molecular mass markers; lane 2, uninduced culture; lanes 3, 4, and 5, induced culture of wt, H121N, and I212N respectively; lanes 7, 8, and 9, purified wt, H121N, and I212N enzymes respectively.

**FIG. 3.** Gel filtration chromatography on Superdex 200 column. The dThd activity with wt (circles), H121N mutant (squares), and I212N mutant (triangles) TK2 enzymes. The elution positions of β-amylase (200 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) are indicated with arrows. Vo, void volume.

The H121N mutant is the loss of negative cooperativity with dThd as substrate (Hill constant $n = 1$).
activity with dCyd as substrate, and an altered cooperativity with dThd as substrate. These mutant enzymes were also characterized with regard to their phosphate donor properties with ATP. The $K_m$ value for wt TK2 was 25 $\mu$M when dThd was the phosphate acceptor, which is similar to the $K_m$ value (38 $\mu$M) reported earlier for native TK2 (24). The $K_m$ values for ATP of the H121N mutant with saturating dThd or dCyd were 3 to 4-fold higher than that of wt TK2, while the $V_{\text{max}}$ values were similar with dThd and reduced by 60% with dCyd (Table II). The I212N mutant showed very low $V_{\text{max}}$ values and 2 to 4-fold increased $K_m$ values for ATP with dThd and dCyd as compared with those of wt TK2 (Table II). The conclusions from these experiments are that the I212N mutant showed very low catalytic rates with all substrates, while the H121N mutant showed a 10-fold reduced capacity to use dCyd as phosphate acceptor when ATP was a limiting phosphate donor.

To further clarify the kinetic properties of the H121N mutant a series of substrate and feedback inhibitor studies were performed (Table III). The most clear cut difference between the mutant and wt TK2 was the competition between dThd and dCyd as alternative substrates. Since the dThd phosphorylation by wt TK2 was negatively cooperative, double reciprocal plots (1/V versus 1/S) in the presence/absence of dCyd were not linear, which made it difficult to interpret the mode of inhibition by dCyd. To simplify this we chose the data sets from the low concentration range where the double reciprocal plots were linear to analyze the mode of inhibition. Plots of the intercepts and slopes versus dCyd concentration were linear, which indicated that the inhibition by dCyd was non-competitive, and the $K_i$ value was 40 $\mu$M (Fig. 4 and Table III). For the H121N mutant enzyme double reciprocal plots of dThd phosphorylation over the entire concentration range were linear. The slope of intercepts versus dCyd concentration plot was zero, which suggested that dCyd competitively inhibited dThd phosphorylation, and the $K_i$ value (4 $\mu$M) was 10-fold lower as compared with wt TK2 (Fig. 4 and Table III). The dCyd phosphorylation by both wt and H121N mutant was competitively inhibited by dThd at similar concentrations; the apparent $K_i$ values were 4.9 and 3.6 $\mu$M, respectively (Fig. 4 and Table III).

The feedback inhibitors, dTTP and dCTP, showed similar inhibition patterns with both wt and the H121N mutant, and the $K_i$ values, with dThd as the substrate, were 2-fold higher with wt TK2 as compared with the H121N mutant (Table III). The results with wt TK2 were similar to what was described earlier with native TK2; dTTP was a competitive inhibitor toward both dThd and ATP, and the $K_i$ values were in the same range (3 to 7 $\mu$M) (20, 23–25). TK2 as well as all other deoxyribonucleoside kinases are feedback-inhibited by their end products, i.e. deoxynucleoside triphosphates. These feedback inhibitors probably act as bistable analogues and not as classical allosteric inhibitors binding to separate effector site on the enzyme (1). Earlier studies with native TK2 and our kinetic data with recombinant enzymes indicated that the deoxynucleoside triphosphates could bind to the phosphate acceptor site since they showed competitive inhibition with the phosphate acceptor. This possibility has been proven by the recent three-dimensional structure determinations for dGK and

### Table I

**Kinetic parameters of human wild type TK2 and the H121N and I212N mutant enzymes**

The values were from at least three independent assays. The $K_m$ and $V_{\text{max}}$ values were calculated using the Michaelis-Menten equation. The concentrations of dCyd and dThd were varied from 1 to 321 $\mu$M, and ATP/MgCl$_2$ concentration was kept constant (2 mM). The Hill constants ($n$) for dThd phosphorylation are given in parenthesis.

| Substrate | $K_m$ (nM) | $V_{\text{max}}$ (nM) | $V_{\text{max}}/K_m$ |
|-----------|------------|----------------------|---------------------|
| dThd      | 13 ± 3 (0.5) | 1288 ± 72 | 99 |
| dCyd      | 11 ± 1 | 789 ± 25 | 72 |
| H121N     | 5.7 ± 0.8 (1.0) | 589 ± 17 | 103 |
| 5.7 ± 1 | 277 ± 10 | 25 |
| I212N     | 18 ± 3 (0.7) | 12 ± 5 | 0.67 |
| 568 ± 160 | 8 ± 1 | 0.01 |

### Table II

**The kinetic parameters of wild type and mutant TK2 enzymes with ATP/MgCl$_2$**

The concentration of dCyd and dThd was 100 $\mu$M, and the ATP/MgCl$_2$ concentrations were varied from 5 to 2000 $\mu$M. The values were calculated as described in Table I. The Hill constants ($n$) are given in parenthesis.

| Substrate | $K_m$ (nM) | $V_{\text{max}}$ (nM) | $V_{\text{max}}/K_m$ |
|-----------|------------|----------------------|---------------------|
| dThd      | 25 ± 0.7 (0.7) | 104 ± 3 | 41.8 |
| dCyd      | 37 ± 9 (0.6) | 716 ± 39 | 19.4 |
| H121N     | 80 ± 14 (0.6) | 760 ± 4 | 9.5 |
| 162 ± 31 (0.8) | 325 ± 18 | 2.0 |
| I212N     | 95 ± 14 (0.7) | 10 ± 0.0 | 0.11 |
| 59 ± 11 (0.7) | 2 ± 0.1 | 0.03 |

### Table III

**The inhibition pattern and $K_i$ values of wild type and the H121N mutant TK2 with substrates and end products**

All the assays were performed with the substrate (dCyd or dThd) concentration varied from 1 to 321 $\mu$M and fixed concentrations of ATP/MgCl$_2$ (2 mM) and inhibitors. The type of inhibition was evaluated with the Sigma Plot Enzyme Kinetic Module v1.1. from a set of experiments with different inhibitor concentrations.

| Substrate | Inhibitor | Wild type | H121N mutant |
|-----------|-----------|-----------|--------------|
| dCyd      | dThd      | 4.9 ± 1 | 3.6 ± 0.2 |
| dCyd      | dCyd      | 0.87 ± 0.1 | 0.89 ± 0.1 |
| dCyd      | dTTP      | 0.5 ± 0.3 | 2.5 ± 0.3 |
| dThd      | dCyd      | 0.40 ± 0.7 | 4.0 ± 0.9 |
| dThd      | dCTP      | 0.79 ± 0.2 | 0.36 ± 0.1 |
| dThd      | dTTTP     | 2.0 ± 0.5 | 0.89 ± 0.3 |
D. melanogaster dNK, which showed that ATP as well as dTTP bound to the phosphate acceptor site of the active enzymes (3, 9). These results showed again relatively subtle alterations in the kinetic properties of the H121N mutant, and it was manifested as an apparently increased capacity of dCyd to compete with dThd as substrate. In case of wt TK2, dCyd is a relatively inefficient competitor of dThd phosphorylation. This result is similar to what was reported earlier with purified lymphoblast TK2 (20), but in that study the $K_i$ value (630 μM) for dCyd was about 10-fold higher than in the present one using wt TK2. We do not know the reason for the difference between the recombinant TK2 used here and the purified lymphoblast enzyme, but it is most likely related to the differences in enzyme preparations. Overall this phenomenon is probably related to the negative cooperativity observed with dThd as substrate for TK2 (1, 2, 20). The mutant enzyme behaved more according to what could be expected by an alternate substrate in a classic enzyme competition experiment.

A major goal in this study was to clarify the molecular background for the mitochondrial location of TK2 by identifying the mt translocation signal in the TK2 gene sequence. Furthermore, we wanted to mimic the situation in patients with point mutations in TK2 who suffered from severe myopathy due to the mtDNA depletion disorder by in vitro mutagenesis and kinetic characterization of pure recombinant TK2 preparations. The kinetic results with the recombinant enzymes strongly support the conclusions made in the earlier study with mitochondrial extracts from the mtDNA depletion patients (8).

The relative rates of dThd and dCyd phosphorylation carried out by TK2 in the physiological situation as well as the action of feedback inhibition of deoxynucleoside triphosphates most likely regulate the size of dTTP and dCTP pools in mitochondria as illustrated in Fig. 5. The I212N mutant enzyme had severely decreased activity, while the H121N mutant had a reduced capacity to use dCyd as substrate, and these changes are apparently due to inefficient ATP/Mg$^{2+}$ binding and catalysis. These results correlated well with the clinical severity of the pathologic effect of specific TK2 mutations: the patients with I212N mutation had severe myopathic changes of muscle histology, while the patients with H121N mutation had relatively mild symptom and later onset of myopathy (8, 26). Recently two novel TK2 mutations, T77M and I22M, were identified in patients with mtDNA depletion syndrome and the...
pathogenicity of these mutations was confirmed by reduced TK2 activity in muscle extracts (27).

Native TK2 had been shown to have negatively cooperative kinetics with ATP as substrate, and the $K_m$ value for ATP was dependent on the concentration of dThd; at physiologically relevant concentration of dThd (5 μM), the $K_m$ value for ATP was 200 μM (25). Although it has not been possible to measure the ATP content in the mitochondria of these patients at the time of biopsy, oligomycin-sensitive ATP synthase (complex V) activity was measured (the direction of ATP hydrolysis) and found to be severely decreased in all patients (web supplement Table A in Ref. 8). Additionally, oxygen consumption of mitochondria from the patient carrying the His mutation was measured in freshly isolated muscle mitochondria. The oxygen consumption was severely decreased with all substrates tested, which imply that the respiratory chain was malfunctioning also in vivo and that the formation of a proton gradient across the mitochondrial inner membrane was hampered. It is therefore reasonable to assume that this will lead to a low mitochondrial ATP content (web supplement Table A in Ref. 8), which in turn may lead to an even lower efficiency of the mutant TK2 enzymes than what was observed in vitro with the recombinant enzymes. Therefore, the H121N mutation probably resulted in very low mt dCTP and dTTP pools, while the H212N mutation most likely leads to normal dTTP pool but very low dCTP pool as compared with the dATP and dGTP pools (Fig. 5). The inability to salvage deoxynucleosides and the imbalance in mt dNTP pools should preferentially affect tissues/or cells where no uptake of dNTPs from the cytosol is possible due to the lack of de novo DNA precursor synthesis (1, 2). Earlier study has suggested that mtDNA copy number may be regulated by tightly controlled mitochondrial dNTP pools (26). The altered mt dNTP pools can be expected to give increased levels of mutations and depletions of mtDNA; the latter has been observed in the patients with myopathy (8, 27–29). The onset of the mtDNA depletion syndrome caused by TK2 mutation is probably dependent on mtDNA turnover and other metabolic situations in the tissues, e.g. the developmental down-regulation of key enzymes such as ribonucleotide reductase. One of the future tasks is to explain why deficiency in TK2 leads to functional defects of muscles. The establishment of a relevant animal model system would be an important step toward this goal and may in the future also enable development of gene and chemotherapies for this type of devastating mt disorders.

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