Mitochondrial deoxyguanosine kinase mutations and mitochondrial DNA depletion syndrome

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Abstract Mitochondrial deoxyguanosine kinase (dGK) catalyzes the initial phosphorylation of purine deoxynucleosides. Mutations in the dGK gene leading to deficiency in dGK activity is one of the causes of severe mitochondrial DNA depletion diseases. We used site-directed mutagenesis to introduce the clinically observed genetic alterations in the dGK gene and characterized the recombinant enzymes. The R142K enzyme had very low activity with deoxyguanosine and no activity with deoxyadenosine. The E227K mutant enzyme had unchanged K_m values for all its substrates but very low V_{max} values. C-terminal truncated dGK proteins were inactive. These results may help to define the role of dGK in mitochondrial DNA (mtDNA) precursor synthesis.

Key words: Deoxyguanosine kinase; Mutation; DNA precursor; Mitochondrial DNA depletion

1. Introduction

The synthesis of mitochondrial DNA (mtDNA) is not cell cycle regulated and a constant supply of deoxyribonucleoside triphosphate (dNTP) is vital for the maintenance of mitochondrial integrity. However, there is apparently no de novo nucleotide biosynthesis in the mitochondria. Nucleotides required for mtDNA replication are either synthesized by the salvage enzymes in the mitochondria or imported from the cytosol [1,2]. Mitochondrial deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK2) are the two enzymes which are responsible for the initial rate-limiting phosphorylation of all the four deoxynucleosides in the synthesis of precursors for mtDNA replication. Both dGK and TK2 are constitutively expressed in all tissues and the levels of these two enzymes are believed to correlate to the number of mitochondria [1,2]. In resting cells and terminally differentiated cells the de novo synthesis of deoxynucleotides is not present nor is the salvage synthesis of thymidine nucleotides via thymidine kinase 1. In this case the import of nucleotides from the cytosol to the mitochondria is not possible, and the salvage of deoxynucleosides by dGK and TK2 is the only source of DNA precursors.

Mitochondrial DNA depletion syndrome (MDS) is a clinically heterogeneous group of disorders characterized by a reduction in mtDNA copy number. Primary mtDNA depletion is inherited as an autosomal recessive trait and may affect single organs, typically muscle or liver, or multiple tissues [3]. Recently, the myopathic form of MDS has been ascribed to mutations in the TK2 gene [4], and the hepatocerebral form has been ascribed to mutations in the dGK gene [5]. Since then, several additional mutations have been identified in either the TK2 or the dGK genes of patients presenting with MDS [6–10].

In order to understand the mechanism resulting in the disease phenotypes caused by dGK deficiency we used site-directed mutagenesis to introduce the mutations, base pair deletions and insertion found in the patients reported recently [5–7]. The recombinant dGK enzymes were expressed in *Escherichia coli*, purified and characterized with the most important substrates.

2. Materials and methods

2.1. Materials

Radiolabelled nucleosides [8-^{3}H]-2′-deoxyguanosine (^{3}H-dGuo; 5.9 Ci/mmol) and [2, 8-^{3}H]-2′-deoxyadenosine (^{3}H-dAdo; 17.1 Ci/mmol) were purchased from Moravek biochemicals Inc. Non-radioactive nucleosides were from Sigma.

2.2. Mutagenesis, expression and purification

Point mutations were introduced by a polymerase chain reaction (PCR)-based method as described [11] and the PCR fragments which contained the desired mutations were subcloned into the pET-9d vector (Novagen) as was wild-type dGK cDNA [12]. The mutations were verified by sequencing using the BigDye™ terminator kit and ABI Prism 310 system (Perkin-Elmer).

The plasmids which contain the desired mutations were transformed into the *BL21* (*DE3*) *pLysS* bacteria (Novagen). All mutant dGK enzymes were expressed and purified by the same procedure as the wild-type dGK as described previously [13] with the exception that no adenosine triphosphate (ATP) was added in the elution buffer. Aliquots of purified enzymes were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and protein concentration was determined by Bio-Rad protein assay using bovine serum albumin (BSA) as standard.

2.3. Enzyme assay

dGK activity was determined by using [^{3}H]dGuo or [^{3}H]dAdo as substrates as previously described [12,14]. The standard reaction mixture contains 50 mM Tris/HCl, pH 7.6, 2 mM ATP, 2 mM $MgCl_2$, 0.5 mg/ml BSA, 5 mM dithiothreitol and 50 μM labelled deoxynucleoside and purified enzyme. For the measurement of the kinetic constants a wide concentration range of each substrate was used. The data were

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Abbreviations: dGK, deoxyguanosine kinase; TK2, thymidine kinase 2; dGuo, deoxyguanosine; dAdo, deoxyadenosine; mtDNA, mitochondrial DNA; MDS, mitochondrial DNA depletion syndrome

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Table 1
Summary of the known genetic defects in the dGK gene

Mutations	Outcome (dGK enzyme alteration)	Disease onset and survival
A (nucleotide 204) deletion[a]	frame shift and premature stop at 80 amino acids	from birth to 6 months, death before 1 year of age
C (nucleotide 313) to T[b]	premature stop at 105 amino acids	From birth, died at 5 months of age
GT (nucleotides 609–610) deletion[c]	premature stop at 213 amino acids	from 2 months, died at 6 months of age
GATT (nucleotides 763–766) duplication[c]	premature stop at 255 amino acids	from 2 months, died at 5 months of age
G (nucleotide 425) to A[c]	point mutation R142K	this patient was compound heterozygous
G (nucleotide 679) to A[c]	point mutation E227K	from 6 months, underwent liver transplantation at 17 months; this patient is alive at 5 years of age

[a]From [5].
[b]From [7].
[c]From [6].

analyzed by the Sigma Plot Enzyme Kinetic Module version 1.1 (SPSS Inc.).

3. Results

3.1. Genetic alteration in the dGK gene of patients with MDS

To date there are five reported alterations in the dGK gene, including deletions, point mutations or base pair insertion, which caused MDS in patients with hepatic failure as summarized in Table 1. Four of these alterations resulted in premature stop and truncation of the dGK proteins and the affected individuals had severe liver diseases and died at early infancy. The point mutations found in a compound heterozygous patient had milder effects and the patient survived after liver transplantation [5–7]. In order to correlate the disease phenotype to dGK enzyme deficiency we used site-directed mutagenesis to introduce these genetic alterations in the dGK gene and expressed the recombinant enzymes in *E. coli*. They were subsequently purified and characterized with regard to substrate specificity and catalytic efficiency.

3.2. Truncations at the C-terminal end of the dGK proteins

A GATT (nucleotides 763–766) duplication in exon 7 resulted in a 22 amino acid deletion at the C-terminus. This C-terminal truncated dGK protein was expressed in *E. coli* and purified. The recombinant protein was inactive. To verify the functional importance of the C-terminal domain of dGK, two other C-terminal deletion mutants were generated in the same way with 11 and 18 amino acid truncations. Both recombinant enzymes were inactive. These results suggested that the C-terminal domain is indispensable for enzyme activity. Thus, one can predict that other truncated dGK proteins (Table 1) are most likely inactive.

3.3. Properties of the R142K mutant enzyme

Recombinant R142K enzyme had very low activity with dGuo and no detectable activity with dAdo. With dGuo as substrate the K_m value was about 30-fold higher and the V_{max} value was about 20-fold lower as compared to wild-type dGK (Table 2). Therefore, the efficiency for dGuo phosphorylation by the R142K mutant enzyme was only about 0.2% of the wild-type enzyme. The ATP/$MgCl_2$ dependence was also examined, the K_m value was about 11-fold higher and the V_{max} value was about 20-fold lower as compared with the wild-type enzyme (Table 3).

3.4. Properties of the E227K mutant enzyme

Recombinant E227K enzyme had low activity with both dGuo and dAdo. The K_m values for dGuo, dAdo and ATP/$MgCl_2$ with either dGuo or dAdo as the fixed substrate were similar to that of the wild-type dGK (Tables 2 and 3). However, the V_{max} values were much lower; 27-, 22-, 31- and 58-fold lower respectively, as compared with the wild-type enzyme (Tables 2 and 3). Thus, the efficiencies for dGuo and dAdo phosphorylation were only 2.8 and 5.5% of the wild-type dGK (Table 2).

4. Discussion

MDS is characterized as quantitative reduction of mtDNA copy number, which impairs the synthesis of respiratory chain components [3]. The synthesis of mtDNA is carried out by several nuclear encoded proteins, e.g. DNA polymerase γ. However, mtDNA copy number has been suggested to be controlled by the mitochondrial dNTP pools [15]. Deficiency in either dGK or TK2 activity would impair the synthesis of DNA precursors in the mitochondria and lead to imbalance

Table 2
Kinetic parameters of wild-type and mutant dGK enzymes with phosphate acceptors

	dGuo			dAdo		
	K_m (μM)	V_{max} (units)	Efficiency (V_{max}/K_m)	K_m (μM)	V_{max} (units)	Efficiency (V_{max}/K_m)
Wild-type[a]	4	43	10.8 (100)	467	429	0.9 (100)
R142K	123 ± 1	2.0 ± 0.01	0.02 (0.19)	NA	NA	
E227K	5.2 ± 1	1.6 ± 0.1	0.3 (2.8)	414 ± 54	19.6 ± 1.6	0.05 (5.5)

For the measurement of phosphate acceptor constants the dGuo or dAdo concentration was varied (4–505 μM) and the concentration of ATP/$MgCl_2$ was constant (2 mM). One unit is defined as the formation of 1 nmol dGMP (or dAMP) per min per mg protein. Data in parentheses are relative values as compared with the wild-type enzyme.
NA: no detectable activity.
[a]Data from [13].

Table 3
Kinetic parameters with ATP/$MgCl_2$ for both wild-type and mutant dGK enzymes

	dGuo (100 μM as acceptor)			dAdo (100 μM as acceptor)		
	K_m (μM)	V_{max} (units)	Efficiency (V_{max}/K_m)	K_m (μM)	V_{max} (units)	Efficiency (V_{max}/K_m)
Wild-type	8.5 ± 1	22 ± 0.6	2.6 (100)	269 ± 28	215 ± 7	0.80 (100)
R142K	97.4 ± 1	1.1 ± 0.01	0.2 (7.7)	NA	NA	
E227K	14 ± 2	0.68 ± 0.01	0.05 (1.9)	242 ± 40	3.7 ± 0.2	0.02 (2.5)

For the measurement of phosphate donor constant ATP/$MgCl_2$ was varied (20–2000 μM) and dGuo or dAdo was at fixed concentration. One unit is defined as the formation of 1 nmol dGMP (or dAMP) per min per mg protein. Data in parentheses are relative values as compared with the wild-type enzyme.
NA: no detectable activity.

of dNTP pools, which eventually would cause mtDNA depletion.

Human dGK has been characterized enzymatically [12,13,16] and the three-dimensional (3-D) structure is known [17]. The C-terminal α-helix number 9 (α9) of dGK is part of the phosphate donor binding site and therefore plays an important role in enzyme catalysis [17,18]. Deletion of the C-terminal 22 amino acid residues resulted in an incomplete phosphate donor binding site. Indeed the C-terminal 22 amino acid deletion mutant is devoid of enzyme activity, which demonstrated the importance of α9 in enzyme catalysis. Additionally, deletions of part of α9, e.g. 11 and 18 amino acid truncations, resulted in virtually inactive enzymes. Therefore, one may assume that mutations that caused premature stops in the dGK gene would lead to dGK deficiency in the tissues of the affected individuals.

Two point mutations, i.e. R142K and E227K, have also been identified in patient with MDS [6]. These mutant enzymes were constructed and characterized. Both residues, R142 and E227, are conserved among the family of deoxynucleoside kinases, including dGK and TK2. R142 is located close to the active site but E227 is not. Residue E227 is located in α8. It forms hydrogen bonds with residues Y191 and Q193 in β4 and appears not to interact directly with any of the substrates [6,17,18]. Experimental results with recombinant E227K enzyme indicated no alteration of substrate binding capacity as revealed by the K_m values but it showed a severely decreased catalytic rate. The hydrogen bonds between α8 and β4 probably stabilize the enzyme active site structure. The so-called LID region is a connecting loop between α7 and α8 [17,18] and it is believed that the LID structure undergoes dynamic movements during catalysis. Substitution of glutamate with a lysine residue in this position, E227K, introduced an opposite charge in the area and also a loss of two hydrogen bonds to Y191 and Q193. Such a disturbance may impair enzyme function which is reflected in low catalytic rates.

In contrast to the E227K mutation, the R142K mutation affected the binding of all the substrates; the mutant enzyme had apparently lost its ability to phosphorylate dAdo and also showed very low efficiency for dGuo phosphorylation. In the dGK 3-D structure R142 is situated in the active site, and interacts by a hydrogen bond with the 5′-OH group of the nucleoside [17]. The other amino acid residue that forms a hydrogen bond with the 5′-OH group is E70 and their contacts with the base largely determine the substrate specificity (Fig. 1) [17,18]. The amino group of K142 is between 1 and 2 Å further away from the 5′-OH group as compared with R142 (Fig. 1). This clearly diminished the catalytic capacity of dGK, possibly by not being able to stabilize the transition state of the reaction intermediates. The R142K mutant is thus providing a start point for detailed studies of the reaction mechanism for this class of deoxynucleoside kinases.

Translation of mtDNA-encoded proteins is dependent on mtDNA copy number since the expression of mtRNA polymerase is directly correlated to mtDNA copy number [19]. Therefore, mtDNA depletion will result in a reduced amount of mtDNA-encoded enzymes and thus low respiratory activity that, in turn, leads to low ATP content. In the in vivo situation the combination of a poor dGK enzyme and low ATP concentration may result in very low deoxyadenosine triphosphate (dATP) and deoxyguanosine triphosphate pools as compared with deoxythymidine triphosphate and deoxycytidine triphosphate pools. Liver, heart, skeletal muscle and

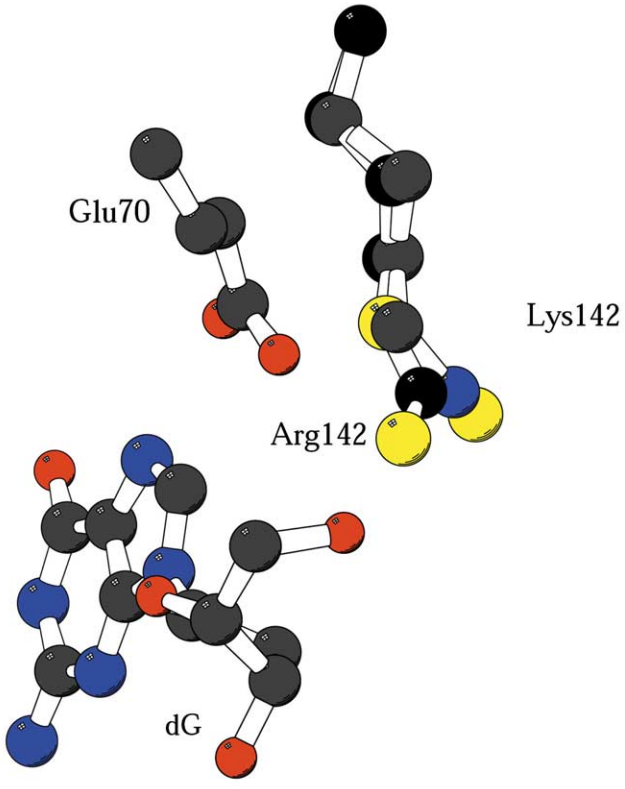

Fig. 1. A model of the dGK structure with the active site residues Glu70 and Arg142 or Lys142 that are in close contact with the 5′-OH group of the substrate (deoxyguanosine: dG). The model is based on the structure of dGK with bound ATP [17], where the adenine base and deoxyribose have been substituted by dG and the mutant Lys142 has been aligned to fit the position of Arg142 as close as possible. Color indications: carbon atoms are in black, oxygen atoms are in red, nitrogen atoms are in blue except that in Arg142 the nitrogen atoms are in yellow for comparison.

brain are among the most energy-dependent tissues of the body and therefore they are vulnerable to mtDNA depletion, most likely through the operation of this vicious circle.

The residual dGK activity seems to play a crucial role in disease progression. Patients who completely lacked dGK activity had in common early onset of the disease and died at earlier infancy. The patient who was compound heterozygous with point mutations, e.g. R142K and E227K, had about 27% dGK activity as compared with control individuals [6]. Even though the mutant enzymes had very low activity, the onset of the symptoms was later as compared with other cases and the symptoms were milder.

The establishment of a correlation between the dGK activity levels and disease development may encourage development of therapeutic regimes to help patients to suppress the disease. Organ transplantation or gene therapy are among the possible approaches but they may not be available to all affected individuals. Therapies which stimulate nucleotide biosynthesis either via the de novo or the salvage pathway to increase the cellular dNTP pools may increase the chance to delay disease development. In line with this assumption, it was recently reported that partial reversion of mtDNA depletion due to dGK deficiency could be obtained by providing resting patient fibroblast with deoxyadenosine monophosphate (dAMP) or deoxyguanosine monophosphate added to the culture medium [20].

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