Studies of Enzymes in Mitochondrial DNA Precursor Synthesis

Regulatory Mechanisms for Human Thymidine Kinase 2 and Deoxyguanosine Kinase

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2013

Acta Universitatis agriculturae Sueciae
2013:64

Cover: Yin yang bagua arrangement
(photo: adopted from Wikimedia Commons)

ISSN 1652-6880
ISBN (print version) 978-91-576-7866-9
ISBN (electronic version) 978-91-576-7867-6

Print: SLU Service/Repro, Uppsala 2013

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Abstract

As important enzymes in mitochondrial nucleotide salvage pathway, thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) are expressed constitutively in almost all cells. These two enzymes catalyze the initial rate-limiting phosphorylation of pyrimidine and purine deoxynucleosides, respectively, providing DNA precursors for mitochondrial DNA (mtDNA) replication.

Inherited genetic defects in TK2 have been associated with infantile myopathic form of mtDNA depletion syndrome (MDS). In study I, two mutations, R225W and a novel T230A, in TK2 are identified as a new genetic cause of adult-onset autosomal recessive progressive external ophthalmoplegia (arPEO) and the kinetic and structural effects of the two mutations on enzyme function have been characterized.

Nucleoside analogs are widely used in anti-viral and anti-cancer chemotherapy, but they can cause severe side-effect such as mtDNA depletion. In study II, the potential mechanism underlying pyrimidine nucleoside analogs-associated mitochondrial toxicities was investigated, and showed that thymidine analogs had opposite effects on dThd and dCyd phosphorylation and thus can inhibit dThd salvage, leading to imbalanced dTTP and dCTP pools. It was found that the mechanism is most likely due to that TK2 normally exists in an inactive form with bound dTTP.

The redox regulation of TK2 and dGK was studied in study III and IV. The activity of both enzymes was sensitive to the cellular redox status. Under oxidative stress, both TK2 and dGK can be reversibly S-glutathionylated by GSSG. The modification of the conserved Cys189 in TK2 was responsible for a partial inactivation and selective degradation of TK2 in mitochondria, most likely via the AAA^+ Lon protease. The oxidative effect of nucleoside analogs was also evaluated. Treatment with 3'-azido-2',3'-dideoxythymidine (AZT) and 2',3'-dideoxyinosine (ddI) led to degradation of mitochondrial TK2 and dGK, whereas uridine and guanosine supplementations to AZT respective ddI treatments prevented both proteins from degradation.

Keywords: Thymidine kinase 2, Deoxyguanosine kinase, Mitochondrial DNA, Progressive external ophthalmoplegia, Nucleoside analogs, AZT (3'-azido-2',3'-dideoxythymidine), ddI (2',3'-dideoxyinosine), S-glutathionylation.

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Dedicated to my family

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I *Tyynismaa, H., ***Sun, R.**, Ahola-Erkkila, S., Almusa, H., Poyhonen, R., Korpela, M., Honkaniemi, J., Isohanni, P., Paetau, A., Wang, L. & Suomalainen, A. (2012). Thymidine kinase 2 mutations in autosomal recessive progressive external ophthalmoplegia with multiple mitochondrial DNA deletions. *Hum Mol Genet* 21(1), 66-75.

* Shared first authorship

II Wang, L., **Sun, R.** & Eriksson, S. (2011). The kinetic effects on thymidine kinase 2 by enzyme-bound dTTP may explain the mitochondrial side effects of antiviral thymidine analogs. *Antimicrob Agents Chemother* 55(6), 2552-8.

III **Sun, R.**, Eriksson, S. & Wang, L. (2012). Oxidative stress induced S-glutathionylation and proteolytic degradation of mitochondrial thymidine kinase 2. *J Biol Chem* 287(29), 24304-12.

IV **Sun, R.**, Eriksson, S. & Wang, L. The role of S-glutathionylation in degradation of mitochondrial thymidine kinase 2 and deoxyguanosine kinase: potential implication for toxicities of nucleoside analogs. M*anuscript*.

Papers not included in the thesis:

V **Sun, R.**, Eriksson, S. & Wang, L. (2010). Identification and characterization of mitochondrial factors modulating thymidine kinase 2 activity. *Nucleosides Nucleotides Nucleic Acids* 29(4-6), 382-5.

VI **Sun, R.** & Wang, L. (2013). Inhibition of *Mycoplasma pneumoniae* growth by FDA-approved anticancer and antiviral nucleoside and nucleobase analogs. *BMC Microbiol* 13, 184.

Abbreviations

Nucleosides

Ado/dAdo	Adenosine/deoxyadenosine
Ino/dIno	Inosine/deoxyinosine
Guo/dGuo	Guanosine/deoxyguanosine
Cyd/dCyd	Cytidine/deoxycytidine
Urd/dUrd	Uridine/deoxyuridine
dThd	Thymidine (deoxythymidine)

Nucleotides

NMP	Nucleoside monophosphate
NDP	Nucleoside diphosphate
NTP	Nucleoside triphosphate
dNMP	Deoxynucleoside monophosphate
dNDP	Deoxynucleoside diphosphate
dNTP	Deoxynucleoside triphosphate

Nucleoside analog reverse transcriptase inhibitors

AZT	3’-azido-2’,3’-dideoxythymidine
ddI	2’,3’-dideoxyinosine
ddC	2’-3’-dideoxycytidine
d4T	2’,3’-didehydrodideoxythymdine
FLT	3’-fluoro-2’,3’-dideoxythymidine

Deoxynucleoside kinases

TK1	Thymidine kinase 1
dCK	Deoxycytidine kinase
dGK	Deoxyguanosine kinase
TK2	Thymidine kinase 2

Other abbreviations

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
GSH	Glutathione
GSSG	Glutathione disulphide
Cys	Cysteine

1 Introduction

1.1 DNA precursors metabolism

Nucleotides, the precursors of RNA and DNA, *i.e.*, ribonucleoside and deoxyribonucleoside phosphates, are involved in almost all aspects of cellular metabolism, including acting as carriers of activated metabolites for biosynthesis, metabolic regulators, storages of chemical energy, and structural moieties of coenzymes either alone or in combination with other molecules. For instance, cyclic adenosine monophosphate (cAMP) acts as a second messenger in the signal transduction pathway, while adenosine triphosphate (ATP) is the ubiquitous intracellular energy currency acting as a cosubstrate in many enzymatic energy-requiring processes (Nygaard & Saxild, 2009).

A nucleotide is composed of a five-carbon sugar - ribose or deoxyribose, one or more phosphoryl groups, and one nitrogenous base which can be divided into two families: purine bases including adenine (A) and guanine (G) and pyrimidine bases including cytosine (C), thymine (T), and uracil (U). Thymine is found exclusively in DNA, while uracil is in RNA. The addition of a base to a ribose or 2-deoxyribose creates a ribonucleoside, which is found in RNA, or a deoxyribonucleoside, which is a constituent of DNA. The attachment of one phosphate group to the 5'-hydroxyl group of the pentose moiety generates a nucleoside monophosphate (NMP/dNMP). The additions of the second and third phosphoryl groups to nucleoside monophosphate result in nucleoside diphosphates (NDP/dNDP) and triphosphates (NTP/dNTP). Different from nucleosides, nucleotides are negative charged because of the phosphate groups.

With the exceptions of some bacteria and parasitic protozoa, nucleotides within most organisms and cells are generated by two distinct biosynthetic routes *i.e.*, the *de novo* pathway and the salvage pathway (Traut, 2001). Nucleotides synthesized *de novo* can meet the large demand for DNA/RNA precursors in growing and proliferating cells, whereas pre-formed nucleosides

and bases can be recycled by the salvage pathway to maintain the nucleotide pools with less energy consumption (Zrenner *et al.*, 2006). Both the synthetic pathways and nucleotide catabolism pathways are presented briefly in the following sections.

1.1.1 The *de novo* biosynthesis of deoxyribonucleotides

Both purine and pyrimidine rings can be synthesized *de novo* from low molecular weight precursors, *e.g.*, CO_2, glutamine and aspartic acid through many enzymatic reactions, which consume considerable energy provided predominantly by ATP. Serving as a source of ribose and phosphate, 5-phosphoribosyl-1-pyrophosphate (PRPP) is a common and essential precursor for purine and pyrimidine nucleotide biosynthesis. It is synthesized from ribose 5-phosphate and ATP, and the reaction is catalyzed by PRPP synthetase (ribose-phosphate pyrophosphokinase). It is notable that PRPP participates in not only the *de novo* pathway of nucleotide biosynthesis, but also the salvage of nucleobases.

Synthesis of purine nucleotides

The heterocyclic purine ring is assembled on ribose 5-phosphate donated by PRPP. The atoms of the base moiety originate from CO_2, glycine, aspartate, glutamine, and N^{10}-formyl-tetrahydrofolate, forming the purine structure. The initial product of purine biosynthesis is inosine 5'-monophosphate (IMP or inosinate), which is generated by ten successive reactions. IMP is then converted in two additional steps to adenosine 5'-monophosphate (AMP) or guanosine 5'-monophosphate (GMP), both of which can be further phosphorylated to the diphosphate forms (NDP) by base-specific nucleoside monophosphate kinases (NMPKs), *i.e.*, adenylate kinase (AMPK) and guanylate kinase (GMPK). Nucleoside diphosphate kinase (NDPK) catalyzes the phosphorylation of NDP to the corresponding NTP and there are several isoforms present in different cell compartments (Figure 1) (Traut, 1988).

DNA precursors, namely 2'-deoxyribonucleotides, are synthesized by the reduction of ribonucleotides. In mammalian cells, this is the committed step in the dNTPs *de novo* synthesis and it is catalyzed by ribonucleotide reductase (ribonucleoside diphosphate reductase, RNR), which reduces ribonucleoside diphosphate (NDP) to deoxyribonucleoside diphosphate (dNDP). The products dADP, dGDP, dUDP and dCDP are converted to dATP, dGTP, dUTP and dCTP by NDPK (Figure 1).

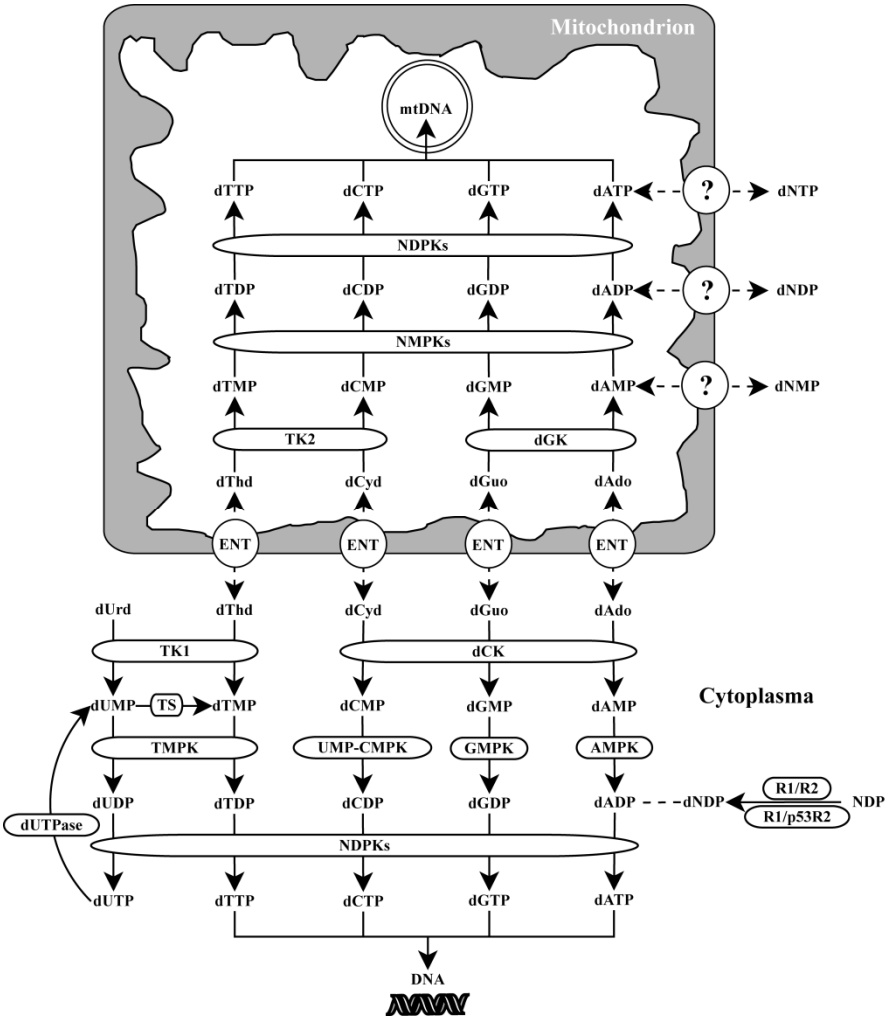

Figure 1. An overview of DNA precursor synthesis. Abbreviations: TK1, thymidine kinase 1; dCK, deoxycytidine kinase; TK2, thymidine kinase 2; dGK, deoxyguanosine kinase; TMPK/GMPK/AMPK and UMP-CMPK, thymidylate/guanylate/adenylate kinase and UMP-CMP kinase; NMPKs, nucleoside monophosphate kinases; NDPKs, nucleoside diphosphate kinases; TS, thymidylate synthase; dUTPase, dUTP nucleotidohydrolase; R1/R2/p53R2, large/small/p53-inducible small subunit of ribonucleotide reductase.

Synthesis of pyrimidine nucleotide

Pyrimidine bases are generated in the form of orotate from CO_2, glutamine, and aspartate, prior to being attached to the ribose 5-phosphate of PRPP. The newly synthesized pyrimidine ring is then converted to orotidine 5'-monophosphate (OMP) by orotate phosphoribosyltransferase with PRPP as the ribose phosphate donor. Thereafter, OMP is decarboxylated to form uridine

monophosphate (UMP), which can be further phosphorylated to uridine di/triphosphate (UDP/UTP). Cytidine triphosphate (CTP) is synthesized from UTP by CTP synthetase (Jones, 1980).

Different from the *de novo* generation of deoxycytidylate described above, thymidine monophosphate (dTMP) is synthesized from dUMP by thymidylate synthase (TYMS or TS) with N^5,N^{10}-methylene tetrahydrofolate as the methyl donor and successively phosphorylated into dTTP by thymidylate kinase (TMPK) and NDPK as shown in Figure 1 (Costi, 1998).

1.1.2 The salvage pathway

Nucleotides can also be synthesized from the salvage of bases and nucleosides originating from the diet and normal turnover of cellular nucleic acids. In organisms lacking the *de novo* pathway, salvage of purine and pyrimidine is an essential process. In most tissues of mammals the salvage pathway also plays a vital role in the maintenance and regulation of intracellular nucleotide pools (Figure 1). Free purine bases can be converted directly to mononucleotides with PRPP as the ribose 5-phosphate source by two enzymes: (i) hypoxanthine-guanine phosphoribosyltransferase which directly converts guanine and hypoxanthine to GMP and IMP, and (ii) adenine phosphoribosyltransferase which salvages adenine to AMP (Nyhan, 2001). Recycling of nucleosides is facilitated by a variety of kinases. Uridine-cytidine kinase is involved in the utilization of pyrimidine nucleosides, catalyzing the phosphorylation of uridine and cytidine to UMP and CMP, and adenosine kinase catalyzes the phosphorylation of Ado to AMP (Van Rompay *et al.*, 2001; Spychala *et al.*, 1996). The salvage of deoxynucleosides is catalyzed by deoxynucleoside kinases, *e.g.*, cytosolic thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK), mitochondrial thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) (Figure 1). TK1 catalyzes the initial phosphorylation of dThd and dUrd, while dCK phosphorylates dCyd, dAdo and dGuo to dCMP, dAMP and dGMP. In mitochondria, purine nucleosides (dAdo, dGuo, and dIno) and pyrimidine nucleosides (dThd, dCyd, and dUrd) are phosphorylated to the corresponding nucleoside 5'-monophosphates by dGK and TK2, respectively. These enzymes have different substrate specificities and subcellular localizations and the characteristics of the enzymes are discussed in detail in the next section (Figure 1) (Arner & Eriksson, 1995).

1.1.3 Purine and pyrimidine catabolism

A fraction of free purines and pyrimidines is recycled via the salvage pathway, whereas excess nucleosides and their metabolites must be catabolized in order to maintain the homeostasis of the nucleotide pools and prevent the

accumulation of free bases within cells. High levels of purines can be toxic to cells and tissues.

The de-phosphorylation of nucleoside monophosphate (NMP/dNMP) is catalyzed by 5'-nucleotidases (5'-NTs), which include a family of enzymes *e.g.*, mitochondrial and cytosolic 5'(3')-deoxynucleotidases (Bianchi & Spychala, 2003). In primates including humans, the end product of purine catabolism is uric acid, which is mainly formed in liver and excreted by the kidney into urine. Several enzymes are involved in purine degradation but the basic structure of the purine ring is not broken down. The ubiquitous purine nucleoside phosphorylase (PNP) enzyme breaks down purine nucleosides adenosine, guanosine, and inosine to purine bases and ribose/deoxyribose-1-phosphate (Bzowska *et al.*, 2000). Guanine and hypoxanthine are then further converted to xanthine by guanine deaminase and xanthine oxidase (XDH), respectively. XDH also catalyzes the oxidation of xanthine to uric acid. In contrast, the catabolism of adenosine nucleotide requires additional step, *e.g.*, deamination of adenosine and AMP by adenosine deaminase and AMP deaminase, respectively (Cristalli *et al.*, 2001).

In contrast to purine catabolism, pyrimidine ring can be completely degraded into β-amino acids, ammonia and CO_2. Prior to the pyrimidine ring cleavage, pyrimidine nucleotides are hydrolyzed by 5'-NTs to the corresponding nucleosides, which are subsequently degraded into uracil (in the cases of CMP and UMP) and thymine (in case of dTMP) by cytidine deaminase, uridine phosphorylase, and thymidine phosphorylase, respectively. The ultimate breakdown of uracil and thymine is catalyzed by dihydropyrimidine dehydrogenase, dihydropyrimidinase, and ureidopropionase, and results in the generation of β-alanine and β-aminoisobutyrate, respectively.

1.2 Basic properties of mammalian deoxyribonucleoside kinases (dNKs)

With distinct intracellular compartmentalization and overlapping substrate specificities, mammalian deoxyribonucleoside kinases (dNKs), including thymidine kinase 1 and 2 (TK1 and TK2), deoxyguanosine kinase (dGK), and deoxycytidine kinase (dCK), catalyze the initial phosphorylation of deoxyribonucleosides as well as their analogs (Figure 1). This provides an alternative route for the synthesis of DNA precursors and contributes to a balanced supply of deoxyribonucleotides complementing the *de novo* synthesis pathway (Johansson *et al.*, 2001; Arner & Eriksson, 1995). This reaction is usually the rate-limiting step in the salvage pathway and the products can be further phosphorylated to dNTP by NMPKs and NDPKs. In terminally

differentiated or quiescent cells, due to the down-regulation of enzymes in the *de novo* biosynthesis of dNTPs, including ribonucleotide reductase (R1-R2) and TS, the dNKs play vital roles in the maintenance of intracellular dNTP pools for mitochondrial DNA (mtDNA) replication and nuclear DNA (nDNA) repair. Actually, dNKs, particularly TK2 and dGK are indispensable for mtDNA maintenance and deficiency in TK2 or dGK caused by genetic alternations results in tissue specific mtDNA depletion syndrome (MDS) and progressive external ophthalmoplegia (PEO) (Ronchi *et al.*, 2012; Tyynismaa *et al.*, 2012; Wang, 2010; Wang & Eriksson, 2010). Meanwhile, many nucleoside analogs used in chemotherapy of viral infections and cancers are administrated as pro-drugs and are dependent on the intracellular phosphorylation by dNKs to be pharmacologically active. Therefore, dNKs have gained special interests in the medical perspective. In the following sections, basic properties of the four mammalian dNKs are described with focus on mitochondrial TK2 and dGK.

1.2.1 Thymidine kinase 1 (TK1, E.C. 2.7.1.21)

Human thymidine kinase 1 (ATP:thymidine 5'-phosphotransferase) is a cytosolic deoxyribonucleoside kinase, encoded by the *TK1* gene, which spans 12.9 kb on chromosome 17q25.2-25.3 and consists of seven exons. TK1 exists as homodimers (ca 50 kDa) and/or homotetramers (ca 100 kDa) with a subunit size of 24-25 kDa (Munch-Petersen, 2010; Munch-Petersen *et al.*, 1993). Compared with the other mammalian dNKs, TK1 has many distinctive characteristics, *e.g.*, this enzyme only catalyzes the phosphorylation of dThd (as well as dUrd), and thus it has a more restricted substrate specificity. The expression of TK1 is highly cell-cycle dependent with a similar profile as the DNA precursors pool: the level of cytosolic TK1 is very low or absent in resting cells or in early G1 phase, increases during G1/S transition, reaches a maximum in S phase, declines in G2/M phase and disappears during mitosis (Munch-Petersen, 2010; Chabes & Thelander, 2003). Ke *et al.* proved that the ubiquitin-proteasome pathway was responsible for the mitotic degradation of human TK1 (hTK1) (Ke & Chang, 2004). The regulation of hTK1 expression occurs at the transcriptional, translational and post-translational levels and is important for the formation of dTTP. However, the mechanism for regulation of TK1 expression is beyond the scope of this thesis and will not be further discussed.

Although TK1 is found in almost all living organisms and has been widely studied, the most likely physiological role of this enzyme has not been resolved until recently when TK1 was found to play an important role in DNA repair (Chen *et al.*, 2010).

In addition, the diagnostic and prognostic potentials of TK1 as a biomarker of cell proliferation have been widely investigated and utilized in both human and veterinary medicine due to the tight correlation between TK1 level and cell proliferation. Increased TK1 activity and protein level in serum (sTK) have been found in different forms of cancers. Accordingly sTK has been used as a useful biomarker in health screening projects, for detection of potential and early-stage malignancies, detection of cancer progression, and as an efficacy marker for antitumor therapy (von Euler & Eriksson, 2011; O'Neill *et al.*, 2001).

1.2.2 Deoxycytidine kinase (dCK, E.C. 2.7.1.74)

Deoxycytidine kinase (dCK, NTP:deoxycytidine 5'-phosphotransferase) phosphorylates deoxycytidine, deoxyadenosine, deoxyguanosine and their analogs to their corresponding monophosphates with nucleoside triphosphates as phosphate donors. dCK plays an important role in overall dNTP metabolism and is a rate-limiting step for the activation of many nucleoside analogs, such as the anti-neoplastic agent 2-chlorodeoxyadenosine and the anti-viral analog 2'-3'-dideoxycytidine (ddC) (Eriksson *et al.*, 1995; Karlsson *et al.*, 1994; Piro, 1992).

Human dCK is encoded by a single-copy gene of 34 kb located on chromosome 4 band q13.3-21.1 with 7 exons, encoding a polypeptide of 261 amino acids (Song *et al.*, 1993). Active dCK purified from various human tissues is a 60 kDa dimer (Bohman & Eriksson, 1988). The expression of dCK occurs in a tissue-specific manner, *i.e.*, highest in lymphoid tissues, intermediate in proliferating cells, and lowest in differentiated tissues *e.g.*, muscle, kidney and brain. The regulation of dCK expression is likely due to both post-transcriptional and post-translational mechanisms (Hazra *et al.*, 2011; Eriksson *et al.*, 2002; Arner & Eriksson, 1995; Hengstschlager *et al.*, 1993).

Similar to TK1, the biological function of dCyd kinase is enigmatic. Toy and coworkers found that T and B lymphopoiesis was impaired in dCK knockout mice, while the development of other tissues and organs was not affected (Toy *et al.*, 2010). Inactivation of dCK was also reported to cause dCTP pool depletion, replication stress, and DNA replication arrest in early S-phase in T and B lymphoid cells in dCK knock-out mice (Austin *et al.*, 2012). That is in agreement with high dCK activity levels in lymphocytic tissues, thus demonstrating the role of dCK-associated dNTP synthesis pathways in certain tissues. Many studies of dCK are focused on its central role in the activation of nucleoside analog pro-drugs widely used in anti-cancer and anti-viral chemotherapy. With its broad substrate specificity dCK can phosphorylate dCyd analogs as well as several purine analogs, such as the anti-neoplastic

drugs cytosine arabinoside (araC), 2',2'-difluorodeoxycytidine (gemcitabine), 2-chlorodeoxyadenosine (cladribine), and β-L-dioxolane-cytidine (troxacitabine), as well as the anti-viral drugs ddC and β-l-2',3'-dideoxy-3'-thiacytidine (lamivudine) (Sabini *et al.*, 2003). The pivotal role of dCK in the clinical pro-drug activation is highlighted by the correlation between drug sensitivity and dCK activity in many target cells. Impaired dCK function has been found in cells resistant to nucleoside analogs, such as araC and gemcitabine (Ruiz van Haperen *et al.*, 1994; Owens *et al.*, 1992).

1.2.3 Thymidine kinase 2 (TK2, E.C. 2.7.1.21)

Thymidine kinase 2 (ATP:thymidine 5'-phosphotransferase), is the mitochondrial thymidine kinase responsible for salvaging pyrimidine deoxyribonucleosides, dThd, dUrd, and dCyd to yield the corresponding 5'-monophosphates, utilizing ATP or CTP as phosphate donors. Both dTTP and dCTP act as feedback inhibitors (Arner & Eriksson, 1995; Berk & Clayton, 1973). TK2 is encoded by a nuclear gene on chromosome 16q22 and mature TK2 is predominantly localized in mitochondria (Munch-Petersen, 2010; Wang & Eriksson, 2000; Johansson & Karlsson, 1997; Willecke *et al.*, 1977). Generally, TK2 is assumed to be expressed constitutively in nearly all tissues, in close correlation to their mitochondrial contents, but irrespective of cell cycles (Wang *et al.*, 1999; Arner & Eriksson, 1995). Wang *et al.* found that rat mt TK2 activity showed a tissue-specific distribution and was highest in brain, lung and spleen, intermediate in heart, kidney and liver, and lowest in skeletal muscle (Wang, 2010). In proliferating cells, TK2 level is much lower than the level of cytosolic TK1, whereas TK2 is the sole pyrimidine deoxyribonucleoside phosphorylating enzyme in post-mitotic tissues, where the *de novo* biosynthesis of dNTPs and cytosolic TK1 are down-regulated (Eriksson & Wang, 2002; Wang *et al.*, 1999). Consequently, TK2 appears to play a significant role in the constant and balanced supply of dNTPs for mtDNA replication and nDNA repair (Wang *et al.*, 2003). Among the four mammalian dNKs, mitochondrial TK2 and dGK are the least investigated enzymes due to their mitochondrial localization and low abundance. Furthermore, the 3D-structure of TK2 has not yet been solved.

Early studies on human TK2 were performed with partially purified proteins from human tissues such as leukemic spleen, placenta, liver, and brain and it was reported that TK2 was a 29 kDa monomer in the presence or absence of ATP or dThd (Jansson *et al.*, 1992; Munch-Petersen *et al.*, 1991). Studies on recombinant mouse TK2 showed that the active form of full-length mouse TK2 was a dimer (Wang & Eriksson, 2000). Analyzed by gel filtration analysis, recombinant human TK2 was also found to oligomerize to dimer and

in rat liver mitochondrial extract several more complex forms were recently described (Sun *et al.*, 2010). Therefore, it is still an open question which form of TK2 exists *in vivo*.

Human TK2 cDNA (1930 bp) was cloned based on amino acid sequence information from a brain cDNA library and an open reading frame for *TK2* (699 bp) corresponding to 232 amino acid residues were found, without any coding region for a mitochondrial target sequence (Wang *et al.*, 1999). The N-terminal truncated recombinant human TK2 (MW 28 kDa), corresponding to the mature mitochondrial TK2, exhibited indistinguishable kinetic properties with the native enzyme. Cloning of full-length mouse TK2 was performed by the same group and the open reading frame of the mouse TK2 cDNA contained an N-terminal mitochondrial targeting signal, which was proven to be essential for the mitochondrial translocation of TK2 (Wang & Eriksson, 2000). The *TK2* gene in human cells exhibits a complex and tissue-specific transcription pattern and four different transcripts were identified. Two major transcripts of 4.0 kb and 2.2 kb were found in most tissues, while two additional transcripts of 1.35 kb and 4.0 kb were discovered in proliferating tissues *e.g.*, testis and thymus (Wang *et al.*, 1999; Johansson & Karlsson, 1997).

TK2 is able to phosphorylate many pyrimidine nucleoside analogs, which are clinically utilized in anti-viral and anti-tumor therapies. Thymidine derivatives with modifications on the sugar moiety such as 3'-azido-2',3'-dideoxythymidine (AZT), arabinofuranosyl thymidine (araT) and 3'-fluoro-2',3'-dideoxythymidine (FLT) could be phosphorylated by TK2, but at much lower rates, while 2',3'-didehydrodideoxy-thymdine (d4T) was an inert substrate for TK2. TK2 showed less than 10% activity with AZT compared with that of dThd (Munch-Petersen *et al.*, 1991). In spite of a poor substrate, the phosphorylation of AZT by TK2 is important for the pharmacological activation of this pro-drug in non-proliferating cells such as macrophages (Arner *et al.*, 1992). TK2 can also recognize several dUrd analogs as substrates, including 5-fluorodeoxyuridine (FdUrd), 1-(2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil (FIAU), 2',2'-difluoro-deoxyuridine (dFdU), and in particular (E)-5-(2-bromovinyl)-2'-dUrd (BvdU). The latter has been used to discriminate the activities of TK1 and TK2 in extracts, because BvdU is an excellent substrate for TK2, exhibiting very low activity with TK1 (Wang & Eriksson, 2008; Wang *et al.*, 1999; Wang & Eriksson, 1996; Eriksson *et al.*, 1991). TK2 can also interact with several uridine derivatives, such as 5-methyl-Urd, 5-(2-bromovinyl)-Urd (5-BV-Urd), and 5-iodo-Urd with high affinity (K_i/K_m of 1-10), but very low catalytic efficacy. Uridine is a very poor substrate for TK2 (Balzarini *et al.*, 2000). In addition, TK2 is able to catalyze

the phosphorylation of a number of dCyd analogs such as 2',2'-difluoro-deoxycytidine (dFdC) and araC.

The first extensive kinetic analysis on TK2 was performed in 1990s by Munch-Petersen and coworkers with enzyme purified from human leukemic spleen. They found that when dCyd and dUrd were substrates, the reactions followed normal hyperbolic Michaelis-Menten kinetics, with K_m (μM) and V_{max}/K_m (units/mg/μM) values of 36 and 25 for dCyd, and 6 and 115 for dUrd, respectively. In contrast, the phosphorylation of dThd exhibited negative cooperativity with Hill coefficients (h or n) in the range of 0.3-0.5. It implies that the affinity of dThd to TK2 declined as its concentration increased as shown by the biphasic Hofstee plot; the K_m value was 0.3 μM, when the dThd concentration was below 8 μM, while above this concentration the K_m value of dThd was 16 μM. AZT phosphorylation was also associated with negative cooperativity (Munch-Petersen, 2010; Munch-Petersen *et al.*, 1991). These kinetic characterizations were in excellent agreement with the results obtained with N-terminally truncated recombinant human TK2 (Wang *et al.*, 1999).

McKee and coworkers investigated the kinetics of exogenous dThd and AZT phosphorylation by intact mouse mitochondria isolated from heart, liver, and brain by measuring the overall rate of total phosphorylation. They demonstrated that mitochondria isolated from all three tissues were capable of transporting dThd into the matrix, which contained all essential enzymes including TK2, TMPK, and NDPK, to convert dThd to mono/di/tri-phosphate forms. The kinetics of dThd phosphorylation showed apparent negative cooperativity, with different catalytic efficiencies. In addition, heart, liver and brain mitochondria could also take up AZT from the medium, but were unable to phosphorylate it beyond the monophosphate form. It was noteworthy that the negative cooperativity of AZT was observed in heart and liver mitochondria but not in brain mitochondria. Instead, mitochondria exhibited non-cooperativity of AZT phosphorylation and negative cooperativity of dCyd phosphorylation (McCann *et al.*, 2012; Susan-Resiga *et al.*, 2007; Lynx & McKee, 2006; McKee *et al.*, 2004). The complex and tissue-dependent kinetic behavior of TK2 contributes to the difficulties in understanding the regulation of this protein in physiological conditions. The mechanism underlying the biphasic kinetics of TK2 towards certain substrates is not clarified, which to a large extent could be due to the lack of structure information regarding TK2. It is unlikely that the TK2 monomer contains multiple substrate binding sites, which are supposedly required for cooperative kinetics. Therefore, it was suggested that TK2 may exist as a homo-multimer with two conformational forms in equilibrium (Radivoyevitch *et al.*, 2011; Munch-Petersen, 2010).

1.2.4 Deoxyguanosine kinase (dGK, E.C. 2.7.1.113)

dGK (NTP: deoxyguanosine 5'-phosphotransferase), also known as *DGUOK*, is an essential component of the dNTP salvage synthesis pathway in mammalian mitochondria and mediates the initial rate-limiting phosphorylation of dAdo, dIno and dGuo with ATP as the most efficient phosphate donor, while dATP and dGTP are feedback inhibitors. Together with TK2, mitochondrial dGK is able to phosphorylate all four deoxynucleosides to supply mitochondria with mtDNA building blocks. Similar to TK2, dGK is also assumed to be constitutively expressed in most tissues and the levels of dGK correlated to the amount of mitochondria (Wang & Eriksson, 2003; Eriksson *et al.*, 2002; Arner & Eriksson, 1995).

Human dGK is encoded by a nearly 32 kb gene (*DGUOK*) with 7 exons localized on chromosome 2q13 and its cDNA codes for a 277 amino acid polypeptide with a mitochondrial import signal (17 amino acids) at the N-terminus. A major 1.35-kb dGK mRNA transcript was reported in most tissues by Northern blot analysis, although four other alternative splice variants have been described (Mousson de Camaret *et al.*, 2007; Eriksson & Wang, 2002; Johansson *et al.*, 1996). The active form of dGK protein is a dimer of two identical 30 kDa subunits and normally located in the mitochondrial matrix. Mitochondrial dGK, however, was found to relocate to the cytosolic compartment at a similar rate as cytochrome c during the course of apoptosis in human epithelial kidney (HEK) 293 and human lymphoblast Molt-4 cell lines. Although the role of the specific redistribution of dGK is not understood, it might contribute to the apoptotic cascade by being involved in the activation of apoptotic nucleotide cofactors *e.g.*, dATP (Jullig & Eriksson, 2001).

Like other dNKs, dGK is able to phosphorylate several cytostatic analogs such as 2-chlorodeoxyadenosine, arabinosyl guanine, and 2',2'-difluorodeoxyguanosine, which are also substrates for cytosolic dCK due to the overlapping substrate specificity between the two kinases (Munch-Petersen & Piškur, 2007). Therefore, dGK may play an important role in the activation of these nucleoside pro-drugs, particularly in the tissues where dCK activity is low.

1.2.5 The structures of dNKs

The X-ray crystallographic three-dimensional structures of the human dCK, dGK, and TK1 have been solved since 2001 (Figure 2B) (Welin *et al.*, 2004; Sabini *et al.*, 2003; Eriksson *et al.*, 2002; Johansson *et al.*, 2001). According to the primary sequences, the dNK enzymes could be roughly divided into two families: TK1 forms a family of its own, indicating that this enzyme has a distinct phylogenetic origin; TK2, dCK, and dGK belong to a homologous

family (the dCK/dGK family), which also includes the dNK in *Drosophila melanogaster* (Dm-dNK). It is noteworthy that Herpes Simplex Virus 1 thymidine kinase (HSV1-TK) is also considered to belong to the dCK/dGK family due to its similar structural folds, although with less than 10% amino acid identity (Sandrini & Piskur, 2005; Brown *et al.*, 1995).

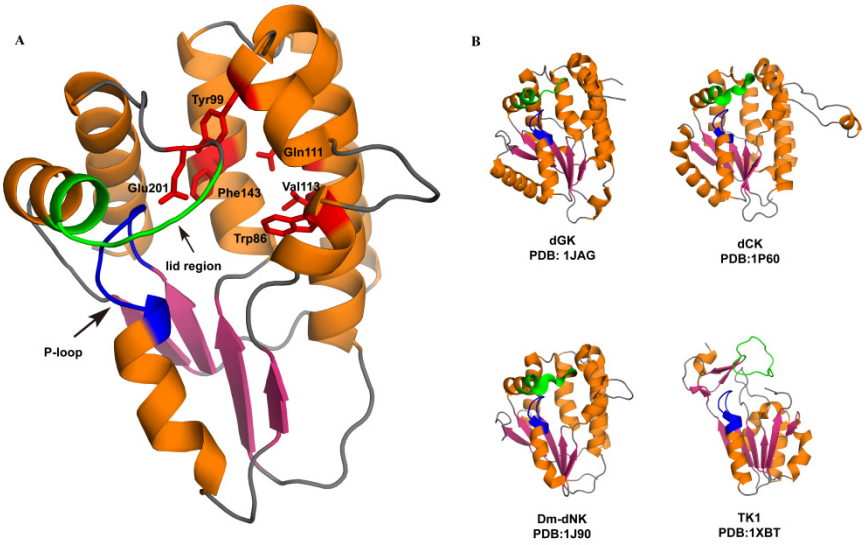

Figure 2. (A) Structure model of human TK2 (SwissPort O00142). This model is built with one subunit of Dm-dNK (PDB ID: 2VPPB) as the template. The side chains of major amino acid residues interacting with deoxynucleoside substrates are shown as sticks (in red) for greater clarity. (B) Monomeric structures of human dGK (PDB: 1JAG), dCK (PDB:1P60), TK1 (PDB:1XBT) and Dm-dNK (PDB:1J90). The α-helices and β-sheets are shown in orange and pink, respectively, while the lid region and P-loop (Lasso for TK1) are shown in green and blue, respectively. All the figures are plotted with PyMOL.

As the only deoxyribonucleoside kinase identified in *D. melanogaster*, Dm-dNK (NTP: deoxyribonucleoside 5'-phophotransferase, EC 2.7.1.145) is able to phosphorylate all four natural deoxyribonucleosides with almost the same high turnover numbers and higher efficiencies than those mammalian dNKs (Eriksson *et al.*, 2002; Knecht *et al.*, 2002). Dm-dNK has markedly higher sequence identity (48%) with TK2, in comparison with dCK/dGK (approximately 40%). Therefore its structure is used as the ideal template to build structural models of TK2 (Tyynismaa *et al.*, 2012; Perez-Perez *et al.*, 2008; Perez-Perez *et al.*, 2005).

As shown in Figure 2, the overall monomer structures of TK2 and other dNKs in the same family, have an α/β-architecture where the active site

consists of a five-stranded parallel β-sheet with the strand order β2, β3, β1, β4, and β5, all of which are surrounded by nine to ten α-helices (Eriksson *et al.*, 2002). Interactions between the hydrophobic residues in the α4 and α7 helices of each subunit (monomer) form a four-helix bundle at the dimer interface, generating the dimer form of dCK, dGK and Dm-dNK. Two sequence motifs *i.e.*, the highly conserved P-loop (consensus sequence GX_4GKS/T) and the arginine-rich lid region with the consensus sequence RX_3RXRX_2E, play a special role in accommodating the phosphate groups of phosphate donors. In the structure models of TK2, the P-loop locates at residues Gly57-Ser63, just in the turn between β1 and α1. It binds and positions the phosphoryl groups of the phosphate donor, while the lid region situated between residues Arg192-Glu199 is a flexible segment, functioning as a cover for the active site cleft, and is critical for ATP binding and catalysis (Figure 2A) (Perez-Perez *et al.*, 2005). The residues Glu133, Arg134 and Ser135 in TK2 are strictly conserved in the dCK/dGK family, form another critical loop (the ERS motif), and play an important role in catalysis. In dCK, Glu127 interacts with the magnesium ion and Arg128 acts as an anchor to bring the 5'-hydroxyl group of dCyd and the γ-phosphate of the phosphoryl donor. Facilitated by the nearby Arg128, Glu53 (in the P-loop) in dCK acts as a base in the de-protonation of the sugar 5'-hydroxyl group (Sabini *et al.*, 2003). The corresponding Glu-Arg pair in human TK2 is residues 81 and 134. The substrate binding cleft locates perpendicularly to the C-termini of the parallel β sheets of the enzymes. A conserved Tyr-Glu pair (TK2, Y99-E201; dNK, Y70-E172; dGK, Y101-E211; dCK, Y86-E197) firmly anchors the deoxyribose moiety of the substrate by two hydrogen bonds to the 3'-hydroxyl group. The tyrosine hydroxyl group is in close proximity to the 2'-sugar position, interfering the binding of ribonucleosides (Sabini *et al.*, 2003).

In TK2, the base moiety of a substrate, such as thymine, is sandwiched between the phenyl ring plane of Phe143 by π-π interaction on one side and Trp86 as well as Val113 on the other side. Two hydrogen bonds are established between residue Gln110 and N3/O4 of the thymine base (Perez-Perez *et al.*, 2008). The substrate selectivity of different dNKs is considered to be largely due to the different amino acid residues located in the plane of the substrate binding pocket. Thus, the interaction between a group of substrates and the side chain of amino acid in the corresponding position determines the discrimination of the dNKs towards different substrates (Sabini *et al.*, 2003; Eriksson & Wang, 2002).

1.2.6 Mitochondrial disorders caused by TK2 and dGK mutations

mtDNA depletion syndrome (MDS)

Human mtDNA is a 16,569 bp circular double-stranded DNA encoding 13 polypeptides, which are subunits of mitochondrial respiratory chain complexes, *i.e.*, the mitochondrial oxidative phosphorylation system. The nuclear genome encodes all the other mitochondrial proteins and peptides (Navarro-Sastre *et al.*, 2012). Accordingly, an adequate amount of mtDNA and its integrity are essential for mitochondrial ATP production, the metabolism of sugar and fatty acids, calcium storage and signaling, the regulation of the cellular redox state, as well as one part of apoptotic machinery. Energy metabolism predominantly relies on cytosolic glycolysis during fetal life and hence functional defects in mitochondria usually manifest only after birth (Suomalainen & Isohanni, 2010). However, the replication and repair of mtDNA proceed throughout the cell cycles and thus demand a continuous and balanced dNTPs supply. Today at least 12 among over 200 nuclear genes involved in mtDNA replication and mtDNA precursor metabolism, are associated with mitochondrial diseases, including *TK2*, *DGUOK*, *TYMP* (thymidine phosphorylase), *RRM2B* (p53R2), *SUCLA2* (ADP-forming succinyl CoA ligase beta subunit), *SUCLG1* (GDP-forming succinyl CoA ligase alpha subunit), *POLG* (DNA polymerase γ), *POLG2* (accessory subunit of DNA polymerase gamma), *PEO1* (or *C10orf2*, Twinkle, mtDNA helicase), *SLC25A4* (or *ANT1,* the adenine nucleotide translocator), *OPA1*, and *MPV17* genes (Uusimaa *et al.*, 2013; Copeland, 2008; Eriksson & Wang, 2008). During the past two decades, eight to nine of them have been identified as the cause of MDS, which is characterized by severe and tissue-specific reduction of mtDNA copy number without qualitative defects in mtDNA. Originating from early childhood or the neonatal period, fatal MDS is one of the most common forms of childhood respiratory chain deficiencies with an approximate frequency of 1/10000, affecting skeletal muscle, brain, liver or several tissues with a variety of symptoms such as progressive myopathy, hepatopathy and encephalopathy (Suomalainen & Isohanni, 2010; Wang, 2010).

Mutations in *TK2*, *RRM2B*, and *SUCLA2* are predominantly associated with myopathic MDS, and defects in *DGUOK*, *SUCLG1*, *POLG*, *PEO1*, and *MPV17* are responsible for hepatocerebral forms of MDS, while encephalomyopathic MDS originates from *TYMP*, *RRM2B*, *SUCLG1*, and *SUCLA2* deficiencies (El-Hattab & Scaglia, 2013; Navarro-Sastre *et al.*, 2012; Suomalainen & Isohanni, 2010; Wang, 2010; Copeland, 2008). Recently, mutations in *MGME1* (*C20orf72*, mitochondrial genome maintenance exonuclease 1), encoding a mitochondrial RecB-type exonuclease, were

reported as a cause of a multi-systemic form of MDS (Kornblum *et al.*, 2013). Notably, the clinical phenotypes of various forms of MDS are overlapping and heterogeneous, and may affect either a specific tissue or a combination of several organs. However, some genes involved in MDS are also associated with other clinical disorders *e.g.*, Parkinsonism and ataxia. In addition, transient mtDNA depletion could result from long-term use of nucleoside analogs, which will be described later.

TK2-related MDS

Deficiency of TK2 due to genetic mutations is associated with clinically heterogeneous myopathic form of MDS. MDS was initially described by Moraes *et al.* in 1991, and 10 years later the first report assigning TK2 mutations to MDS was published by Saada and coworkers, who identified two missense mutations His90Asn and Ile181Asn in four individuals with devastating isolated skeletal myopathy, mtDNA depletion and death at early age (Saada *et al.*, 2001; Moraes *et al.*, 1991). Since then, more than 50 individuals suffering from MDS with both molecularly confirmed diagnosis and histological or biochemical analysis have been linked to a variety of genetic alternations in the *TK2* gene (Chanprasert *et al.*, 2013). In addition to myopathy, neurological phenotypes and multi-tissue pathology have been discovered recently in patients carrying defect TK2 (Bartesaghi *et al.*, 2010; Gotz *et al.*, 2008). Most patients have an uneventful initial phase, but present progressive onset of muscle disorders characterized by generalized hypotonia, fatigue, proximal muscle weakness, impaired physical stamina, poor feeding, respiratory difficulties, and gradual loss of spontaneous activity from birth to about 30 months of age (El-Hattab & Scaglia, 2013). Some other symptoms less commonly reported include hepatic disorder, facial weakness, bulbar weakness, hepatic disorder, cardiomyopathy, kidney tubulopathy, ptosis, encephalomyopathy, and dysarthria (Lesko *et al.*, 2010; Zhang *et al.*, 2010; Galbiati *et al.*, 2006).

Until 2012, twenty-eight patients died from respiratory failure at 0.3 to 16 years of age, while the majority of the living patients still suffer from severe respiratory insufficiency (El-Hattab & Scaglia, 2013). Midler forms with longer survival and diverse MDS phenotypes caused by TK2 mutations were also described, including late onset proximal muscle weakness (Oskoui *et al.*, 2006), progressive sensory neural hearing loss (Marti *et al.*, 2010), and adult-onset progressive myopathy (Behin *et al.*, 2012). Therefore, residual TK2 activity in the tissues affected by MDS is sufficient to support survival. Particularly, the severity of the TK2 deficiency is correlated with the phenotype in patients, namely, severe impairment of TK2 resulted in

encephalomyopathy whereas partial reduction of TK2 was associated with myopathy (Zhou *et al.*, 2013). However, the tissue specific pathogenesis of MDS caused by genetic alternations in *TK2* as well as other genes remains largely unexplained.

In order to investigate the molecular mechanism of mtDNA depletion due to *TK2* mutations and the physiological role of TK2, TK2-deficient mouse strains were generated by two groups in 2008 (Akman *et al.*, 2008; Zhou *et al.*, 2008). It was found that *TK2*$^{-/-}$ mice showed growth retardation and died within 2 to 4 weeks of life with mtDNA depletion in the brain and adipose tissues. Immediately after birth progressive reduction of mtDNA in skeletal muscle, heart, liver and spleen was observed but without elevated mtDNA mutation rates. Pronounced hypothermia and loss of hypodermal fat and abnormal brown adipose tissue were also observed (Zhou *et al.*, 2008). The gradual hepatic mtDNA depletion was accompanied by increasingly hypertrophic mitochondria and accumulation of fat vesicles and affected liver function and lipid metabolism (Zhou *et al.*, 2013). In the same TK2-knockout mouse strain, the importance of TK2 in neuronal homeostasis was emphasized by the findings that after 10-12 days of life these mice had a severe ataxic phenotype with histopathological neurodegeneration of cerebellum. Impaired electron transport chain complexes and mtDNA depletion (~70% on day 12) in the brain were the most likely mechanism (Bartesaghi *et al.*, 2010). Recently, Paredes *et al.* analyzed skeletal muscle and heart muscle isolated from TK2 negative mouse strain during postnatal development. Compared with those of normal mice, the mtDNA levels were depleted in both tissues and the skeletal muscles appeared be significantly underdeveloped. In contrast, the heart muscle was not apparently affected. Furthermore, primary myoblasts from the *TK2*$^{-/-}$ mouse strain exhibited slow proliferation and reduced ability to differentiate. Hence, they proposed that TK2 deficiency could disturb the function of myogenic progenitor cells in postnatal skeletal muscle tissue and this might contribute to the abnormal phenotype appearing in the animal model (Paredes *et al.*, 2013). On the other hand, a knockin murine model harboring an H126N TK2 mutation analogous to human H121N TK2 mutation was developed and reported to have negligible TK2 activity in many organs. At day 10, the *TK2*$^{-/-}$ pups exhibited growth deceleration, rapidly developed progressive and severe encephalomyelopathy with unbalanced dNTP pools, particularly in brain and heart. mtDNA depletion was also found in many tissues (Paredes *et al.*, 2013; Dorado *et al.*, 2011; Akman *et al.*, 2008).

dGK-related MDS.

Deficiency of dGK is associated with hepatocerebral forms of MDS (multisystem disorders with pronounced involvement of liver in neonates) and hepato-specific diseases in late infancy or childhood (Poulton *et al.*, 2009; Mandel *et al.*, 2001). To date, approximately 100 individuals from diverse ethnic origins have been reported to suffer from MDS caused by over 40 distinct mutations in *DGUOK*. The majority of these individuals manifest liver dysfunction with or without accompanying neurologic impairment at birth or within the first month of life with subsequent developmental regression, rotary nystagmus, and hypotonia (El-Hattab & Scaglia, 2013; Suomalainen & Isohanni, 2010; Poulton *et al.*, 2009). Most patients die from progressive hepatic dysfunction within the first year of life (Dimmock *et al.*, 2008). It was reported that early liver transplant might be helpful to patients who have only isolated liver disorders (Mousson de Camaret *et al.*, 2007; Freisinger *et al.*, 2006).

TK2 and dGK-related PEO

In contrast to infantile-onset MDS associated with severe mtDNA depletion, progressive external ophthalmoplegia (PEO) is a manifestation of multiple mtDNA deletions, which accumulate with age and cause late-onset symptoms in post-mitotic tissues such as nervous system and skeletal muscle. PEO is caused by mutations in nuclear genes encoding proteins that are involved in mtDNA replication and nucleotide metabolisms (Brandon *et al.*, 2013; Zeviani *et al.*, 1989). Patients suffering from PEO usually manifest variable clinical symptoms including adult-onset weakness of the external eye muscles, bilateral ptosis, exercise intolerance, proximal muscle weakness, wasting, hearing loss, hypogonadism, optic atrophy and Parkinsonism (Tyynismaa *et al.*, 2009; Copeland, 2008). The autosomal dominant form of PEO (adPEO) has been associated with mutations in *POLG1*, *POLG2*, *PEO1*, *SLC25A4*, *RRM2B*, *OPA1 and MFN2* (Rouzier *et al.*, 2012; Tyynismaa *et al.*, 2009; Hudson *et al.*, 2008; Longley *et al.*, 2006; Spelbrink *et al.*, 2001; Van Goethem *et al.*, 2001; Kaukonen *et al.*, 2000). In contrast, the autosomal recessive PEO (arPEO) has only been associated with mutations in *POLG1* (Van Goethem *et al.*, 2001). In 2012, Ronchi *et al.* identified autosomal recessive mutations in the *DGUOK* gene as a novel genetic cause of arPEO by next-generating sequencing of known mitochondrial targets (MitoExome) (Ronchi *et al.*, 2012). This was previously only associated with the infantile hepatocerebral form of MDS. Mutations in *TK2* gene have also been reported to cause arPEO (Tyynismaa *et al.*, 2012). The overlaps of genetic backgrounds among different forms of

MDS and PEO imply that defects in the same gene might cause strikingly diverse manifestations.

1.3 Nucleoside analogs used in antiretroviral treatment

Analogs of natural nucleosides play important roles in anti-viral and anti-tumor chemotherapy. These synthetic compounds are widely administrated in the treatment and prevention against a vast number of viral infections and cancers, including but not limited to human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), leukemia, as well as cancers in skin, breast and lung. As the first anti-viral drug, 5-iodo-2'-deoxyuridine (idoxurine) was synthesized in 1959 and found to be effective for topical treatment of herpes keratitis (De Clercq & Field, 2006; Prusoff, 1959). Since then, several nucleoside analogs have been developed and approved for clinical use. Particularly, nucleoside analog reverse transcriptase inhibitors (NRTIs) are the backbones in highly active antiretroviral therapy (HAART), which is a combination of three or more antiretroviral drugs, including NRTIs, nonnucleoside analog reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), and lately fusion inhibitors. Among anti-HIV agents approved during the past decades by FDA, there are 8 NRTIs: the thymidine analogs zidovudine (AZT/ZDV) and stavudine (d4T), the deoxycytidine analogs: lamivudine (3TC), zalcitabine (ddC) and emtricitabine (FTC), the guanosine analog: abacavir (ABC), the adenosine analogs: didanosine (ddI) and tenofovir (TDF) which in fact is a nucleotide analog. Due to severe toxicity, ddC is no longer marketed (Warnke *et al.*, 2007). In this section, some general characteristics of NRTIs are briefly summarized, followed by a description of the metabolism of two classical analogs: AZT and ddI.

1.3.1 General characteristics of NRTIs

Administered as un-phosphorylated pro-drugs, NRTIs can penetrate into cells and be activated by several cellular kinases, eventually leading to the formation of the corresponding triphosphates. These are membrane impermeable and exert their anti-viral activity. Activation of the purine analogs ddI and ABC requires an extra enzymatic deamination step to ddA and carbovir (CBV), respectively (Vivet-Boudou *et al.*, 2006; Johnson & Fridland, 1989). Hence, the efficiencies of pro-drug activation determine the intracellular levels of active NRTIs. Referring to the structural similarities, the NRTI triphosphates are capable of competing with the corresponding natural substrates for the active site of the viral reverse transcriptase (RT), which catalyzes the synthesis of viral DNA using RNA as template. Since the 3'-hydroxyl group of the

pseudo-ribose moiety in NRTI monophosphates is missing, the essential 3'-5' phosphodiester bond cannot be generated. Thus the replication of HIV is terminated. In contrast, non-nucleoside analog reverse transcriptase inhibitors (NNRTIs) inhibit the RT activity in a non-competitive manner by binding to another sites rather than the active site. Accordingly, no phosphorylation is required for this type of drugs.

NRTIs can suppress HIV replication and delay the acquired immune deficiency syndrome (AIDS) progression, and therefore, transform the lethal HIV infection into a clinically-tolerable chronic illness. Toxic adverse effects of these drugs include cardiomyopathy, encephalopathy, lactic acidosis, hepatic steatosis, lipoatrophy, and peripheral neuropathy, which are most likely due to mitochondrial dysfunctions similar to the inherited mitochondrial diseases as described above (Lund *et al.*, 2007; Kakuda, 2000). Different NRTIs give adverse effects in a tissue-specific manner, *e.g.*, AZT affects skeletal muscle and causes hematological toxicity and myopathy, while d4T causes lipodystrophy and peripheral neuropathy. The lack of correlation between structural similarity and tissue-specific side-effect of these analogs implies that the metabolism of NRTIs is complex and very likely multiple mechanisms are involved in the NRTIs-induced mitochondrial toxicity.

Another drawback of NRTIs in anti-HIV therapy is the rapid emergence of drug resistance that limits the clinical benefits of NRTIs and ultimately leads to treatment failure. The *pol* gene encoding HIV RT, which is the target of NRTIs, has a high mutation rate because the reverse transcriptase is error-prone (Vivet-Boudou *et al.*, 2006). In addition, the increased mutation rate of NRTI-resistant RT contributes to emergency of drug resistant viruses (Jewell *et al.*, 2003).

1.3.2 3'-azido-2',3'-dideoxythymidine (AZT)

Discovery and clinic application

AZT (zidovudine, ZDV, Retrovir®, IUPAC: 1-[(2R,4S,5S)-4-azido-5-(hydroxymethyl) oxolan-2-yl]-5-methyl-pyrimidine-2,4,-dione) was originally synthesized in 1964 as a potential anti-cancer agent (Horwitz *et al.*, 1964). AZT was found to be able to inhibit the replication of the retrovirus which leads to AIDS (Mitsuya *et al.*, 1985). Since then, application of AZT has delayed disease progression in HIV-infected individuals and prolonged survival in patients with AIDS. In the United States, AZT has protected more than one thousand children every year by significantly suppressing the mother-to-child transmission of HIV (Poirier *et al.*, 2003).

The metabolism of AZT

Prior to intracellular activation, cellular uptake across plasma membrane is the initial step of pharmacological action of NRTIs, including AZT. Different from most nucleosides, AZT was found to permeate cell membranes as well as the blood-brain barrier and the blood-placenta barrier by passive diffusion, partly due to the lipophilic nature of the azido group (D'Andrea *et al.*, 2008; Dancis *et al.*, 1993; Zimmerman *et al.*, 1987). AZT is a substrate for a variety of nucleoside transporters, *i.e.*, CNT1, CNT3, and ENT2, belonging to concentrative and equilibrative nucleoside transport protein families (CNT and ENT, respectively) (Errasti-Murugarren & Pastor-Anglada, 2010; Purcet *et al.*, 2006; Pastor-Anglada *et al.*, 2005). Once inside cells, AZT is phosphorylated by three successive steps to AZT-TP. HIV does not encode any nucleoside kinase, thus the activation of AZT and other NRTIs completely relies on host cell enzymes: TK1 and TK2 catalyze the first phosphorylation of AZT to AZT-MP, which could be further phosphorylated by TMPK to AZT-DP (Cihlar & Ray, 2010; Van Rompay *et al.*, 2000; Arner & Eriksson, 1995). The final phosphate group is added to AZT-DP by NDPK to generate AZT-TP (Krishnan *et al.*, 2002). The bottleneck in AZT activation is the second phosphorylation step catalyzed by TMPK rather than the first reaction which usually limits the activation of other nucleoside analogs (Lavie *et al.*, 1997). Meanwhile, most enzymes involved in AZT activation are active in all cell cycle phases, whereas TK1 is down-regulated in resting cells. Consequently, AZT preferentially exerts anti-viral activity in proliferating cells like lymphoblasts (Gao *et al.*, 1993).

Adverse effects associated with AZT

In early clinical chemotherapy against HIV, AZT was administrated alone in relatively high concentrations and often resulted in severe mitochondrial dysfunction, limiting its clinical effectiveness (Dalakas *et al.*, 1990). Although the doses of AZT are much lower in the modern HAART regime, long-term exposure to this analog still causes adverse effects including anemia, lactic acidosis, neutropenia, skeletal muscle myopathy, and cardiomyopathy with mtDNA depletion (Scruggs & Dirks Naylor, 2008; Samuels, 2006). All of these side effects were attributed to AZT-mediated mitochondrial toxicity, which was believed to be a class-wide major adverse effect associated with NRTIs (Warnke *et al.*, 2007). Early studies focused on defective mtDNA replication in cells with AZT-induced side effects. The AZT toxicity was ascribed to the inhibition of mtDNA polymerase-γ, *i.e.*, the DNA pol-γ hypothesis, which states that AZT-TP inhibits DNA pol-γ by incorporation (Lewis & Dalakas, 1995; Lewis *et al.*, 1994). AZT-MP acts as a chain

terminator because it lacks the 3'-OH group and it cannot be removed efficiently by the 3'-5' proofreading exonuclease of mtDNA polymerase (Lee *et al.*, 2003).

Accumulating experimental evidences indicated that the inhibition of DNA pol-γ by many anti-viral NRTIs, such as ddC, ddI and d4T, was a primary factor involved in the NRTI-related mitochondrial toxicity, since the active forms of these analogs were found to be strong inhibitors of pol-γ. Furthermore, the strength of the interactions with pol-γ correlated with their mitochondrial toxicity (Bienstock & Copeland, 2004; Lewis *et al.*, 2003). However, AZT seemed to be an exception. As a poor pol-γ inhibitor, this thymidine analog showed unexpectedly high mitochondrial toxicity. Hanes *et al.* found that at least 100 μM AZT-TP was required to reach 50% inhibition of pol-γ, whereas the intracellular AZT-TP level was far too low to explain the mitochondrial toxicity (McCann *et al.*, 2012; Hanes & Johnson, 2007; Lynx *et al.*, 2006; McKee *et al.*, 2004; Lee *et al.*, 2003).

An alternative mechanism underlying the AZT-associated mitochondrial toxicity has been suggested to be the inhibition of TK2 and induction of oxidative stress as described below.

AZT is a potent inhibitor of the dThd phosphorylation catalyzed by TK2 and inhibition of TK2 activity could result in a depleted mitochondrial dTTP pool which limits mtDNA replication and ultimately leads to mtDNA depletion (Susan-Resiga *et al.*, 2007; Lynx *et al.*, 2006). Compared with proliferating tissues, most post mitotic tissues mainly rely on the TK2-catalyzed dThd phosphorylation for the maintenance of dTTP pool and therefore exhibit higher sensitivity to AZT. Altered dTTP pool (>50% reduction) has been confirmed in perfused rat heart with short-term AZT exposure (Morris *et al.*, 2009). These manifestations are similar to those found in patients suffering from TK2 deficiency as described in the previous sections. Thus these facts strongly imply that the inhibition of intramitochondrial dThd phosphorylation exerted by TK2 contributes to the mitochondrial toxicity of AZT and other related NRTI drugs.

To release and revert mt toxicity related to AZT and other NRTIs, uridine supplementation has been tested both *in vitro* and in clinical trials. Uridine can fully abrogate the side effects of AZT and other pyrimidine NRTIs analogs and normalize cell function and mtDNA levels in human HepG2-hepatocytes, bone marrow progenitor cells and HIV-infected peripheral blood mononuclear cells (Walker & Venhoff, 2005; Walker *et al.*, 2003; Sommadossi *et al.*, 1988). In mice, oral uridine administration was able to antagonize the AZT-induced myopathy, peripheral neuropathy, and encephalopathy (Venhoff *et al.*, 2010; Lebrecht *et al.*, 2008). Banasch *et al.* investigated the short-course metabolic

effects of uridine supplementation on lipoatrophic HIV-infected individuals receiving dThd analog drugs and found that the uridine-enriched food, NucleomaxX, appeared to increase hepatic mitochondrial function (Banasch *et al.*, 2006). Oral uridine supplementation could also significantly increase limb fat mass during HAART therapy in HIV-patients (Sutinen *et al.*, 2007). The exact mechanism underlying the effect of uridine on AZT toxicity is not understood. Uridine supplementation could possibly enhance the synthesis of thymidine nucleotides and thus help replenish the depleted dTTP pool (Lebrecht *et al.*, 2008; Walker & Venhoff, 2005).

1.3.3 2',3'-dideoxyinosine (ddI)

As the second FDA-approved drug against HIV, ddI (2',3'-dideoxyinosine, DDI, didanosine, Videx® EC, IUPAC: 9-[(2R,5S)-5-(hydroxymethyl)oxolan-2-yl]-6,9-dihydro-3H-purin-6-one) is administrated in combination with other NRTIs in the HAART regimen. ddI is a selective and potent drug against HIV in human lymphoid cells and monocytes/macrophages. It is able to raise the circulating $CD4^+$ T cells and decrease plasma HIV RNA levels in HIV-infected patients (Yarchoan *et al.*, 1989; Mitsuya & Broder, 1986). Similar to other nucleoside drugs, ddI is administrated orally as a pro-drug and demands multiple intracellular phosphorylation steps to exert anti-HIV activity. ddI is unstable in acidic environments and thus could be easily degraded in the stomach, whereas the new enteric-coated capsule formulation of the drug has improved its bioavailability (Barreiro *et al.*, 2004). Gibbs *et al.* showed that ddI could cross the blood-brain barriers but had much lower efficiency compared to AZT, leading to poor uptake into the central nervous system (CNS) (Varatharajan & Thomas, 2009; Gibbs *et al.*, 2003).

The metabolism of ddI within target cells

The activation of ddI has an unusual initial step since it is phosphorylated by the cytosolic 5'-nucleotidase, cN2, producing ddI monophosphate (ddI-MP). This nucleotide is subsequently converted to dideoxyadenosine monophosphate (ddA-MP) by adenylosuccinate synthetase and adenylosuccinate lyase. The anti-viral active moiety of ddI, ddA-TP is generated by successively adding the second and third phosphates to ddA-MP by AMPK and NDPK, respectively (Ahluwalia *et al.*, 1993; Franssen *et al.*, 1992). Due to a longer intracellular half-life than AZTTP, ddA-TP is able to compete with dATP and thus terminate viral DNA replication (Pruvost *et al.*, 2005). ddA was also tested, but was found to be metabolized rapidly to ddI (Hartman *et al.*, 1990). ddI can be degraded to hypoxanthine and uric acid by PNP and xanthine oxidase, respectively. PNP is expressed in most mammalian tissues such as endothelial

cells of blood-brain barrier, intestinal epithelium and erythrocytes at high levels. Thus this enzyme could be responsible for most of the intracellular elimination of ddI (Singhal *et al.*, 1998). In human blood, the catabolism of ddI was found to be extensive and this might explain the short half-life of ddI in patients receiving this drug (Back *et al.*, 1992).

Side effects associated with ddI

Adverse effects caused by ddI are primarily peripheral neuropathy, lactic acidosis, pancreatitis, non-cirrhotic portal hypertension, and retinal toxicity (Gabrielian *et al.*, 2013; Chang *et al.*, 2012). The presence of ddI results in time- and dose-dependent mtDNA depletion, cell growth inhibition, and elevation of intracellular lactate and lipids in cultured HepG2 cells, CEM cells, human muscle cells, and healthy primary human T lymphocytes (Setzer *et al.*, 2005; Lewis *et al.*, 2001; Benbrik *et al.*, 1997). These ddI-induced side effects are regarded to be due to mitochondrial toxicity via inhibition of mtDNA polymerase γ, *i.e.*, the DNA pol-γ hypothesis (Warnke *et al.*, 2007; Lee *et al.*, 2003).

1.4 Protein oxidation and removal of oxidatively damaged proteins

In organism, chemically reactive molecules such as free radicals and related oxygen/nitrogen containing species are generated during aerobic metabolism, aging, and pathophysiological states. Generally, when the production of reactive molecules exceeds their catabolism a state of oxidative stress exists. Pro-oxidants are able to react with various biomolecules, which include lipids, carbohydrates, nucleotides, and proteins, causing a number of adverse alternations *e.g.*, loss of enzyme activity and mutagenesis. On the other hand, many of these reactive molecules have been recognized to perform important physiological functions during normal metabolisms, including cellular signaling, proper development and proliferation. In this section, the effects of reactive species derived from oxygen will be described. Attention will be mainly focused on the reactive species-mediated protein modifications, particularly S-glutathionylation, which has been widely recognized as one of the most important protein post-translational modifications. Cells employ a variety of defense mechanisms including antioxidants and intracellular protein degradation pathways to minimize the deleterious effects of reactive molecules and prevent the accumulation of damaged proteins. Therefore, cellular antioxidants and protein degradation pathways will also be described.

1.4.1 ROS and RNS

Free radicals, such as superoxide anion radicals ($O_2^{\bullet -}$), are molecules or molecular fragments with odd (unpaired) valence electrons in the outer orbitals and they are very reactive towards other substances, including themselves (Valko *et al.*, 2007). Together with reactive nitrogen species (RNS), oxygen-free radicals, also called reactive oxygen species (ROS), are produced in normal cellular metabolism and represent the most significant classes of radicals generated in living systems. Although without unpaired electrons, hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) are still ascribed to ROS. Studies on free radicals in biological system date from the 1950s when oxygen toxicities were found to be associated with the formation of oxidizing free radicals (Gerschman *et al.*, 1954). Since then, the effects of ROS/RNS in biological system have been widely studied.

1.4.2 Sources of ROS

Exogenous ROS can be caused by ultraviolet exposure, radiation, heat stress, smoking, excessive exercises, infections, xenobiotics, and drugs (Marchetti *et al.*, 2012). In mammalian cells, ROS is produced during normal cellular metabolism in various intracellular compartments such as mitochondria. Primary endogenous ROS sources include the mitochondrial respiratory chain, oxidases in peroxisomes, NAD(P)H oxidase (NOX) isoenzymes, cytochrome P450s, the flavoenzyme ERO1 in the endoplasmic reticulum, xanthine oxidase, and nitric oxide synthases (Nathan & Cunningham-Bussel, 2013; Kregel & Zhang, 2007).

In eukaryotic cells, mitochondrial ATP production is the most important source of ROS (Cadenas & Sies, 1998). During electron transport in aerobic respiration, single electrons leak to molecular oxygen (O_2) which is reduced to $O_2^{\bullet -}$. This superoxide product is further converted to other ROS including H_2O_2 and hydroxyl radicals ($^{\bullet}OH$) (Chance *et al.*, 1979). Based on studies with electron transport inhibitors, eight sites in mitochondria have been identified to generate $O_2^{\bullet -}$, although the detailed mechanism is still unclear (Sena & Chandel, 2012; Brand, 2010).

Despite of its lower reactivity, H_2O_2 generated by either endogenous or exogenous routes has a longer half-life than other ROS. Therefore, it can diffuse through the mt membranes and contribute to cytoplasmic oxidative stress. Especially in the presence of transit metal ions, such as Fe^{2+} and Cu^{+}, H_2O_2 can be converted to $^{\bullet}OH$, which is highly reactive via the Fenton reaction (Valko *et al.*, 2007).

1.4.3 Beneficial effects of ROS

At low or moderate concentrations, ROS/RNS play beneficial roles in various processes, including inter-/intracellular signaling and defence against infectious agents. For instance, nitric oxide (NO•), which is produced by specific nitric oxide synthases (NOSs) *in vivo*, is an abundant membrane-permeable small molecule affecting vascular pressure, neuronal transmission, immune regulation and many other physiological processes (Hancock *et al.*, 2001; Bergendi *et al.*, 1999; Chiueh, 1999). Therefore, NO• was appointed as the molecule of the year in 1992 (Koshland, 1992).

1.4.4 Intracellular targets of ROS

Oxidative stress is the harmful effect of ROS (nitrosative stress in the case of RNS) on lipids, nucleic acids and proteins. It takes place when ROS is overproduced and/or enzymatic and non-enzymatic antioxidants are deficient. Thus, oxidative stress can be defined as an imbalance between ROS production and degradation. It is known that oxidative stress is correlated with the normal aging process and many severe diseases, *e.g.*, type-2 diabetes mellitus, cardiovascular diseases, Alzheimer's disease, and cancers. It is clear that mt disorders contribute, at least in part, to elevated free radical formation (Johns, 1995).

Lipid peroxidation

One of the cellular ROS targets is the polyunsaturated fatty acid of phospholipids, which can be oxidized by protonated superoxide anions (HOO•) and •OH into polar lipid hydroperoxides through autocatalytic lipid peroxidation. The presence of transition metals, like cadmium, accelerates the reaction. As a result, ROS increases the fluidity and permeability of the damaged membranes and affects the viability of cells or organelles by triggering signaling pathways (Avery, 2011).

ROS induced DNA damage

ROS can also generate DNA lesions, which include purine/pyridine oxidative damage, apurinic/apyrimidinic sites and DNA strand(s) breaks. mtDNA suffers from higher levels of oxidative damage than nDNA, due to its localization and the less repair capacity of mtDNA pol-γ. Among the bases, guanine is most susceptible and can be oxidized by •OH into 8-oxo-7,8-dihydroguanine (8-oxo-dG), which is very stable and abundant (one 8-oxo-dG per million nucleotides) and thus often used as an oxidative stress marker (Kryston *et al.*, 2011). During DNA replication, 8-oxo-dG causes GC to TA transversions since dATP can be

incorporated opposite to where the lesion occurs. Unrepaired DNA lesions generate mutations and may ultimately lead to cell death.

Protein oxidation

Effects of oxidative stress on protein function and structure are very diverse. Different proteins have distinct sensitivities to ROS, depending on the localization and abundance within cells, the number of redox-sensitive amino acids and metal-binding sites, the conformation and turnover rate. Proteins are more ROS-sensitive when they are newly synthesized and the length of the protein and its amino acid composition are also crucial (Avery, 2011).

The protein backbone can be oxidized by •OH on the α-hydrogen atom, ultimately resulting in protein fragmentation and cross-linkage to other proteins (Berlett & Stadtman, 1997). The alternations induced by oxidative stress in amino acid residues might be reversible *e.g.*, S-glutathionylation or irreversible such as protein carbonylation, depending on the characteristics of the side chains. Although oxidation of side chain may occur to almost all amino acid residues, sulfur-containing amino acids, cysteine (Cys) and methionine (Met) are particularly vulnerable to various ROS and their oxidation is usually reversible, generating cysteine mixed disulphides and methionine sulfoxide (MetO), respectively. The oxidized products can be reduced via enzymatic or non-enzymatic mechanisms. Therefore, cyclic oxidation-reduction of these two amino acid residues plays an important role in the protection and regulation of cellular proteins.

Cysteine oxidation and S-glutathionylation

Cysteine residues are susceptible to oxidative modification due to the reactive thiol group. Those cysteine residues on protein surfaces play important roles in protein defence against oxidative damage and redox homeostasis. Requejo *et al.* reported that the concentration of exposed protein thiols in mitochondrial matrix was 60-90 mM and approximately 26-fold higher than the mitochondrial GSH concentration (Requejo *et al.*, 2010). The cysteine residues in the active sites of enzymes like glutaredoxin (Grx) detoxify ROS/RNS and reduce other oxidized protein thiols. The pKa value of sulfhydryl (-SH) of free cysteine is 8.3, which is higher than the physiological pH value 7.4 of normal cells. Therefore many cysteine residues exist in protonated forms and will not react with oxidants spontaneously under normal conditions. However, the pKa value of the -SH group of a protein cysteine is also dependent on its vicinal amino acids. For instance, the presence of basic amino acid residues *e.g.*, histidine (His) around cysteine residue could lower the pKa value and convert the thiol group to redox reactive thiolate anion (Dalle-Donne *et al.*, 2007).

High ROS levels leads to protein cysteinyl residue (PSH) oxidation, generating sulphenic acid (PSOH), which is a labile intermediate. PSOH is readily reduced back or oxidized into sulphinic acid (PSO_2H) and irreversibly into sulphonic acid (PSO_3H). For some proteins, formation of transient PSOH in specific cysteine residue is also essential to generate proper intracellular disulfide bonds during folding.

Alternatively, oxidation of cysteine residues leads to the formation of disulfide bonds between PSHs (intra/intermolecular disulfide bonds, PSSP/PSSP) or between PSH and the thiol group of low molecular weight molecules *e.g.*, cysteine (S-cysteinylation), GSH and GSSG (S-glutathionylation). Protein S-glutathionylation is a reversible posttranslational modification in which one GSH is added to the protein specific cysteine residue. Since free GSH is the predominant intracellular low molecular weight thiol-containing molecule, S-glutathionylated proteins are the primary intracellular mixed disulfides, while the extracellular proteins like albumin are mainly S-cysteinylated due to the abundance of free cysteine in plasma.

Although the exact mechanism of protein S-glutathionylation has not been resolved, some mechanisms of glutathionylation have been described (Cooper *et al.*, 2011; Dalle-Donne *et al.*, 2009; Mieyal *et al.*, 2008). As illustrated in Figure 3, proteins S-glutathionylation can occur via (i) thiol-disulfide exchange between PSH and GSSG. This exchange reaction is unfavorable due to the high intracellular GSH/GSSG ratio. However, some proteins, such as c-Jun, have unusually high thiol redox potential and thus could be glutathionylated by GSSG (Klatt *et al.*, 1999). Under severe oxidative stress, the GSH/GSSG ratio can decrease drastically, and approximately half of PSH could be converted into PSSG if the GSH/GSSG ratio declined to 1 (Gilbert, 1990). In mitochondria, the physiological pH is around 8, which is close to the pKa value of PSH. As a result, the thiol-disulfide exchange triggered by GSSG can be facilitated in the mt matrix (Hurd *et al.*, 2005). This is the only pathway requiring GSSG accumulation. (ii) thiyl radical intermediates interactions, *i.e.*, $PS^{\bullet}$ and $GS^{\bullet}$ produced by one-electron oxidation of PSH and GSH form mixed disulfide bond by radical recombination. In the presence of oxygen, $PS^{\bullet}$ and $GS^{\bullet}$ can also react with GSH and PSH, respectively, leading to the formation of PSSG and a superoxide radical. (iii) sulfenic acid intermediates. PSOH is an early intermediate during PSH oxidation and glutathionylation may readily occur between PSOH and GSH and subsequently produce PSSG and H_2O. Therefore, this protein post-translational modification is important to prevent PSOH from further detrimental oxidation to PSO_2H or PSO_3H. (iv) induction by nitric oxide (NO). Nitrosative stress or in the presence of NO, PSH and GSH can be converted to PSNO and GSNO, respectively. These

products are reactive towards thiol groups. Consequently, glutathionylated protein is generated by reactions between PSH and GSNO or between PSNO and GSH. The ease of protein S-glutathionylation is influenced by factors similar to those affecting protein cysteine residue oxidation, *e.g.*, location and accessibility, the pKa value of the –SH group, vicinal amino acids, and divalent cations. Furthermore, the GSH/GSSG ratio determines the equilibrium of the reversible protein S-glutathionylation reaction.

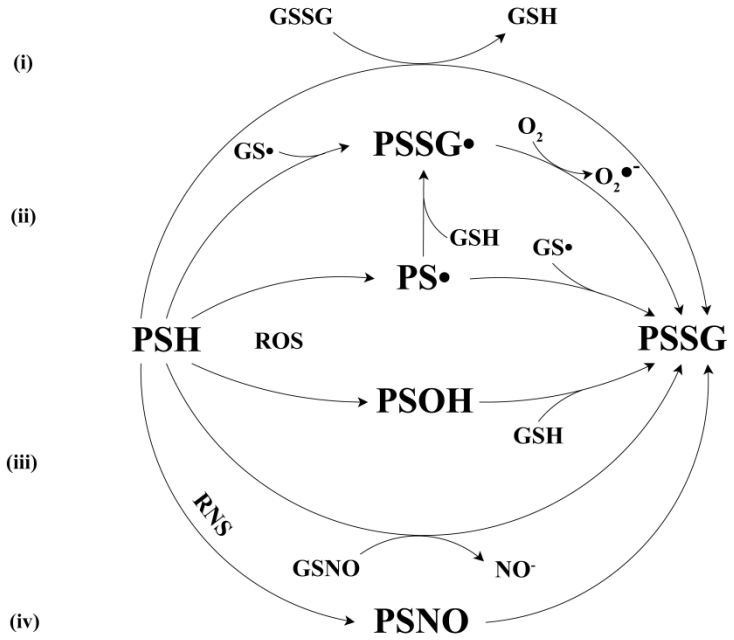

Figure 3. Potential mechanisms of protein S-glutathionylation. The protein S-glutathionylation could occur via (i) thiol-disulfide exchange between P-SH and GSSG; (ii) thiyl radical intermediates; (iii) sulfenic acid intermediates; (iv) induction by nitric oxide.

Protein S-glutathionylation is fully reversible and de-glutathionylation is catalyzed specifically and efficiently by Grx in mammalian cells, but not by other thiol-disulfide oxidoreductase like thioredoxin (Chrestensen *et al.*, 2000). Grx promotes the reduction of PSSG, since it has unique cysteine thiol groups (pKa=3.5) in the active site. These can react with PSSG to form a relatively stable catalytic S-glutathionyl intermediate (Grx-SSG), which can be reduced by GSH to Grx and GSSG (Peltoniemi *et al.*, 2006). Starke *et al.* demonstrated that Grx also catalyzes protein S-glutathionylation in the presence of the GS-thiyl radical generating systems (Fe^{2+}-ADP/H_2O_2+GSH or HRP/H_2O_2+GSH) (Starke *et al.*, 2003).

1.4.5 Antioxidants

In order to minimize the effects of high ROS, antioxidant enzymes *e.g.*, superoxide dismutases (SODs), catalase (CAT) and glutathione peroxidase (GPx), and non-enzymatic antioxidants such as glutathione (GSH) and vitamin E, are employed as one of the most crucial defence mechanisms. It is essential for organisms to maintain a balance between ROS production and their scavenging by antioxidants.

Glutathione

In nearly all prokaryotic and eukaryotic cells, except some gram-positive bacteria, tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is the most abundant intracellular low-molecular-mass non-protein thiol containing antioxidant. It exists in two forms, reduced GSH (MW 307.33) and oxidized GSSG (MW 612.63). The special peptide bond formed by the γ-carboxyl group ensures relative high stability of GSH against cellular peptidases. Glutathione disulfide (GSSG), is the main oxidation product of glutathione and could be reduced back to GSH in response to diminished oxidative stress spontaneously or catalyzed by GSSG reductase. Accordingly, the ratio of the redox couple GSH/GSSG is an indicator of intracellular redox status. In contrast to the low concentration in plasma (10-30 μM in human), the intracellular GSH level is generally in the millimolar range (0.2-15 mM) and GSH/GSSG ratio within cells is more than 100:1, but they vary among different types of cells and various subcellular compartments. For instance, in the cytosol, nuclei and mitochondria, GSH is predominantly in the reduced form, while in the endoplasmic reticulum (ER) the GSH/GSSG ratio ranges from 1:1 to 3:1 (Hwang *et al.*, 1992). Brain cells have lower GSH concentration (1-3 mM) than non-nervous cells (1-10 mM), thus is more susceptible to ROS/RNS. The GSH concentration is highest in liver and kidney, in particular in the liver, which determines the glutathione level in plasma (Purucker *et al.*, 1995).

The GSH and GSSG couple is also present extracellularly but at low concentrations, approximately 2.8 μM and 0.14 μM, respectively. In contrast, free cysteine (Cys) and cystine (CySS) are the major extracellular low molecular weight thiol/disulfide redox couple, with predominantly cystine (>40 μM) compared to cysteine (8-10 μM) (Banerjee, 2012). Accordingly, the extracellular compartment is in a more oxidized redox status compared with that inside cells.

Enzymatic antioxidants

In addition to non-enzymatic antioxidants, organisms can benefit from an enzymatic strategy to keep redox homeostasis, *e.g.*, by modulation of the

steady-state concentrations of peroxides and lower energetic cost. Since enzymes can be regulated at multiple levels, *e.g.*, transcription, translation and posttranslation, these catalytic antioxidants can be induced, activated or inhibited under distinct conditions to regulate the levels of ROS/RNS. The most efficient enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

Superoxide dismutase (EC 1.15.1.1) catalyzes the one-electron dismutation of primary ROS superoxide into less reactive hydrogen peroxide and oxygen. Therefore SOD protects cells from the damage caused by superoxide and its more aggressive derivatives. In human cells, three different SOD isoforms have been discovered in different compartments, *i.e.*, Cu/Zn-SOD/SOD1 in cytosol and mitochondrial intermembrane space, mitochondrial Mn-SOD/SOD2, and extracellular (EC)-SOD3 (Valko *et al.*, 2007).

Hydrogen peroxide generated by SODs or other metabolic pathways is further detoxified into water and oxygen by the heme-containing catalase (EC 1.11.1.6), an enzyme found in almost all aerobic organisms. Mammalian catalase is considered to be located mainly in the peroxisomes and usually exists as a homotetramer (~240 kDa) with NADPH bound as a protector (Kirkman & Gaetani, 2007).

Glutathione peroxidase (GPx, EC 1.11.1.19) is a general name for a family of phylogenetically related enzymes, which catalyze the reduction of hydrogen peroxide and organic peroxides into water and alcohols, respectively, using GSH as an electron donor. In humans, eight different isoforms of GPx have been identified in different intra/extracellular locations. GPx1-4 as well as GPx6 contain selenocysteine (Sec) residue at the active center, which is essential for catalysis (Brigelius-Flohe & Maiorino, 2013).

There are some other reductases and non-enzymatic antioxidants in cells balancing the redox milieu, such as thioredoxin and thioredoxin reductase. This topic is beyond the scope of this thesis and therefore, will not be discussed further.

1.4.6 AZT-mediated oxidative stress

Nucleoside analogs like AZT, d4T, ddI, and 3TC are as mentioned potent antiretroviral drugs and some of them are still used in anti-HIV therapy. In this section, recent evidence for the involvement of ROS as an important cause for the severe adverse effects associated with the clinical use of nucleoside analogs will be described.

The oxidative stress caused by AZT is well studied in animal models, cultured cells, and also patients during AZT treatment. At the beginning of 1990s, Hayakawa *et al.* found that AZT caused dose-dependent increase of 8-

oxo-dG in mouse liver mtDNA and proposed that the oxidative damage of mtDNA was the major cause of AZT-associated mitochondrial myopathy (Hayakawa *et al.*, 1991). Viña and coworkers reported that mice administrated with AZT had higher levels of mitochondrial 8-oxo-dG, lipoperoxidation, and GSH oxidation in skeletal muscle, liver and heart. Supplementation with vitamins C and vitamins E could prevent and reverse the AZT-caused oxidative damage. They also demonstrated that HIV-infected individuals receiving AZT treatment had much higher 8-oxo-dG level in the urine than healthy individuals and HIV-positive untreated patients (de la Asuncion *et al.*, 2004; de la Asuncion *et al.*, 1999; de la Asuncion *et al.*, 1998). Instead of depleting mtDNA, short-term (2 weeks) exposure of growing rats to AZT led to ROS and peroxynitrite production and subsequent single-strand DNA break, lipid peroxidation, and protein carbonylation in rat cardiac muscle mitochondria. It was suggested that the AZT-triggered ROS were important factors in the AZT-associated myopathy and cardiomyopathy prior to mtDNA damage (Szabados *et al.*, 1999). Oxidative stress has also been observed in cultured human placental cell, adipocytes, macrophages, HepG2, and fibroblasts receiving AZT treatment (Caron *et al.*, 2008; Lund *et al.*, 2007). It is noteworthy that the effect of AZT on intracellular ROS generation is enhanced in the absence of serum. Actually, in most investigations, including those mentioned above, the intracellular/mitochondrial reactive species were determined by indirect ROS detection methods, *e.g.*, GSH/GSSG level, carbonylated protein products, 8-oxo-dG, and fluorescent redox indicators like CM-H_2DCFDA. Real-time measurement of reactive species in single macrophage cell was done by Woo and coworker in 2010. They used electroanalytical techniques, which identify and quantify various electroactive species as efficiently as 1000 molecules per millisecond to analyze the pro-oxidative properties of the azido moiety electrochemically with microelectrodes by pretreating the cells with AZT and its structural isomer 5'-azido-5'-deoxythymidine (5'-AZT). It was shown that both AZT and 5'-AZT stimulated the production of $O_2^{\bullet -}$, NO• and their reactive derivatives. The oxidative and nitrosative properties of these two thymidine analogs were due to the presence of the azido moiety because the pro-oxidative activities were lost when the azide group was blocked (Amatore *et al.*, 2010). These results strongly suggest that the oxidative toxicity of AZT is independent on the inhibition of mtDNA polymerase-γ, since 5'-AZT cannot be phosphorylated to mono/di/triphosphates because it lacks a hydroxyl group in the 5' position.

As a vital part of the mitochondrial antioxidant defense system, mitochondrial Mn-SOD activity was suppressed nearly 60% by AZT in mice liver, whereas cytosolic Cu/Zn-SOD was much less affected (Prakash *et al.*,

1997). Azidyl radicals were generated from azido-containing inorganic/organic molecules (Komarov *et al.*, 2004). Passarella *et al.* demonstrated that the addition of AZT to isolated rat heart mitochondria stimulated the generation of superoxide anions in a concentration-dependent manner, in particular when mitochondrial oxygen consumption was inhibited (Valenti *et al.*, 2002).

All these results suggest that AZT can promote ROS generation and induce oxidative stress in mitochondria, at least in part, due to the pro-oxidative nature of the azido moiety. During long-term administration AZT-triggered ROS production may lead to deleterious alternation in mitochondrial membrane potentials, electron-transport chain, oxidative phosphorylation, mtDNA replication, *etc*.

Other nucleoside analogs such as ddI, ddC and d4T were also reported to induce oxidative stress in cultivated cells or tissues (Caron *et al.*, 2008; Opii *et al.*, 2007; Ferraresi *et al.*, 2006). However, the mechanisms underlying the ROS production induced by these analogs is still not resolved (Day & Lewis, 2004).

1.4.7 Intracellular protein degradation pathways

Most proteins in cells are constantly being synthesized and degraded to maintain moderate protein turnover rates. Different from other macromolecules like DNA, little (if any) precise mechanism has been identified in organisms to repair or rescue damaged proteins, in particular those modified/damaged irreversibly. It is well-known that the elevation and accumulation of abnormal proteins are associated with many diseases. Therefore, the removal of misfolded and damaged proteins is as essential as protein synthesis to maintain protein homeostasis and the integrity of cellular metabolism. In animal cells, two major protein degradation pathways have been identified in the cytosol, namely the proteasome and lysosome pathways. In mitochondria several different proteolytic strategies are employed to control mitochondrial protein quality.

Lysosome and proteasome

The lysosomal degradation pathway is mainly responsible for the turnover of intracellular materials by autophagy such as mitophagy, and the catabolism of alien components like pathogens captured by endocytosis or phagocytosis (Boya, 2012; Saftig & Klumperman, 2009). However, most cytosolic endogenous protein degradations occur via the ubiquitin-proteasome system in eukaryotic cells. Proteins to be degraded are tagged covalently by the conserved ubiquitin polypeptide via three consecutive catalytic reactions of which the initial one requires ATP. The proteasome complex is able to degrade

proteins and peptides conjugated with polyubiquitin chains in an ATP-independent manner (Clague & Urbe, 2010; Sorokin *et al.*, 2009). The short oligopeptide products are then further degraded by various peptidases to single amino acids with expenditure of ATP. Thus, proteasomes play a very important role in intracellular protein homeostasis (Sitte *et al.*, 1998).

Recently, it has been shown that the cytosolic ubiquitin-dependent proteasome pathway is also involved in the degradation of some mitochondrial proteins. The majority of the mitochondrial proteins (~1500 in humans) are encoded by nuclear genes and among them over 100 proteins carrying out diverse functions can be ubiquitinated as shown by proteomic studies (Jeon *et al.*, 2007).

Mitochondrial proteolytic systems are composed of three classes of proteases, namely processing peptidases, ATP-dependent proteases, and oligopeptidases. These proteases exert diverse functions, including mitochondrial protein folding, assembly, maturation and turnover, and elimination of defective proteins.

Lon protease

Short-lived regulatory proteins, oxidatively damaged proteins and misfolded proteins in mitochondrial matrix can be degraded by the Lon protease, a classic mitochondrial ATP-dependent protease. Human mitochondrial Lon protease is a huge homo-oligomeric complex, which contains three types of domains, an N-terminal domain, a central ATPase domain and a C-terminal proteolytic domain. The N-terminal domain is conceivable to be related to the selective binding of target substrates and oligomeric assembly of the whole protease complex, while the C-terminal domain exerts serine protease activity. The ATPase domain is involved in unfolding of substrates and ensuring the specificity of proteolysis with energy derived from ATP hydrolysis (Garcia-Nafria *et al.*, 2010). A group of mitochondrial chaperons can assist the recognition of substrates, prevent selected proteins from aggregation, and promote the proteolytic efficiency. Protein aggregation and accumulation have been found in mitochondria of cells with deficient Lon protease. Therefore, Lon proteases are crucial for mitochondrial protein quality surveillance, especially under stressed conditions, when the levels of abnormal and damaged proteins are increased (Kaser & Langer, 2000). It has been reported that Lon-protease degrades aconitase oxidized by H_2O_2 in the mitochondrial matrix in beef heart muscle (Bota & Davies, 2002).

ClpXP protease

The ClpXP protease is the other ATP-dependent proteases in the matrix of mammalian mitochondria. In contrast to Lon proteases, little is known about their physiological role, but ClpXP proteases are still included in the mitochondrial protein quality control system. As hetero-oligomer, ClpXP protease consists of ClpP subunit exhibiting serine peptidase activity and ClpX subunit with ATPase and chaperone activity.

AAA proteases

Mitochondrial AAA proteases are ATP-dependent proteases and anchored within mitochondrial inner membrane. These proteases can degrade defective membrane-embedded polypeptides. Usually, AAA proteases exist in huge molecular mass complexes (approximately 1 MDa), consisting of closely related or identical subunits, which contain ATPase domains with similar functions as those in the Lon and ClpXP proteases (Kaser & Langer, 2000; Langer, 2000).

All mitochondrial proteases described above constitute the first line of defense for the protection of mitochondrial integrity at molecular level. When the capability of these proteases is insufficient, other strategies are employed, *i.e.*, mitochondrial fusion and fission. Constant fusion and fission provide a dynamic nature to mitochondria and supply an extra and essential protection against stress-mediated damage. Irreversibly damaged components can be segregated by fission and eliminated by autophagy. Severely impaired mitochondria are eliminated by autophagy, namely mitophagy, to protect cells from apoptosis triggered by abnormal mitochondria (Lionaki & Tavernarakis, 2013; Youle & van der Bliek, 2012; Tatsuta & Langer, 2008).

2 Present investigations

2.1 *TK2* mutations in autosomal recessive progressive external ophthalmoplegia (Paper I)

2.1.1 Aim of this study

Two female patients were siblings of healthy, non-consanguineous parents and manifested typical PEO-associated symptoms from late 40s to early 50s. The aim of this study was to identify the genetic defects underlying the pathology and clinical features of these two patients. We also attempted to establish genotype-phenotype correlations in this form of arPEO.

2.1.2 Results and discussion

The mtDNA copy number in skeletal muscle sample of patient II:4 was determined and a relative mild mtDNA reduction (decreased by 40%) was observed in skeletal muscles, but it did not fulfill the consensus criteria (>60% impairment) of mtDNA depletion. By Southern blot analysis, multiple mtDNA deletions were detected in skeletal muscle tissue of patient II:5. Likewise, deleted mtDNA molecules were found in skeletal muscle and several regions of the brain but not in heart, liver or cerebellum in patient II:4. By direct sequencing, no pathogenic mutations were detected in neither the mitochondrial genome nor in the coding regions of any nuclear genes, which were known to be associated with PEO.

To elucidate the genetic background of PEO in these two patients, whole-exome next-generation sequencing technology was employed to sequence the coding regions of the nuclear genome of patient II:4. Compound heterozygous missense mutations c.673C>T and c.688A>G, which resulted in p.R225W and p.T230A, respectively (refseq accession no. NM_004614.3) in the *TK2* gene were identified in both patients. The amino acid substitution at T230 was a novel mutation, while the R225W mutation has been reported previously as

homozygous or compound heterozygous with T106M or R172W in Finnish and Swedish patients suffering from the myopathic form of MDS (Gotz *et al.*, 2008). The allele frequencies of the two mutations were assessed by screening 400 healthy Finnish controls and only one heterozygous carrier of the T230A was identified.

Multiple sequence alignment of TK2 exhibited that both T230 and R225 residues were located within a highly conserved sequence regions. Due to the lack of structure information for TK2, we built a structure model by comparative protein modeling using the SWISS-MODEL computer algorithm with the structure of its homolog Dm-dNK (PDB entry 1OT3) as the template. In the structure model, R225 was located on the tip of β4 (Figure 4) and not involved in substrate binding directly. Instead, the substitution of R225 with a tryptophan is predicted to affect the binding affinity of the base moiety of ATP, due to the occupation of space and disturbance of hydrogen bond patterns by the bulkier side chain of the altered amino acid. Similarly, T230 located in α8, which forms the lid region with α9, was not directly engaged in substrate binding (Figure 4). Nonetheless, it interacted with R234 of the lid region, which is essential for the binding of the phosphate groups of ATP by hydrogen bonds. Thus, the substitution with alanine may very likely impair the catalytic efficiency of TK2.

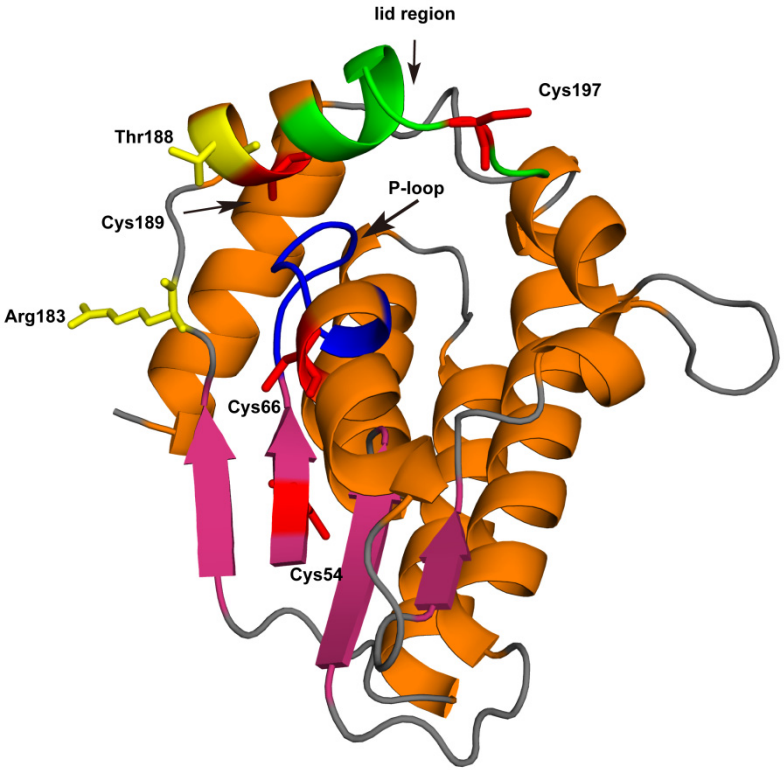

Figure 4. Structure model of truncated human TK2 (SwissPort O00142). Four cysteine residues are shown in red, while the Cys264 is not shown due to the truncation at the C-terminus of TK2 model. The two residues, R183 and T188 (refseq accession no. NM_004614.4), discovered in the two patients suffering from arPEO, are presented in yellow.

To evaluate the effects of the two mutations on the catalytic function of TK2, we constructed and expressed recombinant TK2 mutants in *E. coli* and characterized their kinetic parameters with the natural substrates dThd, dCyd and ATP, respectively. Compared with the wild type enzyme, both recombinant mutants had higher K_m values for dThd and similar K_m values when dCyd was the substrate. Due to the significant impairment in the V_{max} values for both substrates, the catalytic efficiencies (V_{max}/K_m) of R225W and T230A decreased to 5.7% and 30.7% with dThd, respectively, and 7.8% respective 52.8% with dCyd. When ATP was the variable substrates, both mutant enzymes demonstrated similar reductions in catalytic efficiencies as those of dThd and dCyd. These results are consistent with predictions based on

the structure model of TK2. On the other hand, the R225W mutation caused near-total loss-of-function of TK2 and thus the presence of homozygous R225W mutation should result in severe mtDNA depletion, which is in agreement with a previous clinical report (Gotz *et al.*, 2008). We also determined the combined effects of the two mutants on total TK2 activity. The specific activity of TK2 mutants was 22% of the wild type TK2 activity at equal ratio, and decreased when the proportion of R225W was elevated (in a range of 2-38%).

In addition, TK2 activity levels in mitochondria isolated from fibroblasts derived from both patients were also determined. The TK2 activities with dThd and dCyd were 22 and 28% of control values for patient II:4, respectively, and 42% respective 54% of control values for patient II:5. The specificities of mitochondrial TK2 in patients towards dThd and dCyd were also altered compared with that of control. All these findings supported the conclusion that the compound heterozygous *TK2* mutations were the cause of adult-onset arPEO in the patients investigated here.

2.2 The kinetic effects on TK2 by enzyme-bound dTTP (Paper II)

2.2.1 Aim of this study

TK2 phosphorylates pyrimidine deoxynucleosides and their analogs, such as AZT to the corresponding 5'-monophosphate forms. AZT was reported to be a potent inhibitor of thymidine phosphorylation in isolated rat liver and heart mitochondria and this was suggested as the mechanism for the depletion of the mitochondrial dTTP pool, which was essential for mtDNA replication (Lynx & McKee, 2006). In this study, we investigated the effects of AZT and some other pyrimidine analogs on the phosphorylation of dThd and dCyd with human recombinant TK2 and partially purified native rat TK2 to test this hypothesis and clarify the mechanism of mitochondrial side effects caused by anti-viral thymidine analogs.

2.2.2 Results and discussion

First, the kinetic parameters of TK2 were determined with AZT and FLT as substrates by phosphoryl transfer assays. The K_m values for AZT and FLT were 4.5 μM and 6.5 μM and the V_{max} values were 28.9 and 23.5 nmol/min/mg, respectively. Thus, the overall catalytic efficiencies (V_{max}/K_m) of TK2 with AZT and FLT were only 2% respective 4% of that with dThd. Despite being poor substrates of TK2, AZT and FLT were able to inhibit competitively the phosphorylation of dThd by TK2, with K_i values of 3.0 and 1.4 μM, respectively. In contrast, approximate 30% stimulation of dCyd

phosphorylation was observed in the presence of AZT or FLT, while slight inhibition was observed when the AZT or FLT concentration exceeded 100 μM. The other thymidine analogs d4T and araT had similar effects on the phosphorylation of dThd and dCyd as AZT, whereas deoxycytidine analogs 3TC and ddC were not effective inhibitors at low concentrations. By multi-inhibitor assays, we found that AZT and FLT completed with dThd for the same binding site of TK2 because the inhibitory effects of them were mutually exclusive. These results suggested that thymidine analogs would exert similar side effects associated with inhibition of pyrimidine nucleotide metabolism.

To ensure that the properties described above were not artifact due to recombinant expression, native mitochondrial TK2 was prepared from rat liver and analyzed by mass spectrometry. It was showed that native rat TK2 had an apparent molecular weight of 28 kDa and 82% sequence identity with human TK2. For the purified native rat TK2, the apparent K_m and V_{max} values were 19 μM and 70 pmol/min/mg for dThd, and 41 μM and 89 pmol/min/mg when dCyd was substrate. AZT and FLT also inhibited the phosphorylation of dThd with IC_{50} value of ~10 μM and stimulated dCyd phosphorylation more than 3 folds. These results demonstrated that recombinant human TK2 and rat mitochondrial TK2 had similar kinetic properties.

dTTP and dCTP are the feedback inhibitors of TK2 and have been shown to bind to recombinant TK2 (Barroso *et al.*, 2005; Wang *et al.*, 2003). Here, we found that TK2 was in most cases in a complex with approximately equimolar of dTTP, but not with other dNTPs. The TK2-bound dTTP could be removed by incubation with dThd or AZT and less efficiently with dCyd. Furthermore, the phosphorylation of dThd and dCyd by the dTTP-free TK2 was inhibited to a similar extent by AZT or FLT. Thus, these findings suggested that the presence of thymidine analogs released the dTTP inhibitor from the enzyme so that it could react efficiently with dThd or dCyd. On the other hand, these dThd analogs were poor substrates for TK2 and thus could not be converted to monophosphates. Consequently, dCyd phosphorylation benefits from the presence of AZT or FLT, leading to the observed stimulation.

On the basis of the estimation from V_{max}/K_m values, TK2 should produce dTMP and dCMP in a ratio of 1:0.7. Due to different impact on dThd and dCyd phosphorylation by AZT, TK2 activity with dThd will decrease by ~50%, whereas phosphorylation of dCyd will be stimulated by ~30%. Therefore, this will eventually change the normal dTTP and dCTP pools and affected mtDNA replication and maintenance. Thus, our results support the hypothesis for the mechanism suggested by the previously findings that the treatment with AZT led to a 50% depletion of dTTP pool but had little effect on dCTP pool in perfused rat heart mitochondria (Morris *et al.*, 2009). The investigation

presented in paper II provided a novel kinetic mechanism concerning the effects of pyrimidine nucleoside analogs on TK2 function and can improve our understanding of nucleosides metabolism in mitochondria and mitochondrial toxicities of nucleoside analogs.

2.3 S-glutathionylation and proteolytic degradation of TK2 (Paper III)

2.3.1 Aim of this study

Reversible protein S-glutathionylation is a posttranslational modification of redox-sensitive protein thiol groups of cysteine residues and plays important roles in regulation of protein stability and activity. Located in mitochondria that are a major site of ROS/RNS generation, mature TK2 contains five cysteine residues which are potential targets of S-glutathionylation. In the present study, we investigated the effects of cysteine-based redox regulation on the activity and stability of TK2.

2.3.2 Results and discussion

The activity of pure recombinant TK2 increased in the presence of reducing agents such as dithiothreitol (DTT) and GSH, but was inhibited by GSSG, in particular at high concentrations. Further studies showed that TK2 could be glutathionylated spontaneously by GSSG but not by GSH and the reaction was reversed by DTT and enhanced by diamide, a thiol oxidizing compound. To determine which cysteine residues reacted with GSSG, each of the five cysteine residues was replaced by an alanine residue by site-directed mutagenesis and subsequent analysis showed that Cys189 and Cys264 were the sites of S-glutathionylation. Extracts from isolated rat liver and brain mitochondria treated with H_2O_2 were added to recombinant TK2 and we found that recombinant TK2 was S-glutathionylated only by extracts from liver mitochondria. These results showed that S-glutathionylation of recombinant TK2 occurred *in vitro* but only with extracts from certain tissues subjected to oxidative stress.

To elucidate whether endogenous mitochondrial TK2 could be glutathionylated under stress condition, mitochondria were isolated from U2OS cells treated with H_2O_2. By using immunoprecipitation and Western-blot analysis, we detected that the level of S-glutathionylated TK2 in mitochondria from H_2O_2 treated cells was 3-5 times higher than that from untreated cells. The TK2 activity and protein level in U2OS mitochondria also decreased with increasing H_2O_2 concentrations. These results imply that under oxidative stress

mitochondrial TK2 is S-glutathionylated, which down-regulates enzyme activity and TK2 protein levels.

In mitochondria isolated from H_2O_2-treated U2OS cells, a time-dependent degradation of GSSG-treated recombinant TK2 was observed, which could be inhibited by the addition of PMSF, a serine protease inhibitor. Meanwhile, in control mitochondria, neither normal nor glutathionylated TK2 was degraded. Therefore, it was S-glutathionylation that caused the selective degradation of TK2 in mitochondria. Further studies demonstrated that S-glutathionylation of Cys189 was responsible for the selective degradation of TK2, which occurred only in mitochondria isolated from cells under oxidative stress.

Enzyme kinetic studies of cysteine mutants and structure modeling exhibited that none of the cysteines was involved in direct binding of the substrates (Figure 4). Since Cys189 residue was located at the α8 helix which formed the lid region, mutations of Cys189 and Cys264 to alanine or serine had no significant effect on the K_m values for dThd, however, the K_m values of C189A, C189S and C264S mutants were lower than that of wild type TK2 when dCyd was the substrate. Thus, S-glutathionylation of these cysteine residues would predominantly generate a structural effect on TK2, resulting in lower catalytic activity, particularly towards dCyd. Redox regulation of TK2 may play a significant role in the synthesis of mtDNA precursors and thus the maintenance of normal mitochondrial function.

2.4 Down-regulation of mitochondrial TK2 and dGK by nucleoside analogs (Paper IV)

2.4.1 Aim of this investigation

During oxidative stress, TK2 is glutathionylated by GSSG on Cys189, which leads to partial inactivation and rapid degradation of TK2. The Cys189 residue is conserved in dGK and dCK, but not in Dm-dNK. In paper IV, we investigated whether dGK and dCK were subjected to the same processes as TK2. Furthermore, the use of nucleoside analogs in anti-cancer and anti-viral therapies has been reported to induce oxidative stress (Amatore *et al.*, 2010; Kline *et al.*, 2009; Ferraresi *et al.*, 2006). Here, we tried to answer the question if oxidative stress induced by AZT and ddI could cause down-regulation of TK2 and dGK, which could be part of the mechanism of the mitochondrial toxicity observed with these nucleoside analogs.

2.4.2 Results and discussion

We found that both human dGK and dCK were glutathionylated by GSSG in a concentration-dependent manner similar to TK2, but had different sensitivities to GSSG. No glutathionylation could be detected with human TK1 or Dm-dNK despite that they contained 11 and 3 cysteine residues, respectively. This modification resulted in decreased activities of TK2 and dGK, but surprisingly increased dCK activity. These results indicate that S-glutathionylation is a common regulatory mechanism for the activity and/or stability of TK2, dGK and possibly dCK.

The effects of oxidative stress on dGK and TK2 were further investigated using U2OS cells and different oxidants *e.g.*, H_2O_2, tertbutyl hydroperoxide (tBHP) and diamide. We observed that the levels of TK2 and dGK decreased significantly in the presence of these oxidants, while the control protein was unaffected. On the basis of these results, we conclude that both TK2 and dGK are affected by oxidative stress, which apparently leads to selectively degradation by mitochondrial proteases. To exclude the involvement of cytosolic proteases, we incubated mitoplasts isolated from U2OS cells with H_2O_2 in the presence or absence of MG132, which is a mitochondrial AAA^+ Lon protease inhibitor. It was observed that MG132 strongly inhibited the degradation of TK2 and dGK. Thus, the down-regulation of TK2 and dGK during oxidative stress was S-glutathionylation dependent and the mitochondrial Lon protease was most likely responsible for the selective degradation.

AZT is known to provoke ROS/RNS production in some tissues and result in mtDNA oxidative damage, lipoperoxidation and GSH depletion, most likely contributing to AZT side effects. In paper IV, we studied whether mitochondrial TK2 and dGK were affected by the oxidative stress induced by AZT. The levels of mitochondrial TK2 and dGK decreased by 30% and 60% in the U2OS cells exposed to 20 μM AZT. In addition, a ~10% decrease was also observed in the level of the cytochrome c oxidase subunit II (COX II) encoded by mtDNA, while nuclear DNA encoded COX IV levels were not changed. The cytosolic TK1 and dCK levels were also not affected. These results strongly indicated that the presence of AZT leads to down-regulation of mitochondrial deoxynucleoside kinases via a similar mechanism as oxidant treatments.

To elucidate whether other nucleoside analogs had similar effects, a purine analog, ddI was utilized for a comparison. ddI is known to induce mitochondrial toxicity but require a different activation mechanism. The treatment with ddI unexpectedly induced similar effects on mitochondrial TK2 and dGK levels as those found with AZT. Although the mechanism of the ddI

induced degradation of TK2 and dGK is unclear, these results suggested that oxidative stress could be also involved in this case.

Uridine supplementation has been used successfully to remit the side effects of AZT in patients and addition of guanosine could protect human neuroblastoma cells against mitochondrial oxidative stress (Dal-Cim *et al.*, 2012). However, the mechanism of the protective effects of the nucleosides is poorly understood.

In paper IV, results are presented of experiments with additions of uridine and guanosine to cells treated with AZT and ddI, respectively. It was found that uridine supplementation of AZT treated cells could restore the mitochondrial TK2 and dGK to original levels. Guanosine supplementation in ddI treated cells had similar effects. The COX II levels were also back to original levels in the presence of uridine and guanosine. These results indicated that AZT and ddI treatments may lead to oxidative stress and severe and specific TK2 and dGK deficiency in mitochondria. Uridine and guanosine were able to protect TK2 and dGK from the down-regulation induced by AZT and ddI, respectively, and thereby prevent mitochondrial toxicities of these nucleoside analogs. However, the mechanism of the generalized protective effect observed after the addition of these nucleosides on mitochondrial function is unknown. This investigation has extended the knowledge regarding the redox regulation of mitochondrial nucleoside kinases and mtDNA precursor metabolism, and may help to prevent the toxicities of nucleoside analogs used in chemotherapy.

3 Conclusions and future perspectives

Investigations carried out in this thesis focused on the role of TK2 in dThd analogs-associated mitochondrial toxicities, TK2 mutations in arPEO and redox regulation, in particular S-glutathionylation of TK2 and dGK. The conclusions are as follows:

- ✓ TK2 mutations are a novel cause of adult-onset arPEO. Mutations in genes involved in mtDNA precursors metabolism and mtDNA replication can cause diverse clinical phenotypes as manifestations of mtDNA depletion and deletion disorders. MDS-related genes should be screened as candidates when studying the genetic background of individuals harboring multiple mtDNA deletion disorders, and *vice versa*.

- ✓ The phosphorylation of dThd and dCyd is inhibited and stimulated by dThd analogs, respectively, but is not affected by dCyd analogs. The apparent opposite kinetic effects on TK2 activity by dThd analogs result from their potencies in releasing enzyme-bound dTTP, which can disturb the relative rates of mt dTTP and dCTP pool synthesis *in vivo*.

- ✓ During oxidative stress, a conserved cysteine residue (Cys189 in TK2), in the dCK/dGK enzyme family is S-glutathionylated by GSSG. This S-glutathionylation decreases TK2 and dGK activity, but increases dCK activity. Furthermore, this oxidative modification leads to proteolytic degradation of TK2 and dGK in mitochondria.

- ✓ Treatment of U2OS cells with AZT and ddI results in selective degradation of mitochondrial TK2 and dGK, most likely as a result of oxidative stress. Uridine and guanosine supplementations antagonize the impairments of the function of both these enzymes *in vivo*.

- ✓ Nucleoside analogs used in chemotherapies inhibit TK2 and dGK activity, alter specificities of TK2 and dGK towards different substrates and induce oxidative stress and S-glutathionylation of TK2 and dGK, leading to degradation. This may be a major reason for depletion of mtDNA levels and mitochondrial toxicity in cells treated with nucleoside analogs.

The results presented in this thesis provide new insights concerning the regulation of enzymes involved in mtDNA precursor metabolism and on the potential mechanisms underlying nucleoside analogs-induced mitochondrial toxicities. Future crystal structure determination of TK2 is needed to understand thoroughly the kinetic effects of nucleoside analogs on pyrimidine nucleoside phosphorylation by TK2 and the correlation of TK2 mutations and functional defects. These will be helpful to improve pro-drugs used in anti-viral and anti-cancer chemotherapy. Meanwhile, the expression and regulation at the transcriptional, translational and post-translational levels of TK2 and dGK still remain unsettled. Future studies may provide insight regarding mechanisms of mtDNA precursor metabolism and homeostasis, and the relations between diverse phenotypes of tissue-specific mtDNA disorders and genetic/pharmacological backgrounds. Furthermore, past investigations on inherited mtDNA disorders mainly focused on the genetic alternations in mtDNA and nuclear genes, which have been identified to be involved in mitochondrial structure and function. More comprehensive whole genome-wide screening and evaluation are essential to ascertain the genetic causes of mitochondrial diseases.

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Acknowledgement

The studies presented in this thesis were performed at the Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences. I would like to give my sincere appreciation to all the people who supported me in all aspects during my study, and express my special gratitude to:

Liya, my main supervisor and mentor. You gave me the opportunity to be a PhD student in your group, introduced me to the field of nucleoside metabolism, tolerated my mistakes and encouraged me with every possible chance. I have learnt much more knowledge and skills than any time before and experienced a fruitful PhD carrier. I could never find a better supervisor than you.

Staffan, my co-supervisor, for your support for my PhD position and great enthusiasm on my projects, helping me to revise my manuscripts and thesis, for your open mind and solid scientific knowledge in valuable discussions about my projects.

Members of the SE group: Elena, thanks for your help with cell cultures. Louise, thanks for the enjoyable time and interesting conversations about our problems as PhD students. Hanan, you are my first colleague from North Africa, I learn many traditions and customs about your home country. Kiran, a wonderful roommate, we have very good collaborations in booking and using facilities.

Current and former colleagues at the department: Fabio, you teach me a lot about flow-cytometry and cell culturing skills; Anders, I'm glad to have the enzyme kinetic lab with you. Gianni, my humorous roommate, thanks for

guiding me in cell culturing and data analysis. Ronnie and Elin, it is my pleasure to have the mitochondria lab with you. Ida, thank you for assist in application for dissertation and grants. Gabriella, Mirjana and Kerstin, for ordering reagents and preparation of pipette tips. Piotr, for fixing everything in the corridor. I also want to thank all the people in our department, Gunnar, Sara W, Iulia, Helena, Osama, Jan, My, Diana, Sara, Mona, Ingrid, Anna, Siwei, Erik, Marlene, Naresh and Hanna.

I would like to acknowledge all the collaborators and co-authors in Professor Anu Suomalainen's group from University of Helsinki, Finland.

My special friends in Uppsala: Chuan Wang and Lin Li, I am pleased to meet both of you in Sweden. Jun Chen, you have been my classmate for ten years, much longer than others. Wei Sun, the efficiency of my experiments improves with your help.

Finally and mostly, I wish to express my deepest thankfulness to my family: Mom and Dad, thanks for your supports and endless love. My wife, Shengnan, you abandoned your well-paid job in China in order to accompany and take care me aboard. Thank you and I love all of you.