Functions and Regulation of eukaryotic NAD kinase – an essential enzyme for NADP biosynthesis

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CONTENTS

LIST OF PAPERS

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

Paper I:

Pollak N, Niere M, Ziegler M. (2007). NAD kinase levels control the NADPH concentration in human cells. *J. Biol. Chem.* 282, 33562-33571.

Paper II:

Pollak N, Berger F, Ziegler M. Calcium/calmodulin-dependent phosphorylation of human NAD kinase. Manuscript.

Paper III:

Pollak N, Niere M, Patel S, Ziegler M. Isoform-specific targeting of NAD kinase by calmodulin. Manuscript submitted.

ABBREVIATIONS

ADP	adenosine diphosphate
ADPr	ADP-ribose
AIP	autoinhibitory peptide
Ca^{2+}	calcium
cADPr	cyclic ADP-ribose
cADPrP	2'-phosphate cyclic ADP-ribose
CaM	calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase II
G6PD	glucose-6-phosphate dehydrogenase
H_2O_2	hydrogen peroxide
IDP	NADP-specific isocitrate dehydrogenase
NAAD	nicotinic acid adenine dinucleotide
NAADP	nicotinic acid adenine dinucleotide phosphate
NAD	nicotinamide adenine dinucleotide, oxidized and reduced form
NADK	NAD kinase
NADP	nicotinamide adenine dinucleotide phosphate, oxidized and reduced form
RNS	reactive nitrogen species
ROS	reactive oxygen species
shRNA	short-hairpin-RNA

ABSTRACT

NAD and NADP, both major carriers of cellular reducing equivalents, are of vital importance in diverse metabolic and signaling processes. Efficient maintenance and regulation of the reduction-oxidation status is therefore crucial within the cell. The only known pathway of NADP generation is the phosphorylation of NAD catalyzed by NAD kinase (NADK). Owing to the multiple roles of NADP these enzymes have gained considerable interest over the past few years. Genes encoding NADKs have been cloned only recently. The enzymatic activity has been shown to be essential for prokaryotic organisms and important for maintenance of the NADP pool in yeast *Saccharomyces cerevisiae* and plant *Arabidopsis thaliana*. While both yeast and plant encode three isoforms, only a single NADK, located in the cytoplasm, has been identified in mammals. Little is known about the function and regulation of NADK in mammalian cells. To investigate the physiological roles of NADK its expression level was altered in human cells. While recombinant NADK was rather specific for the oxidized form, NAD^+, as substrate, modulation of cellular NADK activity was reflected in significant changes of the NADPH content. Overexpression of NADK caused a 4-5 fold increase in NADPH but provided only moderate protection towards oxidative stress conditions. Short-hairpin-RNA-mediated downregulation of NADK had surprisingly little impact on cell proliferation, endogenous reactive oxygen species levels and cell viability after oxidant treatment. However, the stable shifts in the NADP redox ratio in the generated cell lines influenced the expression of genes known to be involved in oxidative stress response. Furthermore, phosphorylation of NADK *in vitro* mediated by calcium/calmodulin-dependent protein kinase II was established. The serine residue 64 was identified as phosphorylation site. The consequence of this modification remains unclear since no influence on the catalytic activity or the subcellular localization of NADK was observed.

Two NADK isoforms, which differ only in their N-terminal sequences, were identified in the sea urchin *Strongylocentrotus purpuratus*. Importantly, for both isoforms a direct activation of NADK by calcium/calmodulin was demonstrated. Although the existence of calmodulin-dependent NADKs has long been appreciated, the molecular basis of this regulation had so far remained unknown. Moreover, one of the isoforms was also specifically phosphorylated by calcium/calmodulin-dependent protein kinase II. The calmodulin-mediated activation and phosphorylation was associated with the different N-termini, while the catalytic domain is found in the identical C-terminal region of the proteins. The results suggest the N-terminal part of NADKs to be important as a regulatory domain.

3

1 INTRODUCTION

1.1 Metabolic roles of NAD and NADP

Nicotinamide adenine dinucleotide (NAD) and its phosphorylated form NADP play an essential role in vertebrate and invertebrate cells being involved in electron transfer in various redox reactions (Figure 1). This electron transfer is not associated with a net consumption of NAD(P). Despite the little difference in the structure by only a single phosphate group, the redox cofactors have distinct roles in cellular pathways. NAD^+ is used in catabolic reactions and the generated NADH provides reducing power for other metabolic pathways or ATP synthesis. In contrast, NADP in its reduced form is used for reductive biosynthetic pathways namely syntheses of fatty acids, amino acids, steroids and deoxyribonucleic acids and NADP is an essential cofactor for one of the fundamental processes of life, the photosynthesis in plant chloroplasts. NADPH is considered to be the reductive power of the cell being an indispensable cofactor of reductases maintaining the pool of reduced glutathione and thioredoxin and for detoxifying enzymes as cytochrome P450. However, NAD(P) was discovered to be not only an electron carrier but also a precursor for molecules involved in protein modification and signaling pathways (Figure 1). The knowledge of the role of "NAD(P)-consuming" enzymes in the regulation of important cellular functions has rapidly increased since then. Thus, the interest in the biosynthetic routes leading to NAD(P) formation and maintaining the nucleotide pools is rising. One of the crucial enzymes in the maintenance of the NAD(P) pool is NAD kinase. It phosphorylates NAD to NADP using ATP as phosphoryl donor. This reaction represents the only known way to generate NADP in all living organisms.

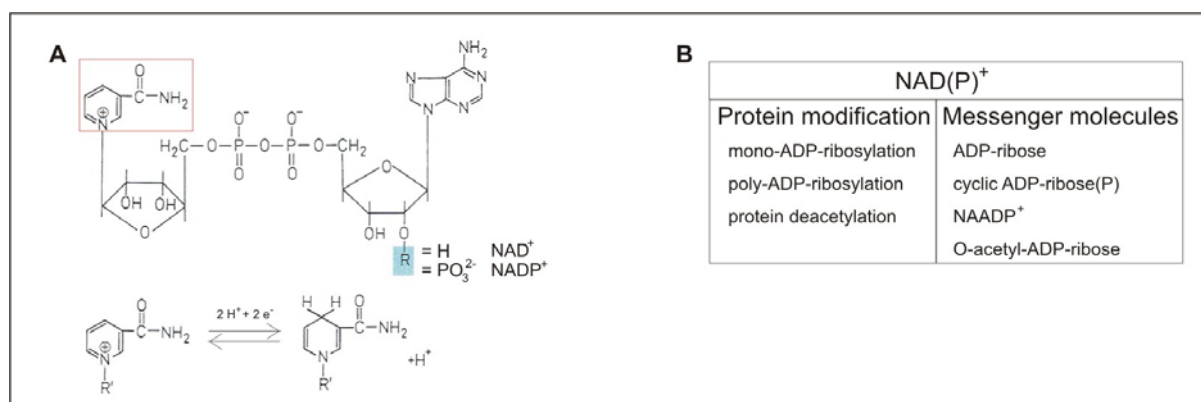**Figure 1: NAD(P) as electron carrier and in regulatory reactions.** (A) Structure of NAD(P). The nicotinamide moiety is boxed in red. The lower part shows the difference in hydrogen atoms in the nicotinamide moiety during reversible conversion between oxidized and reduced forms. (B) NAD(P) serves as precursor of messenger molecules and as substrate for covalent modifications of target molecules.

1.2 NAD(P)-mediated signaling

1.2.1 NAD$^+$ as a substrate for protein modification

NAD$^+$-dependent protein modification involves the cleavage of the glycolytic bond between nicotinamide and ADP-ribose and the subsequent ADP-ribosyl-transfer either onto proteins (ADP-ribosylation) or on acetate (NAD$^+$-dependent protein deacetylation). ADP-ribosylation reactions are associated with two classes of enzymes: mono-ADP-ribosyltransferases (ARTs) and poly-ADP-ribose polymerases (PARPs).

The family of PARPs catalyze the polymerization of ADP-ribose units from NAD$^+$ on target proteins. This poly-ADP-ribosylation (Figure 2) introduces negative charges and can thereby alter the functional properties of modified proteins. Interestingly, poly-ADP-ribosylation occurs in multicellular organisms, but it is absent in prokaryotes and yeast. Poly-ADP-ribose metabolism regulates various biological processes, including DNA repair, maintenance of genome integrity, transcriptional regulation, centromer function, telomere dynamics and cell death (reviewed in (Bürkle, 2005; Schreiber et al., 2006)). Cell death depending on the overactivation of PARP-1, a nuclear enzyme, has been demonstrated to play an important role in a variety of pathophysiological and inflammatory conditions (Virag, 2005). Thus, the development of PARP-inhibitory compounds as therapeutic agents is of major interest (Horvath and Szabo, 2007). Recently, new PARP proteins have been identified in mammalian cells and have already been established as important regulators of various cellular events (Schreiber et al., 2006). The investigation of their physiological functions will increase the understanding of the cellular PAR metabolism.
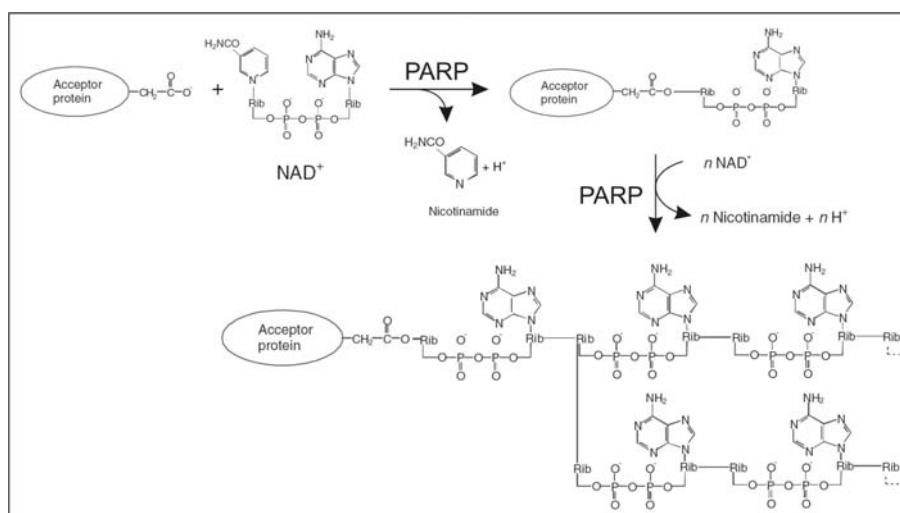

Figure 2: Poly-ADP-ribosylation by PARP. PARPs cleave the glycosidic bond of NAD$^+$ between nicotinamide and ribose followed by the covalent modification of mainly glutamate residues of acceptor proteins with an ADP-ribosyl unit. PARPs also catalyze the elongation, giving rise to linear polymers with chain lengths of up to

about 200 ADP-ribosyl units. Some of the PARP family members catalyze a branching reaction. Modified from (Bürkle, 2005).

Mono-ADP-ribosylation (Figure 3A) was originally identified as a catalytic activity of bacterial toxins, including diphtheria, cholera, pertussis and clostridial toxins (Corda and Di Girolamo, 2003). The modification of crucial host cell proteins interferes with their physiological function (Corda and Di Girolamo, 2003; Di Girolamo et al., 2005). Several ARTs are characterized in eukaryotes (Glowacki et al., 2002; Di Girolamo et al., 2005). Interestingly, ART1-ART4 are glycosylphosphatidylinositol-anchored membrane proteins where the catalytic domain is extracellularly located and ART5-ART7 are secreted proteins (Glowacki et al., 2002). These findings suggest the targets of ARTs to be primarily extracellular proteins. Recently, unusual intracellular ARTs were identified (Liszt et al., 2005; Haigis et al., 2006). They are members of the SIR2 (silent information regulator 2) family and do not share obvious sequence homology with ART1-7. Protein mono-ADP-ribosylation is involved in several regulatory processes such as signaling, immune response, cytoskeleton modification and membrane traffic (Corda and Di Girolamo, 2003). The consequence of this transfer reaction appears to be associated with an inhibition of the target proteins (Di Girolamo et al., 2005). For example, the enzymatic activity of mitochondrial glutamate dehydrogenase is inhibited by the transferase SIRT4 (Herrero-Yraola et al., 2001; Haigis et al., 2006) and CD38, an ectoenzyme involved in calcium signaling, is inhibited by ADP-ribosylation (Han et al., 2000).

ADP-ribose transfer has also a function in tRNA splicing. The 2'-phosphate of the splice intermediate is transferred to NAD^+ thereby forming ADP-ribose 1''-2'' cyclic phosphate and nicotinamide (Culver et al., 1993). Whether this product has a physiological function remains to be established.

It should be noted that $NADP^+$ can also be used as substrate for covalent protein modifications. 2´-phospho-adenylation and 2´-phospho-ADP-ribosylation were reported (Hilz et al., 1986; Hilz, 1988) but the relevance of both was not further investigated. A recent study reported $NADP^+$-dependent phospho-ADP-ribosylation of dinitrogenase reductase from *Azotobacter vinelandii*, an aerobic soil bacterium able to fix atmospheric nitrogen by converting it to ammonia (Ponnuraj et al., 2005).

The deacetylation of acetyl-lysine-modified proteins (Figure 3A) is catalyzed by Sir2-homologues, sirtuins (Landry et al., 2000; Smith et al., 2000), a family of proteins conserved from bacteria to humans (Frye, 2000). Originally identified in yeast, the proteins encoded by SIR genes are responsible for repression of transcription at yeast mating-type loci (Rine and Herskowitz, 1987), telomeres (Aparicio et al., 1991) and ribosomal DNA (Fritze et al., 1997). Silencing is associated with deacetylated histones (Braunstein et al., 1993) and causes a tightly packed, inaccessible regional chromatin structure (Loo and Rine, 1994; Bi and Broach, 1997). The requirement for NAD^+ as co-substrate couples sirtuins to the metabolic status of the cell. Each reaction cycle generates 2' and 3'-O-acetyl ADP-ribose (OAADPr; Figure 3A), nicotinamide and the deacetylated substrate (Figure 3A, (Tanner et al., 2000; Sauve et al., 2001)). The by-product OAADPr is suggested to be a new messenger molecule (Borra et al., 2002; Kustatscher et al., 2005; Liou et al., 2005; Grubisha et al., 2006) that may be linked to sirtuin-mediated pathways. Hydrolysis of OAADPr (Rafty et al., 2002; Ono et al., 2006) generates free ADP-ribose which may have signaling functions.

Sir proteins regulate the lifespan in multiple model organisms. In yeast, an extra copy of the *SIR2* gene increased the replicative lifespan, while deletion of *SIR2* shortens lifespan (Kaeberlein et al., 1999). Increase in the dosage of *Caenorhabditis elegans and Drosophila melanogaster* Sir2 orthologues also extends lifespan (Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004). Whether sirtuins similarly influence human aging is not known. Calorie restriction (CR) is a dietary regimen which is also known to mediate lifespan extension in yeasts, worms, flies and mammals (reviewed in (Sinclair, 2005)). CR positively affects age-related diseases such as cancer and auto-immune disorders (Barger et al., 2003). Sir2 is required for lifespan extension by CR in yeast, worms and flies (Lin et al., 2000; Rogina and Helfand, 2004; Wang and Tissenbaum, 2006) but Sir2-independent lifespan extension is also discussed (Kaeberlein and Powers, 2007). The mechanisms by which CR promotes NAD^+-dependent activities of Sir2 might include reducing of inhibitory nicotinamide levels (Anderson et al., 2003), elevating NAD^+ and increasing levels of Sir2.

Mammals have seven sirtuins (SIRT1-7). However, mono-ADP-ribosyl transferase activity, rather than protein-deacetylation, has been reported to be the main enzymatic activity of at least two proteins (Liszt et al., 2005; Haigis et al., 2006). Sirtuins regulate multiple processes in mammalian cells (Figure 3B; reviewed in (Haigis and Guarente, 2006; Michan and Sinclair, 2007)) and may also be connected to CR and longevity in mammalian cells (Rose et al., 2003; Cohen et al., 2004; Chen et al., 2005; Nisoli et al., 2005; Zhang et al., 2007). Thus, sirtuins could represent novel therapeutic targets for metabolic and neurodegenerative

diseases. In particular, sirtuin activators may positively influence health and vitality (Baur and Sinclair, 2006; Lagouge et al., 2006).

Figure 3: Sirtuin enzymatic activities and cellular functions of mammalian sirtuins. (A) Sirtuins are NAD$^+$-dependent deacetylases and mono-ADP-ribosyl transferases that regulate a wide array of proteins involved in metabolism and cell survival. The ε-acetyl lysine residues of the target protein serve as substrates for sirtuin-mediated deacetylation, which generates 2' and 3'-OAADPr as a by-product. NAM, nicotinamide. (B) Sirtuins regulate a variety of processes in mammalian cells. AC, acetylation. Adapted from (Michan and Sinclair, 2007).

1.2.2 NAD(P)$^+$ as precursor for molecules involved in calcium signaling

The divalent cation calcium (Ca^{2+}) is the most common signal transduction element in prokaryotic and eukaryotic cells controlling many processes (Berridge et al., 2000). Unlike other second messenger molecules, Ca^{2+} cannot be metabolized therefore cells have to tightly regulate intracellular Ca^{2+} levels. The cell has two sources of Ca^{2+} entry, either from the external medium or release from internal stores, mainly the endoplasmatic/sarcoplasmatic reticulum (ER/SR). These Ca^{2+} influx mechanisms are balanced by Ca^{2+} pumps to remove the Ca^{2+} signal. Both processes are often organized to produce short spikes and waves of Ca^{2+} to avoid the cytotoxic effects of prolonged high intracellular Ca^{2+} levels.

The release of Ca^{2+} from internal stores is stimulated and modulated by Ca^{2+} itself or the second messenger molecules myo-inositol 1,4,5-trisphosphate (IP$_3$), cyclic ADP-ribose (cADPr; Figure 4), and nicotinic acid adenine dinucleotide phosphate (NAADP; Figure 4). IP$_3$ releases Ca^{2+} from the ER (Streb et al., 1983) after binding its receptor (IP$_3$R). Ca^{2+} mobilization by the pyridine nucleotide derivatives was discovered shortly after (Clapper et al., 1987; Lee et al., 1989; Lee and Aarhus, 1995) and has been detected in several organisms and cell types (Lee, 2001; Guse, 2004).
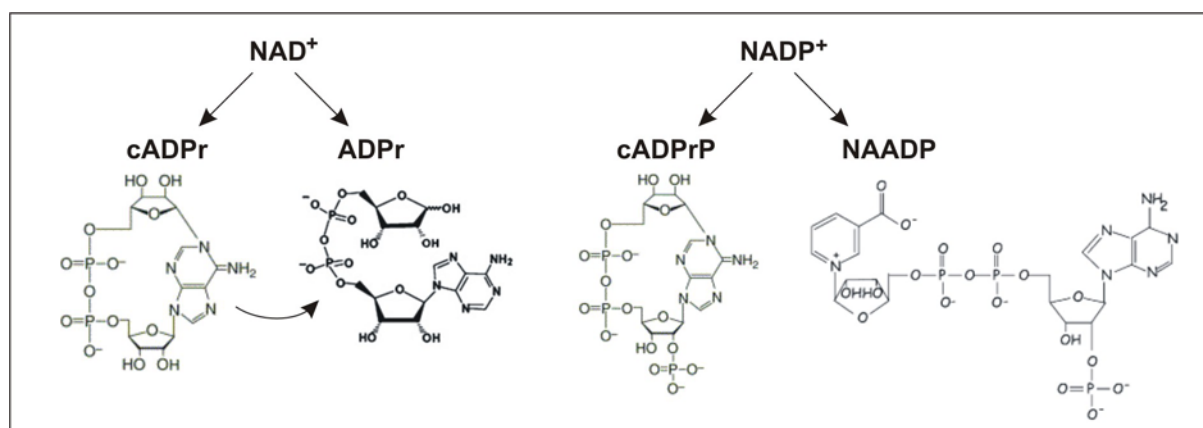
Figure 4: Structures of Ca^{2+}-mobilizing derivatives of NAD(P)$^+$. Cyclic ADPr is formed from NAD$^+$ by ADPr cyclase. ADPr is generated directly by NAD$^+$ hydrolysis or by cADPr hydrolysis. NAADP formation by an base-exchange reaction, substituting nicotinamide for nicotinic acid, is also catalyzed by ADPr cyclase and the same class of enzymes synthesize cADPrP from NADP$^+$. ADPr, ADP-ribose; cADPr, cyclic ADPr; cADPrP, 2'-phosphate cADPr; NAADP, nicotinic acid ADP.

Cyclic ADPr was shown to be an endogenous activator of Ca^{2+}-induced Ca^{2+} release by ryanodine receptor (RyR) in the ER/SR (Galione et al., 1991) while NAADP activates intracellular Ca^{2+} channels distinct from those that are sensitive to IP$_3$ and cADPr (Clapper et al., 1987; Lee and Aarhus, 1995). In the sea urchin egg, the NAADP-sensitive store appears to be an acidic, lysosome-related compartment (Churchill et al., 2002; Galione, 2006). This has been confirmed in some mammalian cell types but sensitivity of the ER/SR toward NAADP has also been reported (Hohenegger et al., 2002; Gerasimenko et al., 2003; Steen et al., 2007). Controversial discussed is the target receptor/Ca^{2+} channel for NAADP. Since inhibitors of the known IP$_3$R and RyR do not block NAADP-mediated Ca^{2+} release (Chini et al., 1995; Lee and Aarhus, 1995), a novel channel was suggested. RyRs are also shown to be involved in NAADP action (Gerasimenko et al., 2003; Langhorst et al., 2004; Dammermann and Guse, 2005). An NAADP receptor of sea urchin eggs has been partially characterized on the biochemical level (Berridge et al., 2002b) and the receptor is present in mammalian tissues (Patel et al., 2000; Zhang and Li, 2007). However, the molecular identity remains to be elucidated for further understanding how the multiple Ca^{2+}-release pathways contribute to the generation of specific Ca^{2+} signals.

The enzymatic activity responsible for synthesizing cADPr was purified from *Aplysia californica* (Hellmich and Strumwasser, 1991) and identified as ADPr cyclase (Lee and Aarhus, 1991). Strikingly, although NAADP is structurally distinct from cADPr, its formation by a base-exchange reaction is catalyzed by the same class of enzymes (Aarhus et al., 1995).

As an alternative, the direct phosphorylation of NAAD by NAD kinase has been proposed (Ziegler, 2000). In mammalian systems, the ecto-enzymes CD38 and CD157 appear to be the major enzymes with ADPr cyclase activity (Schuber and Lund, 2004), although additional cADPr-forming enzymatic activities have been detected in brains from CD38 knockout mice (Ceni et al., 2003). Degradation of cADPr is also associated with ADPr cyclase activity acting as a glycohydrolase thereby generating ADPr. Inactivation of the messenger NAADP is catalyzed by a specific Ca^{2+}-dependent phosphatase (Berridge et al., 2002a).

During the last years, even more Ca^{2+}-mobilizing molecules were linked to ADPr cyclases. 2'-phospho-cADPr is generated from $NADP^+$ (cADPrP; Figure 4) and likely acts by a similar mechanism as cADPr (Vu et al., 1996). Adenylic dinucleotides, synthesized from cADPr in the presence of adenine (Basile et al., 2005) may have cytotoxic effects (Bruzzone et al., 2007). ADPr, generated directly by NAD^+ hydrolysis or by cADPr hydrolysis (Figure 4), activates transient receptor potential melastatin 2 (TRPM2), a Ca^{2+} channel in the plasma membrane (Perraud et al., 2001). ADPr and TRPM2 are proposed to mediate oxidative stress-induced cell damage (Hara et al., 2002; Perraud et al., 2005; Yang et al., 2006).

1.3   Antioxidant defense systems – a key role for NADPH

The term redox state is often used to describe the balance of thiol redox (GSH/GSSG, protein sulfhydryls) and pyridine nucleotide redox ($NADPH/NADP^+$, $NADH/NAD^+$) in the cell. The redox state influences many metabolic, signaling and transcriptional processes and is kept in a reducing state to deal with free radical intermediates, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS). Some are highly reactive, can diffuse freely in the cell and oxidize cellular macromolecules thereby modulating their function. ROS include superoxide, hydroxyl radical and hydrogen peroxide (H_2O_2). Nitric oxide (NO) or its derivatives, in particular peroxynitrite, are considered as the major RNS. "Oxidative stress" results from a disturbance of the intracellular redox balance and is an important contributor in inflammatory processes, ischaemia/reperfusion injury, the onset of human diseases and the aging process (Houstis et al., 2006; Loh et al., 2006; Droge and Schipper, 2007; Valko et al., 2007).

ROS were classically described as harmful byproducts of aerobic metabolism. ROS, mainly superoxide anions, are generated in the mitochondria of eukaryotic cells during the process of oxidative phosphorylation (Cadenas et al., 1977) and also to a lesser extent outside the mitochondria. Mitochondrial ROS homeostasis plays a key role in the life and death of eukaryotic cells as mitochondria not only respond to ROS but also release ROS in response to

10

a number of pro-apoptotic stimuli (Jezek and Hlavata, 2005; Zorov et al., 2006). Additionally, cells encounter exogenous stress conditions including UV- or γ–irradiation, temperature changes, nutrient limitation, hypoxia and exposure to various drugs or toxins. On the other hand, there exist enzymes dedicated to ROS/RNS production as NAD(P)H oxidase, particularly in activated neutrophils and macrophages, other superoxide-producing enzymes and NO synthases (Nathan and Xie, 1994; Forman and Torres, 2002; Harrison, 2004). Furthermore, it is now obvious that transient increases of ROS and RNS act as normal intracellular messengers during cell growth, differentiation and even cell death (Sauer et al., 2001). For example, H_2O_2 at subtoxic concentrations modifies the function of several proteins including transcription factors, protein kinases and phosphatases (Burdon, 1995; Thannickal and Fanburg, 2000; Martindale and Holbrook, 2002). The reversible oxidation of protein thiols in form of cysteine and methionine residues plays an important role in this redox regulation of cellular signaling pathways (Ghezzi, 2005).

All cells are equipped with a variety of protective mechanisms against ROS-induced oxidative stress. Non-enzymatic reductants include the major thiol antioxidant glutathione (GSH), the small protein thioredoxin (Trx), ascorbate (Vitamin C), α-tocopherol (Vitamin E), carotenoids and flavonoids. Antioxidant enzymes include mitochondrial and cytosolic superoxide dismutases, peroxisomal catalase, and peroxidases in the mitochondria and the cytosol. These proteins are involved in removal of superoxide anion and H_2O_2 and their activities can be regulated according to the need of the cell to respond to increased levels of ROS.

GSH and Trx act also as cofactors for detoxifying enzymes as GSH peroxidases, Trx peroxidases, peroxiredoxins, glutaredoxins, sulfiredoxin and methionine sulfoxide reductases (Wood et al., 2003; Chang et al., 2004; Holmgren et al., 2005; Moskovitz, 2005). All these systems rely on the reduced form of glutathione and thioredoxin, their regeneration is catalyzed by GSH reductase and Trx reductase at the expense of NADPH. Accordingly, NADPH is regarded as the principal cellular reductant. NADPH is also needed for formation of active catalase (Kirkman and Gaetani, 1984) and can act directly as an antioxidant with scavenging and repairing capabilities (Kirsch and De Groot, 2001).

1.4 Biosynthesis of NAD$^+$

Four different precursors derived from the diet and the cellular catabolism contribute to NAD$^+$ synthesis (Figure 5; (Rongvaux et al., 2003; Bieganowski and Brenner, 2004; Magni et al., 2004)). The pathways leading to NAD$^+$ synthesis can be divided in *de novo* pathway, nicotinic acid (NA) import pathway and salvage pathways from nicotinamide (Nam) and nicotinamide riboside (NamR). Since NAD$^+$-consuming enzymes, as PARP, CD38 and ART/sirtuin, can be inhibited by Nam, the salvage of Nam is of major importance. In addition, recycling of Nam can save the energy which is required for *de novo* NAD$^+$ synthesis. Tryptophan, an essential amino acid, is used in the *de novo* generation of NAD$^+$ in most living organisms. It is converted in several steps to quinolinic acid (QA) and further to nicotinic acid mononucleotide (NAMN). NAMN is also formed from NA by NA phosphoribosyltransferase (NAPRT). NAMN is then adenylated by NMN/NAMN adenylyltransferase (NMNAT) and resulting desamido-NAD (NAAD) is converted into NAD$^+$ by glutamine-dependent NAD synthase (NADS).

Apparently, the Nam salvage pathway diverged in eukaryotes. Nam may represent the main source of NAD for most mammalian cells. It is converted by Nam phosphoribosyltransferase (NamPRT) to NMN, which is further adenylated to NAD$^+$ by NMNAT. In contrast, eukaryotes including yeast, plant, worm and fly and many bacteria convert Nam to NA through nicotinamidase (NDase). No obvious homologues of NDase have been found in vertebrates (Rongvaux et al., 2003).

Recently, it was shown that fungi and vertebrates salvage NamR, a vitamin precursor of NAD, which occurs in milk (Bieganowski and Brenner, 2004). NamR is phosphorylated by NamR kinase (NRK) and the generated Nam mononucleotide (NMN) is converted to NAD$^+$ by NMNAT. At least in yeast, NamR can also be cleaved to yield free Nam (Belenky et al., 2007).
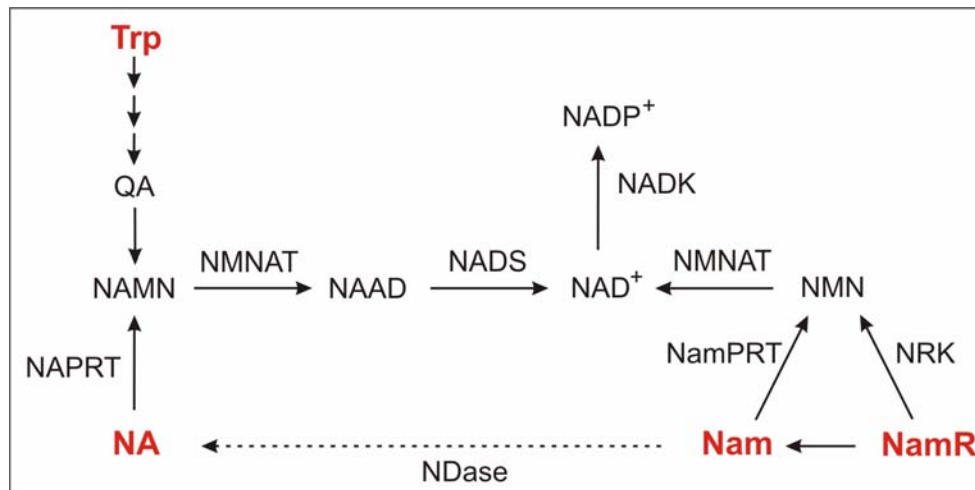
Figure 5: Biosynthesis of NAD(P)$^+$. Four different precursors (red) contribute to the known pathways of NAD$^+$ biosynthesis. Nicotinamidase (NDase) is found in yeast, plant and *C. elegans* but is absent in mammals. NA, nicotinic acid; NAAD, NA adenine dinucleotide; Nam, nicotinamide; NAMN, NA mononucleotide; NamR, Nam riboside; NMN, nicotinamide mononucleotide; QA, quinolinic acid; Trp, tryptophan.

The primary structures of eukaryotic enzymes involved in NAD$^+$ biosynthesis are well known (Berger et al., 2004; Bieganowski and Brenner, 2004; Magni et al., 2004). Interestingly, three NMNAT isoforms are identified in humans and shown to be expressed in the nucleus, the Golgi apparatus and the mitochondria (Berger et al., 2005) thus suggesting organelle-specific NAD$^+$ biosynthesis. Increased NMNAT-1 activity did not result in changes of cellular NAD$^+$-levels (Anderson et al., 2002; Revollo et al., 2004) but can protect axons from mechanical and toxic insults (Araki et al., 2004). SIRT1 was suggested as effector of this axonal protection (Araki et al., 2004) however SIRT1-independent local mechanisms might also be involved (Wang et al., 2005). NamPRT, also known as pre-B-cell colony-enhancing factor (PBEF), is located in the cytosol and extracellular (Rongvaux et al., 2002). While overexpression of NamPRT increases NAD$^+$ concentrations and has cell-protective benefits (Revollo et al., 2004; van der Veer et al., 2005), extracellular NamPRT might convert Nam produced by extracellular cADPr synthases. Increased dosage of NaPRT and NDase increased longevity of yeast but had no influence on cellular NAD$^+$ levels (Anderson et al., 2002; Anderson et al., 2003). Knock-down of NDase in *C. elegans* is associated with decreased lifespan (van der Horst et al., 2007) and NDase-deficient plants have lower levels of NAD(P) (Wang and Pichersky, 2007). Increasing the dosage of NDase increased the survival of *C. elegans* under conditions of oxidative stress (van der Horst et al., 2007).

In conclusion, NAD$^+$ or its precursors might have protective effects for health or in a variety of human diseases and enzymes involved in NAD$^+$ metabolism are suggested to be attractive

targets for drug discovery (Khan et al., 2007). However, further studies are needed to completely understand the contribution of NAD$^+$-mediated pathways.

1.5 NADK – the essential enzyme for NADP biosynthesis

NAD kinase (NADK) catalyzes the phosphorylation of NAD to NADP using ATP or inorganic polyphosphate as phosphoryl donors. This reaction represents the only known way to generate NADP in prokaryotic and eukaryotic cells. NADP synthesis was first reported in yeast homogenates (Vestin, 1937; von Euler and Adler, 1938). Later, the enzyme was partially purified and characterized from yeasts, mammals, plants and sea urchin (McGuinness and Butler, 1985). However, conclusive structural information has only recently become available after identification of the amino acid sequences of *Mycobacterium tuberculosis* and *Micrococcus flavus* NADKs (Kawai et al., 2000). To date, NADKs of *Homo sapiens*, yeast *S. cerevisiae*, plant *Arabidopsis thaliana* and multiple prokaryotes have been characterized (reviewed in (Magni et al., 2006; Pollak et al., 2007)). NADK is an essential enzyme as has been demonstrated in *Escherichia coli*, *M. tuberculosis*, *Bacillus subtilis* and *Salmonella enterica* (Gerdes et al., 2002; Kobayashi et al., 2003; Sassetti et al., 2003; Grose et al., 2006) and in the yeast *S. cerevisiae* (Bieganowski et al., 2006; Shianna et al., 2006). Furthermore, embryonic lethality upon loss of both alleles in mouse is reported as an unpublished observation (Shianna et al., 2006).

1.5.1 Structural properties of NADK

The comparison of known NADK amino acid sequences shows a highly conserved region, the catalytic domain, within the C-terminus of the proteins whereas the N-terminal parts vary and are significantly longer in eukaryotic NADKs (Figure 6). The catalytic domain (Pfam PF01513) is characterized by two well-conserved motifs, the GGDG motif and a glycine-rich motif. The GGDG motif is also found in diacylglyceride kinase, sphingosine kinase and 6-phosphofructokinase thereby proposed to be a feature of a kinase superfamily including NADK (Labesse et al., 2002). The importance of both motifs for the enzymatic activity was confirmed by site-directed mutagenesis of amino acid residues within the motifs (Labesse et al., 2002; Raffaelli et al., 2004; Mori et al., 2005b) and was further supported by three-dimensional structures of prokaryotic NADKs (Garavaglia et al., 2004; Liu et al., 2005; Mori et al., 2005b; Oganesyan et al., 2005; Poncet-Montange et al., 2007).
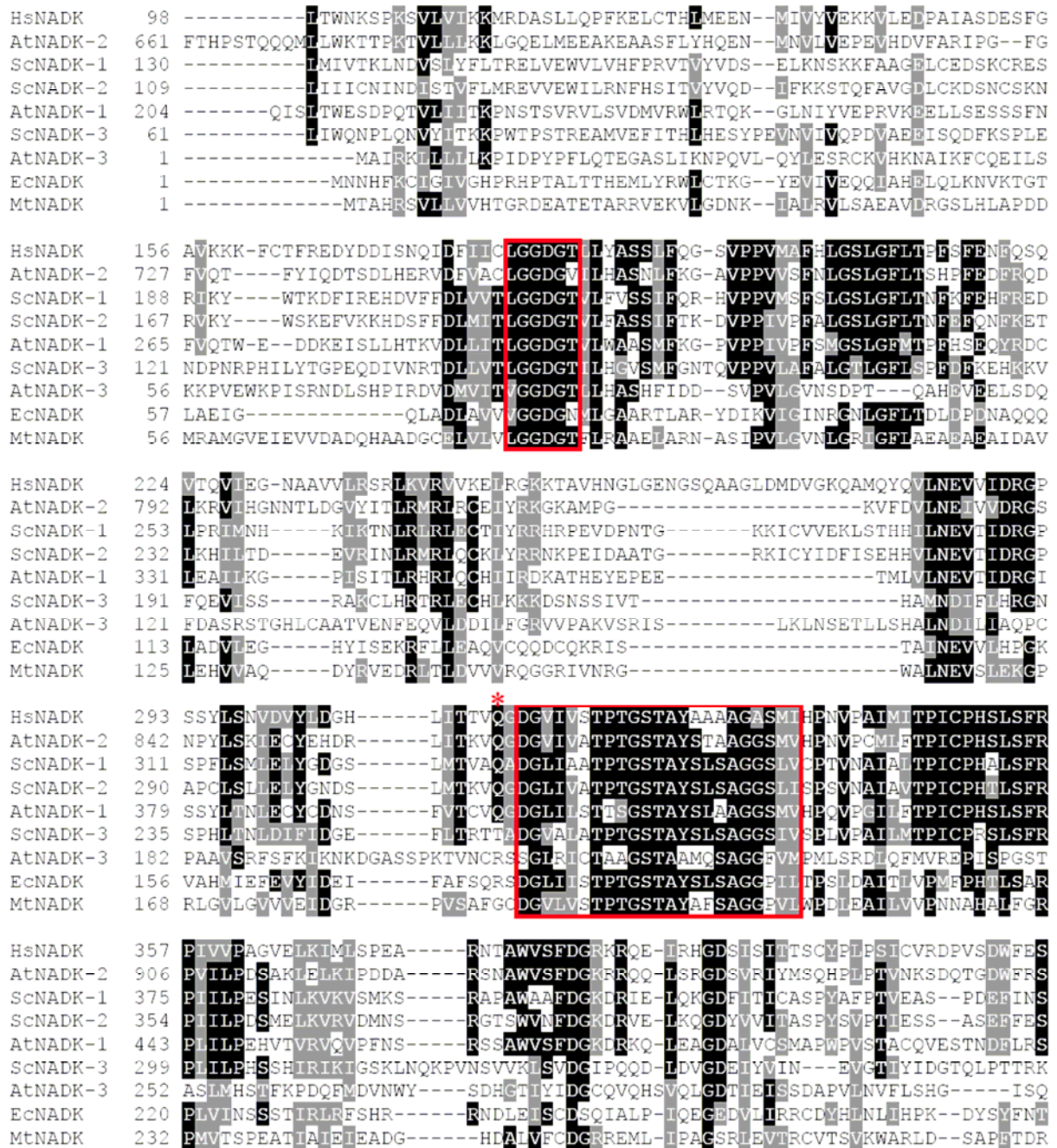
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HsNADK    98  ----------ITWNKSPKSVLVIRKMRDASLLQPFKELCTHLMEEN--MIVYVEKKVLELPAIASDESFG
AtNADK-2 661  FTHPSTQQQMLLWKTTPKTVLLIKKLGQELMEEAKEAASFLYHQEN--MNVLVEPEVHDVFARIPG--FG
ScNADK-1 130  ----------IMIVTKLNDVSLYFLTRELVEWLVHFPRVTVYVDS--ELKNSKKFAAGELCEDSKCRES
ScNADK-2 109  ----------LIIICNINDISTVFLMREVVEWILRNFHSITVYVQD--IFKKSTQFAVGDLCKDSNCSKN
AtNADK-1 204  -------QISLTWESDPQTVLIITKPNSTVRVLSVDMVRWLRTQK--GLNIYVEPRVKEELLSESSSFN
ScNADK-3  61  ----------IIWQNPLQNVYITKKPWTPSTREAMVEFITHLHESYPEVNVIVQPDVAEEISQDFKSPLE
AtNADK-3   1  --------------MAIRKLLLILKPIDPYPFLQTEGASLIKNPQVL-QYLESRCKVHKNAIKFCQEILS
EcNADK     1  ------------MNNHFKCIGIVGHPRHPTALTTHEMLYRWLCTKG--YEVIVEQQIAHELQLKNVKTGT
MtNADK     1  ------------MTAHRSVLLVHTGRDEATETARRVEKVLGDNK--IALRVLSAEAVDRGSLHLAPDD

HsNADK   156  AVKKK-FCTFREDYDDISNQIDFIICLGGDGTLLYASSLFQG-SVPPVMAFHLGSLGFLTPFSFENFQSQ
AtNADK-2 727  FVQT----FYIQDTSDLHERVDFVACLGGDGVILHASNLFKG-AVPPVVSFNLGSLGFLTSHPFEDFRQD
ScNADK-1 188  RIKY----WTKDFIREHDVFFDLVVTLGGDGTVLFVSSIFQR-HVPPVMSFSLGSLGFLTNFKFEHFRED
ScNADK-2 167  RVKY----WSKEFVKKHDSFFDLMITLGGDGTVLFASSIFTK-DVPPIVPFALGSLGFLTNFEFQNFKET
AtNADK-1 265  FVQTW-E--DDKEISLLHTKVDLLITLGGDGTVLWAASMFKG-PVPPIVPFSMGSLGFMTPFHSEQYRDC
ScNADK-3 121  NDPNRPHILYTGPEQDIVNRTDLLVTLGGDGTILHGVSMFGNTQVPPVLAFALGTLGFLSPFDFKEHKKV
AtNADK-3  56  KKPVEWKPISRNDLSHPIRDVDMVITVGGDGTVLHASHFIDD--SVPVLGVNSDPT---QAHEVEELSDQ
EcNADK    57  LAEIG------------QLADLAVVVGGDGNMLGAARTLAR-YDIKVIGINRGNLGFLTDLDPDNAQQQ
MtNADK    56  MRAMGVEIEVVDADQHAADGCELVLVLGGDGTFLRAAELARN-ASIPVLGVNLGRIGFIAEAEAEAIDAV

HsNADK   224  VTQVIEG-NAAVVLRSRLKVRVVKELRGKKTAVHNGLGENGSQAAGLDMDVGKQAMQYQVLNEVVIDRGP
AtNADK-2 792  LKRVIHGNNTLDGVYITLRMRLRCEIYRKGKAMPG-------------------KVFDVLNEIVVDRGS
ScNADK-1 253  LPRIMNH-----KIKTNLRLRLECTIYRRHRPEVDPNTG------KKICVVEKLSTHHILNEVTIDRGP
ScNADK-2 232  LKHILTD-----EVRINLRMRIQCKIYRRNKPEIDAATG-------RKICYIDFISEHHVLNEVTIDRGP
AtNADK-1 331  LEAILKG-----PISITLRHRIQCHIIRDKATHEYEPEE----------------TMLVLNEVTIDRGI
ScNADK-3 191  FQEVISS-----RAKCLHRTRLECHLKKKDSNSSIVT--------------------HAMNDIFLHRGN
AtNADK-3 121  FDASRSTGHLCAATVENFEQVLDDILFGRVVPAKVSRIS--------LKLNSETLLSHALNDILIAQPC
EcNADK   113  LADVLEG-----HYISEKRFILEAQVCQQDCQKRIS-----------------------TAINEVVLHPGK
MtNADK   125  LEHVVAQ-----DYRVEDRLTLDVVRQGGRIVNRG-----------------------WALNEVSLEKGP

HsNADK   293  SSYLSNVDVYLDGH------LITTVQGDGVIVSTPTGSTAYAAAAGASMIHPNVPAIMITPICPHSLSFR
AtNADK-2 842  NPYLSKIECYEHDR------LITKVQGDGVIVATPTGSTAYSTAAGGSMVHPNVPCMLFTPICPHSLSFR
ScNADK-1 311  SPFLSMLELYGDGS------LMTVAQGDGLIAATPTGSTAYSLSAGGSLVCPTVNAIALTPICPHALSFR
ScNADK-2 290  APCLSLLELYGNDS------LMTKVQGDGLIVATPTGSTAYSLSAGGSLISPSVNAIAVTPICPHTLSFR
AtNADK-1 379  SSYLTNLECYCDNS------FVTCVQGDGLILSTTSGSTAYSLAAGGSMVHPQVPGLIFTPICPHSLSFR
ScNADK-3 235  SPHLTNLDIFIDGE------FLTRTTAGDGVALATPTGSTAYSLSAGGSIVSPLVPAILMTPICPRSLSFR
AtNADK-3 182  PAAVSRFSFKIKNKDGASSPKTVNCRSSGLRICTAAGSTAAMQSAGGFVMPMLSRDLQFMVREPISPGST
EcNADK   156  VAHMIEFEVYIDEI------FAFSQRSDGLIISTPTGSTAYSLSAGGPILTPSLDAITLVPMFPHTLSAR
MtNADK   168  RLGVLGVVVEIDGR------PVSAFGDGVLVSTPTGSTAYAFSAGGPVLWPDLEAILVVPNNAHALFGR

HsNADK   357  PIVVPAGVELKIMISPEA-----RNTAWVSFDGRKRQE-IRHGDSISITTSCYPLPSICVRDPVSDWFES
AtNADK-2 906  PVILPDSAKLELKIPDDA-----RSNAWVSFDGKRRQQ-LSRGDSVRIYMSQHPLPTVNKSDQTGDWFRS
ScNADK-1 375  PIILPESINLKVKVSMKS-----RABAWAAFDGKDRIE-LQKGDFITICASPYAFPTVEAS--PDEFINS
ScNADK-2 354  PIILPDSMELKVRVDMNS-----RGTSWVNFDGKDRVE-LKQGDYVVITASPYSVPTIESS--ASEFFES
AtNADK-1 443  PLILPEHVTVRVQVPFNS-----RSSAWVSFDGKDRKQ-LEAGDAIVCSMAPWPVSTACQVESTNDRLFS
ScNADK-3 299  PLILPHSSHIRIKIGSKLNQKPVNSVVKLSVDGIPQQD-LDVGDEIYVIN---EVGTIYIDGTQLPTTRK
AtNADK-3 252  ASLMHSTFKPDQFMDVNWY----SDHGTIYIDGCQVQHSVQLGDTIEISSDAPVLNVFLSHG-----ISQ
EcNADK   220  PLVINSSSTIRLRFSHR------RNDLEISCDSQIALP-IQEGEDVLIRRCDYHLNLIHPK--DYSYFNT
MtNADK   232  PMVTSPEATIAIEIEADG------HDAIVFCDGRREML-IPAGSRLEVTRCVTSVKWARLD--SAPETDR
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Figure 6: Partial multiple alignment of NADKs from several organisms. Amino acid sequences of human NADK (protein ID NP_075394); *A. thaliana* NADK-1, NADK-2 and NADK-3 (NP_974347, NP_564145 and NP_177980); *S. cerevisiae* NADK-1, NADK-2 and NADK-3 (P21373, NP_010873 and NP_015136); *E. coli* NADK (NP_417105); and *M. tuberculosis* NADK (BAB21478) were compared using Clustal W. Identical and similar residues are highlighted in black and grey respectively. The two conserved NADK motifs are boxed in red. Modified from (Pollak et al., 2007).

While several three-dimensional structures of prokaryotic NADKs have been resolved (Figure 7), no eukaryotic one is yet reported. *M. tuberculosis* NADK is a biological tetramer and corresponds to a dimer of dimers (Garavaglia et al., 2004; Mori et al., 2005b). The overall

structure is organized into an N-domain, a C-domain and a C-terminal tail. The N-terminal domain resembles a classical Rossmann fold, known to be involved in dinucleotide binding (Rossmann et al., 1974), consisting of a single parallel β-sheet flanked by α-helices. The C-terminal domain adopts a novel fold with structural similarity to the human Ki67 fork-head-associated domain (Garavaglia et al., 2004). The structures of *Archaeoglobus fulgidus* and *Listeria monocytogenes* NADKs show similarities, two dimers assemble a tetramer (Liu et al., 2005; Poncet-Montange et al., 2007). The substrates NAD^+ and ATP and the product $NADP^+$ are all bound to a cleft between the N- and C-domains. Subunit interactions are required for ligand binding (see Figure 7B; black broken line). Indeed, known NADKs are homo-oligomeric proteins as determined by gel filtration experiments.

A phosphate transfer mechanism based on substrate-assisted catalysis was suggested (Poncet-Montange et al., 2007), however further studies are needed to completely understand the reaction mechanism for NADKs. In particular, it will be necessary to determine the crystal structure of the human NADK. Comparison of its structural, catalytic and kinetic properties with NADKs of highly pathogenic organisms could represent an important step for development of novel antibacterial drugs.
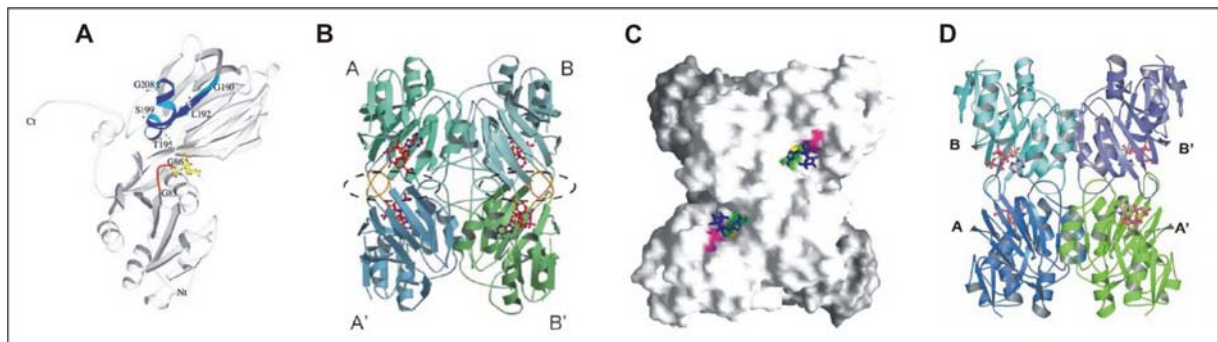
Figure 7: Crystal structures of prokaryotic NADKs. (A) Stereo ribbon representation of the MtNADK subunit. Residues of the GGDG sequence fingerprint and of the second conserved region are colored in *red* and *blue*, respectively. Residues proposed to be involved in the NAD binding are drawn in a *ball-and-stick* representation. Ct, C terminus; Nt, N terminus. Modified from (Garavaglia et al., 2004). (B) Ribbon model of quaternary structure of MtNADK-NAD. An asymmetric unit contained 2 subunits (MtNADK-NAD-A/-B, or –A'/B'). NAD is indicated in *red* (nitrogen, *blue*; phosphorus, *pink*). The A-A' and B-B' contact regions are enclosed by *black broken line*. MtNADK-flexible loops are shown in *yellow*. Modified from (Mori et al., 2005b). (C) Molecular surface model of quaternary structure of MtNADK-NAD as shown in (B), but was rotated by 90°C. NAD is shown in *light blue*. Surfaces of residues involved in NAD binding are colored in *green* (Thr200-Ser205), *yellow* (Asn159, Glu160), and *pink* (Asp85, Gly86). Modified from (Mori et al., 2005b). (D) Ribbon diagram of the AfNADK tetramer in complex with ATP. The individual monomers are labeled as A, A', B and B'. Modified from (Liu et al., 2005).

1.5.2 Enzymatic properties of NADK

NADK has an absolute requirement for divalent metal ions. The enzymes from both, prokaryotic and eukaryotic organisms utilize nucleoside triphosphates, preferentially ATP, as phosphoryl donor for catalysis. Interestingly, some bacterial enzymes, for example from *M. tuberculosis*, *M. flavus* and *B. subtilis*, can also utilize inorganic polyphosphate (poly(P)) (Magni et al., 2006; Pollak et al., 2007). Poly(P) is present in nearly all living organisms and considered to be an 'ancient' energy carrier preceding ATP (Kornberg, 1999). Even glucose-6-phosphate-dependent bacterial NADKs have been described (Bark et al., 1993), but were not investigated further. The question how the bacterial NADKs distinguish between the phosphoryl donors has not been answered yet but may represent a key to the development of new specific antibacterial drugs. In particular the mycobacterial enzyme could be an attractive and novel drug target for the treatment of tuberculosis. NADK is essential for *M. tuberculosis* (Sassetti et al., 2003) and exhibits higher affinity towards poly(P) than ATP (Raffaelli et al., 2004). Relatively high K_M values for ATP have been reported for *E. coli*, *S. enterica* and human NADKs, suggesting a regulation of their activity according to physiological changes of the cellular ATP concentration, which is in the millimolar range.

Several recombinant enzymes have been shown to phosphorylate only NAD^+, but hardly NADH, thus displaying strict substrate selectivity. Others exhibit a far less stringent substrate specificity and use both the oxidized and reduced nucleotide however with different efficiency (reviewed in (Magni et al., 2006; Pollak et al., 2007)). Significant differences in substrate selectivity as reported for plant NADKs may have arisen from the assay conditions used. What determines this difference in specificity regarding the substrate NAD? A previous report suggested that a single amino acid residue in the conserved domain of NADK homologues is responsible for substrate specificity (Mori et al., 2005a). Relaxed substrate specificity is associated with a glycine or polar amino acid residue while an arginine, as well as charged and hydrophobic residues might be responsible for strict NAD^+-specificity. Indeed, substitution of the corresponding arginine residue in NAD^+-specific NADK from *E. coli* resulted in activity towards NADH but accounted only for ~3% of the original activity (Mori et al., 2005a). Multiple sequence alignment analysis reveals dominantly polar amino acid residues in eukaryotic homologues including human NADK at the investigated position (see Figure 6, red asterisk). This suggests mainly relaxed substrate specificity for these NADKs. Human NADK was shown to efficiently phosphorylate NAD^+ (Lerner et al., 2001). Whether the enzyme does utilize NADH was not investigated before.

1.5.3 Modulation of NADK activity

The enzymatic activity of NADKs is modulated in different ways. Several NADKs are inhibited by the reduced pyridine nucleotides (Kawai et al., 2001a; Kawai et al., 2001b; Raffaelli et al., 2004; Shi et al., 2005; Grose et al., 2006) and by high concentrations of $NADP^+$ (Garavaglia et al., 2003; Ochiai et al., 2004; Raffaelli et al., 2004). NADP and NADH inhibit *S. cerevisia*e NADKs to different extents suggesting a possible regulation by these compounds (Shi et al., 2005). NADK of *S. enterica* is inhibited by NADPH during normal growth and is released from the inhibition in response to metabolic changes induced by UV irradiation or oxidative stress (Grose et al., 2006). Quinolinate, a metabolite in NAD biosynthesis, was shown to inhibit NADK of *S. enterica* serotype Typhimurium (Cheng and Roth, 1994) and to activate *B. subtilis* NADK (Garavaglia et al., 2003), but had no effect on human NADK (N. Pollak and M. Ziegler, unpublished work). Several NADKs are strongly inhibited by thiol-group modifying reagents, indicating that an SH-group of the enzyme is likely involved in catalysis (Delumeau et al., 2000; Kawai et al., 2001b; Garavaglia et al., 2003; Ochiai et al., 2004; Shi et al., 2005; Turner et al., 2005).

A connection between NADK activity and calcium/calmodulin (Ca^{2+}/CaM) is well known for decades, at least for the enzymes from sea urchin eggs (Epel et al., 1981), plants (Anderson et al., 1980) and human neutrophils (Williams and Jones, 1985). Surprisingly, the molecular mechanism of this activation has not been elucidated yet. NADK in sea urchin eggs is activated by Ca^{2+}/CaM early after fertilization, resulting in a shift in the NADPH/$NADP^+$ ratio from 1:1 to 3-6:1 (Epel, 1964; Schomer and Epel, 1998). Increased levels of NADPH may be important for DNA and protein syntheses (Whitaker and Steinhardt, 1981; Standart et al., 1985; Akkaraju et al., 1991). Moreover, increased NADPH levels lead to an increase of H_2O_2 production via NADPH oxidase (Foerder et al., 1978; Heinecke and Shapiro, 1989). H_2O_2 has a role in hardening of the fertilization membrane (Foerder and Shapiro, 1977). NADK activity has been partially purified from sea urchin eggs (Blomquist, 1973) but the molecular identity of the enzyme is not known.

Plants possess both CaM-dependent and –independent NADK isoforms that differ in their subcellular localization (Simon et al., 1982; Dieter and Marme, 1984; Simon et al., 1984; Pou De Crescenzo et al., 2001). However, the activity of the three recombinant NADK isoforms from *A. thaliana* was not influenced by Ca^{2+}/CaM although AtNADK-2 binds CaM in a Ca^{2+}-dependent manner (Turner et al., 2004; Turner et al., 2005). CaM has also no detectable effect on the activity of human recombinant NADK (Lerner et al., 2001). In contrast, a partially purified NADK from human neutrophils was shown to be activated by Ca^{2+}/CaM (Williams

and Jones, 1985). These observations suggest that the activation of NADK by Ca^{2+}/CaM might be indirect and requires additional factors or the existence of so far not identified NADK isoforms in human cells and plants.

1.5.4 Subcellular localization and physiological roles of eukaryotic NADKs

Mammals – NADK activity has been reported in several fractions of various mammalian tissues (McGuinness and Butler, 1985). However, enrichment of NADK activity in bovine liver was only detectable after a high-speed centrifugation step of the homogenate (Lerner and Ziegler, unpublished). The subcellular localization of the characterized enzyme from *H. sapiens* (Lerner et al., 2001) has yet not been established. The protein is predicted to be localized to the cytosol (PSORT II, (Nakai and Horton, 1999)) as no putative subcellular targeting sequences are found. It should be noted that the human genome harbours two putative NADK homologues. One cDNA sequence (GenBank AF250320) could represent an alternatively spliced form. Attempts to isolate this respective nucleotide sequence from various human cell lines have not been successful (Berger and Ziegler, unpublished). The second cDNA sequence (GenBank NM_153013) obviously lacks the 5'-region since the highly conserved GGDG motif is not present. Attempts to isolate additional 5'-sequence have not been successful yet (Berger, Pollak and Ziegler, unpublished). Tissue-specific expression of the NADK mRNA revealed only one band at the expected size in most tissues (Lerner et al., 2001).

Relatively little is known about the physiological role of NADK in mammals. NADK activity showed dramatic temporal and spatial variations during development of rat conceptus (Akella and Harris, 2001), reflecting the importance of maintaining the cellular redox state during organogenesis. Indeed, mouse embryonic lethality upon NADK loss was reported as an unpublished observation (Shianna et al., 2006). Furthermore, NADK activity may have an important role in the mitogenic response of immune cells (Williams and Jones, 1985; Berger et al., 1987; Iqbal and Zaidi, 2006).

Yeast – The presence of NADK activity in the cytoplasm and mitochondria of yeast is long established (McGuinness and Butler, 1985). Three NADK isoforms in *S. cerevisiae* have been characterized recently. ScNADK-1 and ScNADK-2 are cytosolic proteins and exhibit relaxed substrate specificity with some preference towards NAD^+ (Kawai et al., 2001b; Shi et al., 2005) whereas ScNADK-3 localizes to the mitochondrial matrix and prefers NADH as substrate (Outten and Culotta, 2003). A global analysis of protein expression in yeast revealed ScNADK-2 to be less present (~300 molecules per cell) compared to ~5000 molecules of

19

isoform 1 and 3 (Ghaemmaghami et al., 2003). In agreement, ScNADK-1 and ScNADK-3 are responsible for almost all NADK activity *in vivo* since the disruption of both is synthetically lethal, with or without the deletion of isoform 2 (Bieganowski et al., 2006; Shianna et al., 2006). The double mutant can be rescued by overexpression of any of the yeast NADK isoforms (Bieganowski et al., 2006) or human NADK (Shianna et al., 2006). Notably, ScNADK-3, the mitochondrial isoform, was established as the most critical generator of NADPH in yeast. Yeast cells deleted for this isoform are highly sensitive towards oxidative stress induced by hydrogen peroxide or hyperoxia (Krems et al., 1995; Outten and Culotta, 2003). ScNADK-3 mutants exhibit increased frameshift mutations in the mitochondrial DNA and increased petite colony formation (Strand et al., 2003). Deletions strains accumulate iron in the mitochondria and are defective in mitochondrial Fe-S cluster-containing enzymes (Outten and Culotta, 2003) leading to upregulation of genes involved in iron transport (Shianna et al., 2006). In contrast, disruption of ScNADK-1 or ScNADK-2 does not result in severe growth defects or in hypersensitivity to high oxygen concentration (Outten and Culotta, 2003). ScNADK-1 is suggested to provide NADP for cytosolic NADP-dependent dehydrogenases and detoxifying systems while the role of isoform 2 remains rather unclear (Bieganowski et al., 2006).

Plants – NADK activity has originally been associated with cytoplasmic (Simon et al., 1982), mitochondrial (Dieter and Marme, 1984) and chloroplastic fractions (Muto and Miyachi, 1981; Jarrett et al., 1982). Based on sequence similarity with NADKs from other organisms, three genes encoding putative NADKs were predicted in the *A. thaliana* genome (Hunt et al., 2004) and subsequently cloned and characterized (Turner et al., 2004; Turner et al., 2005). AtNADK-1 and AtNADK-3 are expressed in most tissues, while isoform 2 is only expressed in leaves (Turner et al., 2004; Berrin et al., 2005). Recent studies on the localization of overexpressed green fluorescent protein-NADK constructs in plant revealed AtNADK-2 to be targeted to the chloroplasts (Chai et al., 2005) while AtNADK-1 and AtNADK-3 are found in the cytoplasm (Chai et al., 2006).

Plant NADK isoforms respond differently to stress conditions. Irradiation or treatment with hydrogen peroxide of plant cell suspensions induced AtNADK-1 mRNA and protein levels (Berrin et al., 2005). The transcript of AtNADK-3 is induced by cold, osmotic stress, a superoxide-generating agent and high salinity whereas the transcript levels of AtNADK-1 and AtNADK-2 were not significantly affected by these conditions (Chai et al., 2006). Plants deficient for AtNADK-1 exhibit increased sensitivity to oxidative stress (Berrin, 2005). The

knockout of NADK-2 in *A. thaliana* delays growth and development, resulting in reduced leaf size and seed production. NADK activity and NADP(H) content was significantly lowered in leaves of the mutant compared to wild-type (Takahashi et al., 2006). AtNADK-2 protects chloroplasts against oxidative stress and plays a vital role in chlorophyll synthesis according to the importance of NADP in photosynthesis (Chai et al., 2005). Knockout of AtNADK-3 enhanced the plants sensitivity to various stress conditions (Chai et al., 2006). Under these conditions the mutant plants show significantly decreased NADPH level and GSH/GSSG ratio and increased expression of stress marker genes compared to wild-type plants.

In summary, these observations establish significant differences for maintenance of the NADP pool in different organelles in yeast compared to human and plant cells. The fact that the yeast *S. cerevisiae* apparently lacks the enzyme transhydrogenase makes a separate NADPH production in the mitochondria necessary. The direct utilization of NADH for generation of NADPH may be reflected by the oxidation state of NADH. In yeast cells ~50% of the total cellular NAD(H) is in the reduced form, and this may also be the case for the mitochondrial NAD(H) pool (Ting et al., 1977). In contrast, only 10-20% of the total cellular NAD(H) is in the reduced form in mammalian cells. Furthermore, yeast mitochondria possess an NADP phosphatase activity more active towards the reduced form (Bernofsky and Utter, 1968) and can take up NAD^+ from the cytosol (Todisco et al., 2006) which might facilitate the recycling of NAD^+ back into the tricarboxylic acid cycle. However, it remains possible that mammalian and plant mitochondria also possess an NADK to generate NADP, although such an enzyme has not been identified to date.

1.6   Redox-reactions generating NADPH

Besides a direct phosphorylation of NADH by NADKs several $NADP^+$-specific dehydrogenases, as well as nicotinamide nucleotide transhydrogenase have been identified as significant contributors to the cellular NADPH pool (Figure 8).

1.6.1   *Glucose-6-phosphate dehydrogenase*

Glucose-6-phosphate dehydrogenase (G6PD), the key regulatory enzyme of the cytosolic pentose phosphate pathway, catalyzes the oxidation of glucose-6-phosphate to 6-phospho-gluconolactone and the production of reducing equivalents in form of NADPH. The importance of G6PD is illustrated by clinical manifestations of its deficiency. This common enzymopathy, affecting over 200 million people worldwide, is the cause of haemolytic

anaemia (Beutler, 1991). Furthermore, G6PD might be linked to diabetes. Hyperglycemia causes inhibition of G6PD activity thus lowered NADPH levels and increases oxidative stress (Xu et al., 2005; Diaz-Flores et al., 2006). Oxidative stress can induce damage of pancreatic β-cells and is believed to be involved in type 2 diabetes (Robertson and Harmon, 2006).

In human cell lines exposed to H_2O_2 or diamide both G6PD activity and mRNA level are rapidly and transiently enhanced (Ursini et al., 1997) and salt stress in plants increased G6PD activity and protein (Valderrama et al., 2006). This suggested G6PD to be part of a mechanism to protect cells against oxidative damage. Indeed, G6PD overexpression confers strong protection against H_2O_2-induced cell death through induction of GSH production (Salvemini et al., 1999; Tian et al., 1999). Conversely, inhibition of G6PD activity potentiated H_2O_2-induced cell death (Tian et al., 1999). Surprisingly, the knockout of the G6PD gene in yeast or mouse embryonic stem cells produced viable cells (Nogae and Johnston, 1990; Pandolfi et al., 1995; Filosa et al., 2003) while mouse embryos lacking G6PD fail to develop normally (Longo et al., 2002). Yeast and mouse cells deleted for the G6PD gene (ΔG6PD) are more sensitive to oxidizing agents than control cells (Nogae and Johnston, 1990; Pandolfi et al., 1995; Filosa et al., 2003). Mouse ΔG6PD cells were able to maintain high [NADPH]/[NADP$^+$] and [GSH]/[GSSG] ratios under normal culture conditions but oxidative stress resulted in a dramatic reduction of both ratios in ΔG6PD cells compared to wild-type cells (Filosa et al., 2003). G6PD-deficient cell lines were shown to undergo premature cellular senescence (Ho et al., 2000).

In summary, G6PD has an important role in antioxidant defense mechanisms. However NADPH produced by a cytosolic enzyme is unavailable to mitochondria, the major site of superoxide production. Thus, other NADP$^+$-specific dehydrogenases have been identified as major sources of NADPH supply.
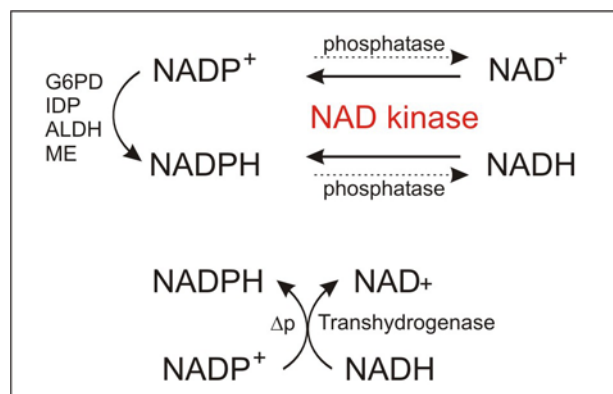
Figure 8: Generation of NADP in eukaryotic cells. G6PD, glucose-6-phosphate dehydrogenase; IDP, NADP$^+$-dependent isocitrate dehydrogenase; ALDH, NADP$^+$-dependent aldehyde dehydrogenase; ME, NADP$^+$-dependent malic enzyme; Δp, electrochemical proton gradient.

1.6.2 Isocitrate dehydrogenase

NADP^{+}-dependent isocitrate dehydrogenase (IDP) catalyzes the oxidative decarboxylation of isocitrate, producing α-ketoglutarate and CO_2. Two isoforms were identified in mammals, localized to the mitochondria (IDPm) and the cytosol (IDPc) (Plaut et al., 1983) although the latter has also been found in peroxisomes (Yoshihara et al., 2001). Interestingly, IDPc in rat liver was shown to be 16-18fold more active in NADPH production than G6PD (Veech et al., 1969). Several reports have confirmed that the isoforms play a key role in the control of cytosolic and mitochondrial redox balance through the supply of NADPH for regeneration of GSH and reduced Trx in both compartments. IDPm activity and protein level are upregulated after treatment with H_2O_2 (Jo et al., 2001) and the activity of both isoforms is increased by γ-irradiation (Lee et al., 2004). The overexpression of either isoform protected the cells from oxidative damage induced by oxidizing agents (Jo et al., 2001; Lee et al., 2002), γ-irradiation (Lee et al., 2004), cadmium (Kil et al., 2006) or heat-shock (Kim et al., 2005). Conversely, downregulation of IDP at the mRNA level potentiated ROS production and oxidative damage. Three IDP homologues were identified in yeast and localized to mitochondria (Haselbeck and McAlister-Henn, 1991), cytosol (Loftus et al., 1994) and peroxisomes (Henke et al., 1998). The mitochondrial and cytosolic isoforms are not essential, even in the context of G6PD deletion (Minard et al., 1998). The peroxisomal IDP is involved in metabolism of unsaturated fatty acids (Henke et al., 1998).

In plants, four IDP isoforms have been described with different subcellular localizations: mitochondria, chloroplast, peroxisomes and cytosol (Galvez et al., 1999), however the cytosolic enzyme is the predominant form (Chen, 1998). This isoform may have a protective antioxidant role since activity and protein level are upregulated under salt stress (Valderrama et al., 2006) and oxidative stress induced by paraquat (Marino et al., 2007).

1.6.3 NADP^{+}-dependent malic enzyme and aldehyde dehydrogenase

Two malic enzyme isoforms have been described in mammalian tissues and were localized to the cytosol and the mitochondria (Frenkel, 1971; Bukato et al., 1995). Cytosolic malic enzyme activity is increased in rat liver cirrhogenesis (Sanz et al., 1997) and affected in acute hepatic injury (Diez-Fernandez et al., 1996), presumably by providing NADPH for detoxification reactions. The mitochondrial isoform plays an important role in the pyruvate-recycling pathway and in maintaining the intramitochondrial GSH in the brain (McKenna et al., 2000). In pancreatic islets, the mitochondrial pyruvate-malate shuttle could contribute far

more to NADPH generation than the cytosolic pentose phosphate pathway (MacDonald, 1995).

Yeast malic enzyme is a mitochondrial protein (Boles et al., 1998). Disruption of the corresponding gene did not result in an observable growth phenotype (Boles et al., 1998), nor did it worsen the growth phenotypes of G6PD and IDP deletion strains (Minard et al., 1998).

Aldehyde dehydrogenases (ALDHs) are a group of $NAD(P)^+$-dependent enzymes that are important for detoxification of aldehyde substrates. ALDHs are upregulated in response to oxidative stress and several mutations in *ALDH* genes have been linked to cancer and Alzheimer's disease (Vasiliou and Nebert, 2005). ALDH isoforms may play a role in cellular defense mechanisms (Pappa et al., 2003; Choudhary et al., 2005). However, whether mammalian ALDHs have a general role in the maintenance of NADPH has to be investigated. There is little information available on the antioxidant properties of $NADP^+$-dependent ALDHs in yeast cells. Initially, disruption of the genes encoding Ald6, a cytosolic enzyme, and G6PD was thought to be lethal (Grabowska and Chelstowska, 2003) but it was shown later that co-disruption of both is not lethal (Minard and McAlister-Henn, 2005).

1.6.4 *Nicotinamide nucleotide transhydrogenase*

Nicotinamide nucleotide transhydrogenases (NNT) of mammalian mitochondria and bacteria are membrane-bound enzymes that catalyze the direct transfer of a hydride ion between NAD(H) and NADP(H) in a reaction that is coupled to transmembrane proton translocation $[H^+_{out} + NADH + NADP^+ \rightleftharpoons H^+_{in} + NAD^+ + NADPH]$. Under physiological conditions the equilibrium of this reaction is shifted towards NADPH formation. The contribution of NNT to the NADPH pool was estimated to be 35-45% in *E. coli* (Sauer et al., 2004). In mammalian mitochondria, the contribution is assumed to be at least as high or higher. An important function of NNT is the generation of a high $NADPH/NADP^+$ and thus GSH/GSSG ratio which is critical for protection of mitochondria against oxidative stress (Hoek and Rydstrom, 1988). Indeed, knockout of NNT in *C. elegans* resulted in mutant nematodes with highly decreased GSH/GSSG ratio and increased sensitivity to conditions of oxidative stress (Arkblad et al., 2005). Recently, a link between NNT and diabetes was demonstrated (Toye et al., 2005; Freeman et al., 2006). The diabetic characteristics of C57BL/6J mice were linked to a mutated *Nnt* gene resulting in lack of the NNT protein (Toye et al., 2005) and an elevated level of ROS in β-cells (Freeman et al., 2006). Thus, the C57BL/6J mice may provide a model for human type 2 diabetes.

2   AIMS OF THE STUDY

NAD kinase is suggested to play a key role in the maintenance of the cellular redox status. Relatively little is known about the functions and regulation of NADK in mammalian cells. Therefore, a major goal of the work presented in this thesis was to identify the physiological roles of the known human NADK and the molecular mechanisms regulating NADK activity in mammalian cells. The cellular function of NADK was investigated by modulation of its abundance by overexpression or short-hairpin-RNA-mediated knock-down with subsequent analyses of the cell's phenotype, the redox-ratio of NADP and the ability to cope with oxidative stress situations.

The regulation of NADK activity by Ca^{2+}/CaM in extracts of plants and human neutrophils has long been known. However, the molecular mechanism of the CaM-dependent activation of NADK has not been established. Neither the recombinantly expressed human enzyme nor the plant NADK isoforms displayed sensitivity towards Ca^{2+}/CaM. Thus I speculated that CaM may act indirectly on NADK. Therefore, I tested the possibility that the human NADK is substrate for a Ca^{2+}/CaM-dependent phosphorylation which could affect the catalytic activity of the enzyme.

Since NADK in sea urchin eggs is also known to be activated in a CaM-dependent manner I was interested in studying the molecular mechanism of this activation. However, the molecular identity of the enzyme was not known at the beginning of this project. In 2006, the complete sequence and analysis of the genome of the sea urchin *Strongylocentrotus purpuratus* was reported ("Science" Vol. 314). I aimed at identifying and cloning the respective sea urchin NADK cDNA sequence and to characterize this enzyme. In particular, I was interested whether CaM regulates sea urchin NADK activity directly.

3 SUMMARY OF RESULTS

3.1 Functional consequences of NADK up- and downregulation in human cells

The study published in Paper I describes the cellular consequences on manipulating the expression level of cytoplasmic NADK in human cells. HEK293 cell lines stably overexpressing NADK or small-hairpin-RNA targeting the NADK mRNA were generated. Cells stably overexpressing an unrelated small-hairpin-RNA directed against the mRNA of enhanced green fluorescent protein were used as control cells. The stable overexpression of NADK in HEK293 cells resulted in an almost 200-fold increase of NADK transcript level, protein level and catalytic activity. On the other hand, stable knock-down of NADK was followed by a decreased level of NADK transcript and protein and consequently, reduced NADK activity. This efficient downregulation of the single known NADK mRNA had no significant effect on cell proliferation under normal conditions.

NADK recombinantly expressed in bacteria was rather specific for NAD$^+$ as substrate and phosphorylated NADH less efficiently. However, modulation of NAD kinase expression in human cells was shown to have an effect on the cellular NADPH content, while the NADP$^+$ concentration was not significantly altered. NADK overexpression was followed by a 4-5 fold increase in the NADPH content, whereas the concentration of this nucleotide was about threefold decreased in the knock-down cells. Next, I investigated whether this significant increase of NADPH resulted from upregulation of NADP$^+$-dependent dehydrogenases. Measurements of the relative mRNA levels of NADP$^+$-reducing enzymes of the cytosolic pentose phosphate pathway revealed only slight changes. The activity of G6PD, the rate-limiting enzyme of this pathway, was also not changed in reponse to NADK overexpression or knock-down.

As one consequence of the altered NADPH concentration, the level of intracellular reactive oxygen species (ROS) correlated with the level of NADK activity. Overexpression of NADK resulted in a decrease of about 20% and knock-down of NADK was followed by an ~25% increase in endogenous ROS amount compared to control cells. To monitor the capability of the three cell lines to deal with oxidative stress, two agents were used to elevate ROS levels: menadione, which generates superoxide radicals through a cycling reaction and hydrogen peroxide, a source of peroxide radicals. Treatment of the NADK knock-down cells resulted in slightly higher ROS level with respect to control cells whereas the ROS level was significantly lower in cells overexpressing NADK.

Secondly, the NADK expression and concomitantly the NADPH levels had surprisingly little effect on ROS-induced damage. The different cell lines were exposed to hydrogen peroxide or menadione and cell survival was determined 24 hours after the treatment. NADK upregulation increased resistance against hydrogen peroxide-induced cell death while this protective effect was not observed for menadione treatment. On the other hand, cell survival after treatment of NADK knock-down cells was comparable to that of control cells.

Thirdly, the presented results provide evidence that the NADK expression level influences the expression of genes known to be involved in oxidative stress response, namely peroxiredoxin 5 (PRDX5) and NF-E2-related factor 2 (Nrf2). PRDX5 is a thioredoxin peroxidase catalyzing the reduction of hydrogen peroxide, alkyl hydroperoxides and peroxynitrite, and is therefore important in cellular antioxidant defense and hydrogen peroxide-mediated signaling pathways. The transcription factor Nrf2 is known to transactivate genes containing the antioxidant response cis-elements (ARE) in their promoter region. Nrf2 regulates the expression of numerous antioxidant proteins including peroxiredoxins in response to pro-oxidant conditions in the cell. The PRDX5 mRNA and protein levels were significantly decreased in NADK overexpressing cells while knock-down of NADK and thus reduced NADPH content increased PRDX5 transcript and protein levels. Significantly elevated Nrf2 amount was found as a consequence of NADK downregulation, whereas NADK overexpression had no significant effect on the Nrf2 transcript level.

3.2 Ca^{2+}/CaM-mediated posttranslational modification of human NADK

A putative posttranscriptional regulation for human NAD kinase is described (Paper II). The protein was shown to be an *in vitro* substrate for Ca^{2+}/CaM-dependent phosphorylation. The serine residue at amino acid position 64 (S64) of NADK was identified as major acceptor site which could be phosphorylated by Ca^{2+}/CaM-dependent protein kinase II (CaMKII). This was shown by the use of specific inhibitors for CaMKs. The CaMK kinase inhibitor STO-609 did not affect phosphorylation of NADK in the presence of Ca^{2+}/CaM while the CaMKII-specific inhibitors KN-93 and autoinhibitory peptide (AIP) almost completely abolished the observed modification.

To address a physiological consequence of NADK phosphorylation at S64 the catalytic activity of NADK incubated with ATP, Ca^{2+}/CaM and either cell extract or recombinant activated CaMKII was determined. No difference in NADK activity could be detected.

An NADK mutant (S64D) was generated, in which S64 was replaced by an aspartate residue, in order to mimic the constitutively phosphorylated form. The NADK S64A mutant could be

used as the constitutively unphosphorylated form. The proteins were expressed in *E. coli* and the catalytic activity of the purified proteins determined. The generated mutant NADK proteins displayed no significant difference in activity compared to the wild-type protein. Further analysis of the NADK amino acid sequence revealed that the negative charge introduced into NADK S64D could be insufficient to mimic phosphorylation due to the adjacent positively charged Arg63. However, the generated recombinant double mutant protein (R63E/S64D) exhibited similar activity as the wild-type NADK. Furthermore, the NADK activity in extracts of human cells overexpressing the generated NADK mutants was assayed. No significant difference could be detected.

Phosphorylation of a protein can lead to a change of the subcellular localization. Transiently overexpressed, FLAG-tagged NADK wild-type and mutant proteins were all detected in the cytoplasm. In addition, FLAG-tagged wild-type NADK was co-expressed with αCAMKII, wild-type or constitutively active form. Double immunostaining showed NADK and αCAMKII to be exclusively expressed in the cytoplasm.

3.3 Identification of two CaM-activated NADK isoforms from sea urchin

Two NADK isoforms (SpNADK-1 and SpNADK-2) in the sea urchin *S. purpuratus* were identified (Paper III). The isoforms are encoded by the same gene and differ only in their N-terminal sequences while the C-terminal regions, which include the catalytic domain, are identical. Presence of the transcripts for SpNADK-1 and SpNADK-2 in sea urchin eggs was confirmed by RT-PCR analysis. The open reading frames of the identified SpNADKs were overexpressed as N-terminally His-tagged proteins in *E. coli*, purified and characterized.

Both SpNADK isoforms catalyzed the phosphorylation of NAD^+ in the presence of ATP at a rate of about 1.8 and 0.6 U/mg for isoform 1 and 2. NADH phosphorylation was hardly detectable suggesting NAD^+ to be the preferred substrate. Similar to NADH, both sea urchin NADKs did not accept NAAD as substrate. Thus, the potent endogenous Ca^{2+}-mobilizing molecule NAADP does not seem to be directly produced by these two NADK isoforms.

Next, I investigated whether sea urchin NADKs are regulated by Ca^{2+}/CaM. Indeed, the purified recombinant SpNADKs were significantly stimulated by CaM in the presence of Ca^{2+}, although the affinity to CaM was remarkably different. The maximal stimulation by CaM (at saturation) was ~4-fold for SpNADK-1 and 2.5-fold for SpNADK-2. Using N-terminally truncated deletion mutants, the CaM-interacting region of SpNADK isoform 1 was shown to be located within amino acids 30-90. Deletion of the first 30 amino acids of

SpNADK-1 neither changed the catalytic activity nor the activation by Ca^{2+}/CaM of the recombinant mutant protein. In contrast, a deletion mutant lacking amino acids 1-91 was catalytically active as SpNADK-1 wild-type but did not respond to CaM.

In addition to a direct CaM-mediated activation, SpNADK-1 was shown to be an *in vitro* substrate for phosphorylation by CaMKII while this modification was not observed for SpNADK-2. Incubation of SpNADK-1 with extracts from human cells overexpressing rodent αCaMKII highly increased the incorporation of ^{32}P-phosphate into isoform 1. The modification of SpNADK-1 was strongly inhibited by CaMKII-specific autoinhibitory peptide. Since SpNADK-2 was not phosphorylated, I speculated the phosphorylation site(s) in SpNADK-1 to be located within the N-terminal part of the protein. The deletion mutant lacking the first 30 amino acids of SpNADK-1 was hardly phosphorylated compared to the full length protein. Alanine mutation of the serine residue at position 18 (S18A) prevented the CaM-mediated phosphorylation of SpNADK-1. Together, these results established S18 of SpNADK-1 as CaMKII-specific phosphorylation site.

The structural differences in the N-termini of the two NADK isoforms could influence their subcellular localization. To investigate this possibility, the cDNA encoding the C-terminal FLAG-tagged SpNADK isoforms was overexpressed in a human cell line. Indirect immunocytochemistry revealed both SpNADK proteins to be localized in the cytoplasm.

The primary structures of human and sea urchin NADKs are highly similar. Thus it was attractive to test whether a substitution of the first 91 amino acids of human NADK by the corresponding N-terminal region of SpNADK-1 could confer sensitivity to Ca^{2+}/CaM. This generated chimeric protein was expressed in *E. coli*, exhibited normal activity but did not respond to Ca^{2+}/CaM.

4 DISCUSSION

4.1 Functional consequences of NADK up- and downregulation in human cells

The study presented in Paper I demonstrates an important role of NADK in the regulation of the cellular NADP pool in human cells. Recombinantly expressed NADK phosphorylated both, NAD^+ and NADH however the oxidized form appeared to be the preferred substrate. This is strenghtened by the fact that the cellular NAD^+ content is ~3-10 fold higher than NADH (Zhang et al., 2002). Since modulation of cytoplasmic NADK expression was only reflected in significant changes of NADPH, it is likely that the generated $NADP^+$ is subsequently reduced by cytoplasmic $NADP^+$-specific dehydrogenases. It is known, that the rather low concentration of NADP in human cells is maintained in the reduced form under normal conditions. Thereby, reducing equivalents are available upon prooxidant conditions.

Stable overexpression of $NADP^+$-dependent dehydrogenases resulted in a 3 - 4.5-fold increase in activity (Salvemini et al., 1999; Jo et al., 2001). Compared to these levels, the approximately 200-fold increase in NADK activity in the overexpressing cell line is very high but resulted only in a 4-5-fold increase in NADPH. This suggests that human NADK might be inhibited by an excess of NADPH which is known for several NADKs (Kawai et al., 2001b; Raffaelli et al., 2004; Shi et al., 2005; Grose et al., 2006).

NADPH is a key player in anti-oxidative defense mechanisms. However, the significant changes of the NADPH content in the generated cell lines affected the endogenous ROS levels only moderately. Furthermore, knock-down of NADK had no impact on cell survival after exposure to oxidative stress conditions. In contrast, an increase of the $NADP^+$-synthesizing capacity provided better protection towards oxidative stress conditions induced by hydrogen peroxide. Another oxidative stimulus, menadione, had no significant effect on the cell viability of HEK293 cells. This might be explained by differences in the metabolism of these agents and therefore the involvement of different pathways as it was reported for *S. cerevisiae* (Thorpe et al., 2004) and *A. nidulans* (Pocsi et al., 2005). It is likely, that the rather moderate extent of protection against hydrogen peroxide-induced damage, which does not correlate with the 4-5 fold increase of the NADPH concentration, is due to the normal content of $NADP^+$-dependent dehydrogenases. The role of these enzymes in the cell response to oxidative stress is well established. They maintain the NADPH pool which is involved in the regeneration of oxidized glutathione and thioredoxin via their reductase activities. Several studies have shown that $NADP^+$-specific dehydrogenases are transiently upregulated under conditions of oxidative stress (Ursini et al., 1997; Jo et al., 2001; Lee et al., 2004; Vasiliou

and Nebert, 2005). In contrast, no increase of human NADK activity or its protein content could be detected following treatment of untransfected HeLa or HEK293 cells with oxidizing agents. An explanation could be that cells, when exposed to oxidants, rather rely on the existing NADP pool and enhance the capacity of keeping it in a reduced state by increased expression of $NADP^+$-dependent dehydrogenases. Although the reason to not additionally increase the $NADP^+$ concentration is not obvious, at least in the human system there is more variety in the sets of enzymes that can re-reduce $NADP^+$ than to produce it.

Additionally, the results indicate the absence of other genes coding for an NADK in human cells which is in striking contrast to yeast and plants (reviewed in (Pollak et al., 2007)). Notably, an NADK with slightly different molecular and kinetic properties is decribed in human neutrophiles (Williams and Jones, 1985). However, knock-down of NADK in HEK293 correlated with the protein level, the activity and the NADP concentrations suggesting that the remaining expression of the known NADK gene is responsible for the residual NADK activity. With regard to this consideration the question remains how the subcellular pools of NADP are generated, compartmentalized and regulated in mammalian cells. Especially the mitochondrial DNA (mtDNA) is highly exposed to oxidative attacks caused by ROS produced by electron leakage during oxidative phosphorylation (Yakes and Van Houten, 1997). Importantly, damage to mtDNA is now emerging as an etiological factor in oxidative stress-related disorders but also normal aging (Melov et al., 1999). To diminish ROS-induced damage, mitochondria contain numerous antioxidant enzymes and a GSH/Trx pool which is dependent on mitochondrial NADPH. In mammals, mitochondrial NADPH is provided by $NADP^+$-dependent isocitrate dehydrogenase (Jo et al., 2001), malic enzyme (Bukato et al., 1995) and nicotinamide nucleotide transhydrogenase (Rydstrom, 2006). Strikingly, yeast cells obviously lack the enzyme nicotinamide nucleotide transhydrogenase (Rydstrom et al., 1976) but possess a mitochondrial NADK isoform highly specific for NADH as substrate (Outten and Culotta, 2003). This isoform was demonstrated to be the most critical generator of NADPH in yeast (Outten and Culotta, 2003; Strand et al., 2003). The plant homologue also prefers NADH but was shown to be a cytosolic protein (Turner et al., 2005; Chai et al., 2006). In contrast to human and yeast, an exchange of NADP across the cytosol and the mitochondria is described for plant cells (Bykova and Moller, 2001). The apparent absence of a mitochondrial human NADK isoform and the lack of evidence for an exchange of NADP across the cytosol and the mitochondria raises the question how the human mitochondrial NADPH pool is maintained. It will be interesting to determine whether

modulation of NADK expression and thus NADP pool affects the expression or activity of mitochondrial NADPH sources.

Finally, the dramatic redox shift in the generated cell lines is accompanied by changes in the transcription levels of PRDX5 and Nrf2. PRDX5 is mainly localized to the mitochondria but also found in other compartments (Knoops et al., 1999; Kropotov et al., 1999; Yamashita et al., 1999). The promoter of the PRDX5 gene contains clusters of AREs, which the transcription factor Nrf2 can bind to (Kropotov et al., 2006). Under normal conditions Nrf2 is complexed with Keap1 in the cytoplasm. Oxidative or electrophilic stress induces translocation of Nrf2 in the nucleus, where it activates transcription of ARE-regulated genes that protect against oxidative cell damage. Therefore, the Keap1/Nrf2 complex was proposed to constitute a cellular redox sensor through which the activation of ARE-dependent genes in response to oxidative stress is regulated (Itoh et al., 1999). A reduced cellular NADPH content, equal to moderate stress condition, resulted in increased expression levels of PRDX5 and Nrf2. These observations suggest the possiblity that expression of PRDX5 can be regulated by Nrf2 in response to the redox state and that PRDX5 might serve as a redox buffer which is adjusted according to the reservoir of the ultimate regeneration source of the oxidative defense systems. Whether the changes in NADPH directly alter PRDX5 or Nrf2 function or whether other mechanisms are involved, remains to be elucidated. Recently it was shown that NAD redox changes can regulate transcription of the NAD^+-dependent protein deacetylase SIRT1 (Zhang et al., 2007). This report and the present study highlight an additional function of pyridine nucleotides, namely a possible role in dynamic and specific transcriptional regulation in response to metabolic changes in the cell.

The presented results suggest striking differences in regulation of the human NADP metabolism by NADK compared to yeast and plant. These include the number of NADK isoforms, their cellular localization and substrate specificity. Further investigations are necessary to complete our understanding of the pathways and molecular mechanisms of NADP generation and its regulatory functions. This could provide new insights how to cope with the many pathological conditions that involve oxidative damage.

4.2 Ca^{2+}/CaM-mediated posttranslational modification of human NADK

The results presented in Paper II establish human NADK to be phosphorylated in response to Ca^{2+}/CaM. The CaMKII-dependent phosphorylation site was located within the N-terminal part of the protein. A deletion mutant of NADK lacking the first 64 amino acids abolished the phosphorylation by CaMKII but fully retained the catalytic activity. This finding together

with the observation that the CaM-mediated regulation of sea urchin NADK isoforms is associated with the different N-termini (Paper III) highlight the importance of the N-terminal region of NADKs as a regulatory domain. In agreement with these results, it was shown that the large N-terminal extension of plant NADK isoform 2 is involved in binding of CaM (Turner et al., 2004) and serine residues within the N-terminal part of mouse NADK were identified as specific phosphorylation sites (Villen et al., 2007).

The results of this study appear to rule out, at least for the known human NADK, that CaM-mediated phosphorylation is sufficient for the stimulation of NADK: the catalytic activity of the enzyme incubated with ATP, Ca^{2+}/CaM and either cell extract or recombinant CaMKII was not significantly altered. Furthermore, the specific activities of mutant NADK proteins, which either mimicked or excluded the phosphorylation at S64, were unchanged compared to that of the wild-type.

As partially purified NADK from human neutrophils is activated directly by Ca^{2+}/CaM (Williams and Jones, 1985) it is likely that multiple human NADK isoforms exist, which are specifically expressed in cells involved in the regulation of immune response. It would be interesting to purify NADK from human neutrophils and to determine the N-terminal amino acid sequence for comparison with the known human NADK. Alternatively, if CaM-dependent phosphorylation has a role in regulating the activity of human NADK, there could be additional regulatory factors involved. Recently, a mass spectrometry-based analysis of mouse liver protein phosphorylation identified two phosphorylation sites in mouse NADK, namely the serine residues 48 and 64 (Villen et al., 2007). This result raises the possibility that synergistic effects are needed to fully activate NADK in response to Ca^{2+}/CaM. A comparison of the amino acid sequences of the human and a putative mouse NADK revealed very high similarity even in the N-terminal part. In particular the region between amino acids 40-70 is nearly identical. It is tempting to speculate the serine residue 48 (S48) of human NADK to be an additional phosphorylation site. Substitution of this residue by alanine had at least no effect on the CaMKII-mediated phosphorylation of NADK.

Phosphorylation can influence the subcellular localization of the target protein (Kwiek et al., 2007). A phosphorylation state-dependent subcellular distribution of the only known human NADK could explain the generation of NADP pools in compartments that do not exchange this nucleotide with the cytosol such as the mitochondria. Overexpression of the mutant NADKs mimicking or excluding phosphorylation (NADK S64A, S64D and R63E/S64D) resulted in cytoplasmic localization which is the same as found for the wild-type protein (Paper I). Furthermore, co-expression of NADK and αCaMKII did also not alter the

subcellular localization of human NADK. These observations are consistent with the predicted subcellular localization of the enzyme (PSORT II algorithm) and with the lack of any putative targeting sequence motifs.

In conclusion, this study clearly established human NADK as a specific target of CaMKII-mediated phosphorylation. Taking into consideration a recent proteomic study ((Villen et al., 2007), this modification occurs in S64 of the protein and is most likely of physiological relevance. However, the actual effect of the modification remains to be identified. Further studies are needed to establish whether the phosphorylation, for example, alters the protein stability or mediates interactions with other proteins.

4.3   Identification of two CaM-activated NADK isoforms from sea urchin

In the study presented in Paper III, two NADK isoforms of the sea urchin *S. purpuratus* were identified. Both isoforms are directly activated by Ca^{2+}/CaM however the affinity to CaM and the extent of the stimulation vary. Furthermore, isoform 1 was shown to be a target for Ca^{2+}/CaM-dependent phosphorylation by CaMKII.

Ca^{2+}-signaling has a critical role in fertilization and activation of sea urchin NADK is known to be one of the earliest Ca^{2+}-mediated events in the activation of the sea urchin egg. Various postfertilization processes depend on NADPH synthesized from $NADP^+$ by the pentose phosphate pathway. The results establish for the first time the molecular basis for this increase in NADPH shortly after fertilization. That is, an increase in Ca^{2+} leads to direct activation of NADKs via CaM and thereby increased production of NADPH.

The major structural differences among known eukaryotic NADKs are found within the N-terminal region (reviewed in (Pollak, 2007)). Remarkably, both sea urchin NADKs share high similarity to each other as well as to the human NADK besides the first 60 amino acids. The CaM-mediated stimulation of both sea urchin NADKs and the phosphorylation of SpNADK-1 were mapped to the N-terminal parts. Deletion of amino acids 1-91 of SpNADK-1 (resulting in the identical protein for both SpNADK isoforms) had no effect on the catalytic activity but abolished both CaM-dependent events. Thus, the different N-terminal parts of SpNADKs were established to be important as a regulatory domain.

Interestingly, the CaMKII-dependent phosphorylation of SpNADK-1 suggests regulation of NADK activity by an additional Ca^{2+}/CaM-mediated pathway. In fact, this possibility could explain the reported stimulation of NADK activity by Ca^{2+}/CaM in tissue or cell extracts from plants and humans (Anderson et al., 1980; Dieter and Marme, 1984; Williams and Jones, 1985). In contrast, none of the purified recombinant NADKs from *A. thaliana* as well as the

34

human enzyme were activated by Ca^{2+}/CaM (Lerner et al., 2001; Turner et al., 2004; Turner et al., 2005). Since measurement of NADK activity requires the addition of ATP, CaM-dependent phosphorylation events could have taken place in the crude extracts or partially purified enzymes.

NAADP is an important calcium releasing messenger in sea urchin eggs (for a review see (Whitaker, 2006)). It was suggested that NAADP could be generated by NADK via phosphorylation of the physiological intermediate NAAD (Ziegler, 2000). However, similar to the human NADK (Lerner et al., 2001), such an activity for both SpNADKs was not detectable whether or not CaM was present. There might be a yet not identified kinase specific for NAAD phosphorylation.

Both, yeast and plant cells encode three NADK isoforms which differ in their subcellular localization and contribution to the subcellular NADP pools (Outten and Culotta, 2003; Berrin et al., 2005; Chai et al., 2005; Bieganowski et al., 2006; Chai et al., 2006). NADK activity in sea urchin eggs was found in the cytoplasmic supernatant fraction (Epel, 1967). Furthermore, the PSORT II algorithm predicts both SpNADKs to be cytoplasmic. Indeed, overexpression of the cDNAs encoding SpNADKs in a human cell line followed by indirect immunocytochemistry revealed both isoforms to be present in the cytoplasm. To confirm this observation, it would be interesting to study the distribution of the endogenous SpNADKs. A polyclonal antibody raised against the purified human NADK did not reveal specific detection of SpNADKs in western blot analysis. Therefore it is necessary to produce specific antibodies against both isoforms.

It will be important to investigate the contribution of the two NADK isoforms to maintain the NADP pool in sea urchin, in particular at fertilization or under oxidative stress conditions. Since activation of NADK is suggested to be an important step for the progress of fertilization, one could speculate that inhibition of SpNADKs blocks or delays postfertilization processes. It will also be interesting to elucidate the physiological role of the CaMKII-mediated phosphorylation of SpNADK-1. CaMKII has been reported as a critical link between sufficiently high NADPH levels and cell survival during Xenopus development (Nutt et al., 2005) and to be required for nuclear envelope breakdown during mitotic division of sea urchin embryos (Baitinger et al., 1990). Moreover, Ca^{2+}-signaling, particularly involving CaMKII, is a major system which is activated under conditions of oxidative stress (reviewed in (Franklin et al., 2006)). Since NADPH is essential to all mechanisms counteracting oxidative damage, the CaMKII-dependent phosphorylation of SpNADK isoform 1 could be a key regulatory step to ensure sufficient amounts of NADPH.

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