REVIEW ARTICLE
The power to reduce: pyridine nucleotides – small molecules with a multitude of functions

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The pyridine nucleotides NAD and NADP play vital roles in metabolic conversions as signal transducers and in cellular defence systems. Both coenzymes participate as electron carriers in energy transduction and biosynthetic processes. Their oxidized forms, NAD$^+$ and NADP$^+$, have been identified as important elements of regulatory pathways. In particular, NAD$^+$ serves as a substrate for ADP-ribosylation reactions and for the Sir2 family of NAD$^+$-dependent protein deacetylases as well as a precursor of the calcium mobilizing molecule cADPr (cyclic ADP-ribose). The conversions of NADP$^+$ into the 2′-phosphorylated form of cADPr or to its nicotinic acid derivative, NAADP, also result in the formation of potent intracellular calcium-signalling agents. Perhaps, the most critical function of NADP is in the maintenance of a pool of reducing equivalents which is essential to counteract oxidative damage and for other detoxifying reactions. It is well known that the NADPH/NADP$^+$ ratio is usually kept high, in favour of the reduced form. Research within the past few years has revealed important insights into how the NADPH pool is generated and maintained in different subcellular compartments. Moreover, tremendous progress in the molecular characterization of NAD kinases has established these enzymes as vital factors for cell survival. In the present review, we summarize recent advances in the understanding of the biosynthesis and signalling functions of NAD(P) and highlight the new insights into the molecular mechanisms of NADPH generation and their roles in cell physiology.

Key words: ADP-ribosylation, calcium signalling, NAD(P)(H), NAD kinase (NADK), oxidative stress.

INTRODUCTION

Cells throughout all organisms have established a universal set of small molecules that carry out versatile functions both in metabolic pathways and in regulatory processes. Among the best characterized of these molecules is ATP. It has been termed the universal energy currency of the cell, but, in addition, serves in signalling pathways as a substrate for protein phosphorylation as well as precursor of cAMP. It appears, however, that another molecule might have even more facets in its repertoire and has become subject of intense investigations, NAD. NAD and its phosphorylated form NADP were well known cofactors of cellular metabolism and were characterized as electron carriers in oxidoreductase reactions before other properties were unravelled and the involvement of the dinucleotides in signalling reactions moved into focus [1].

Although it is recognized to have important regulatory roles, NADP has so far received less attention. It is generally accepted that its reduced form, NADPH, is vital to the cellular oxidative defence systems and reductive syntheses. How delicately these processes are regulated by the availability of NADPH has become of increasing interest over the past few years. In particular, the mechanisms and subcellular compartmentation of NADP$^+$ and NADPH generation appear to critically influence the physiological state of the cell and thereby cellular survival.

In the present review, we will briefly summarize the biosynthetic pathways and signalling mechanisms of NAD(P) and then elaborate on the importance and pathways of NADPH generation. Finally, we will focus on the key enzyme of NADP synthesis, NADK (NAD kinase), whose vital role has been highlighted in a number of recent studies.

BIOSYNTHESIS OF NAD(P)

The participation of NAD(P) in electron transfer reactions, that is, the reversible conversion between oxidized (NAD$^+$, NADP$^+$) and reduced (NADH, NADPH) forms does not result in a net consumption of the nucleotides:

$$\text{Metabolite} + \text{NAD(P)}^+ \rightleftharpoons \text{Metabolite} + \text{NAD(P)H} + \text{H}^+$$
$$\text{(reduced)} \qquad\qquad\qquad \text{(oxidized)}$$

Consequently, except for cell divisions, the constant requirement for pyridine nucleotide re-synthesis does not arise from their function as redox carriers, but rather from their involvement in signalling reactions. The latter are accompanied by the cleavage of the glycosidic bond between the nicotinamide and ADP-ribose moieties. The liberated nicotinamide is presumed to be recycled by a salvage pathway (Figure 1). Two principal routes of NAD biosynthesis are known (Figure 1; reviewed in [2,3]). The *de novo* biosynthesis includes several steps to generate QA (quinolinic acid) from either L-tryptophan in animals and some bacteria, or L-aspartate in some bacteria and plants [4]. The

Figure 1   Schematic overview of NAD(P) biosynthetic pathways

The major known pathways of NAD(P) synthesis are presented. Main endogenous precursors of NAD(P) synthesis are Nam, NA and L-tryptophan. Nicotinamidase is only found in yeast. NamR, Nam riboside; NA/NamPRT, NA/Nam phosphoribosyltransferase; NADS, NAD synthetase.

subsequent enzymatic step catalysed by QAPRT (QA phosphoribosyltransferase) appears to be common, at least in eukaryotes. The salvage pathways utilize degradation products, namely NA (nicotinic acid) and Nam (nicotinamide), to regenerate NAD (Figure 1). Both pathways converge at the transfer of NMN or its acid form NAMN (NA mononucleotide) on to the adenylyl group of ATP under pyrophosphate release. The reaction is catalysed by N(A)MNAT (Nam/NA mononucleotide adenylyltransferase). In humans, three isoforms of N(A)MNAT were identified and recently shown to be expressed in the nucleus, in the Golgi apparatus and the mitochondria [5]. When NAMN is used in the transfer reaction, the product is NAAD (NA–adenine dinucleotide), which is subsequently amidated to NAD by NAD synthetase. Despite the presence of the same enzyme activities, mammalian cells exhibit a clear preference towards Nam compared with yeast, for which NA appears to be the preferred precursor. In fact, yeast express an enzyme, nicotinamidase, which deamidates Nam to NA before it can be recycled into NAD (Figure 1). With regard to the overall activity of NAD synthesis, the *de novo* synthesis from tryptophan (via QA) is of minor importance.

Niacin or vitamin B$_3$, which is synonymous with both Nam and NA, was long thought to be the only external precursor for *de novo* synthesis of NAD. Recently, an alternative pathway has been established by the molecular identification of NRK (Nam riboside kinase) [6]. Although such an activity had been detected in mammalian tissues before [7], the recent study [6] demonstrated the importance of this enzyme for NAD biosynthesis in yeast and potentially in humans. Moreover, the presence of nicotinamide riboside in food sources was demonstrated, further supporting the importance of this novel pathway.

Finally, some of the cellular NAD is converted into NADP by NADK. As for all other enzymes of NAD(P) biosynthesis, this enzymatic activity has long been known, but the molecular identification and characterization has only been achieved within the past few years. It turns out that, at least by exerting a direct influence on NAD(P) turnover, many of these enzymes have a critical influence on major cellular events. Specifically, as will be highlighted in a subsequent section, the activities of NADKs determine the availability of NADPH and are therefore vital.

REGULATORY REACTIONS INVOLVING NAD$^+$ AND NADP$^+$: ADP-RIBOSE – A MODULE OF VARIOUS SIGNALLING REACTIONS

Both NAD$^+$ and NADP$^+$ are involved in signal transduction as precursors of messenger molecules. In addition, NAD$^+$ serves also

as substrate for covalent modifications of target molecules. All known derivatives share structural similarity owing to their ADP-ribose backbone (Figure 2). Thus, NAD$^+$-mediated signalling reactions can be referred to as ADP-ribosyl transfers, because the initial cleavage of Nam (leaving the ADP-ribosyl moiety) is common to all of them. The eventual acceptors of the transfer reaction are rather diverse, including macromolecules such as proteins, or small molecules such as water (yielding ADP-ribose), acetate [yielding OAADPr (*O*-acetyl ADP-ribose)] or the adenine ring leading to intramolecular cyclization into cADPr (cyclic ADP-ribose; Figure 2).

The first observations of NAD$^+$-mediated protein modification by poly-ADP-ribose date back to the 1960s [8,9]. Mono-ADP-ribosyltransferase activities were identified originally in a group of bacterial toxins [10] and were later detected in eukaryotic cells [11]. The target molecules are usually proteins, but modification of DNA has also been described [12]. The physiological consequence of mono-ADP-ribosylation appears to be inhibition of the target proteins [13]. For example, modification by the mitochondrial transferase SIRT4 [14] inhibits glutamate dehydrogenase [15].

PARsylation [poly(ADPribosyl)ation] constitutes the attachment of ADP-ribose polymers to target proteins and therefore represents a rather elaborate protein modification. It is catalysed by PARPs [poly(ADP-ribose) polymerases], a protein family comprising 17 members in humans [16,17]. The founding and most catalytically active member, PARP-1, is a nuclear enzyme involved, among others, in DNA repair and apoptotic pathways [1,17,18]. PARsylation has also important functions in telomere dynamics [19], transcriptional regulation [20], cell division [21] and trafficking of endosomal vesicles [22].

ADP-ribose transfer has also an important function in tRNA splicing, which is mediated by NAD$^+$. The 2′-phosphate of the splice intermediate is transferred to NAD$^+$ thereby forming ADP-ribose 1″-2″ cyclic phosphate and Nam [23]. Whether this product has a physiological function remains unknown.

Several NAD(P)$^+$ derivatives act as second messenger molecules. Following cleavage of the Nam, the ADP-ribose moiety undergoes a reaction with a small modifying group (e.g. water, NA or an intramolecular group, cf. Figure 2) resulting in a biologically active messenger. Remarkably, so far all known NAD(P)$^+$ derivatives have been associated with calcium signalling. Even ADP-ribose itself triggers Ca^{2+} influx into the cytosol by activating TRPM2 (transient receptor potential cation channel melastatin 2), a Ca^{2+} channel in the plasma membrane [24].

ADP-ribosyl cyclases generate cADPr and 2′-phospho-cADPr from NAD$^+$ and NADP$^+$ respectively. Both molecules trigger
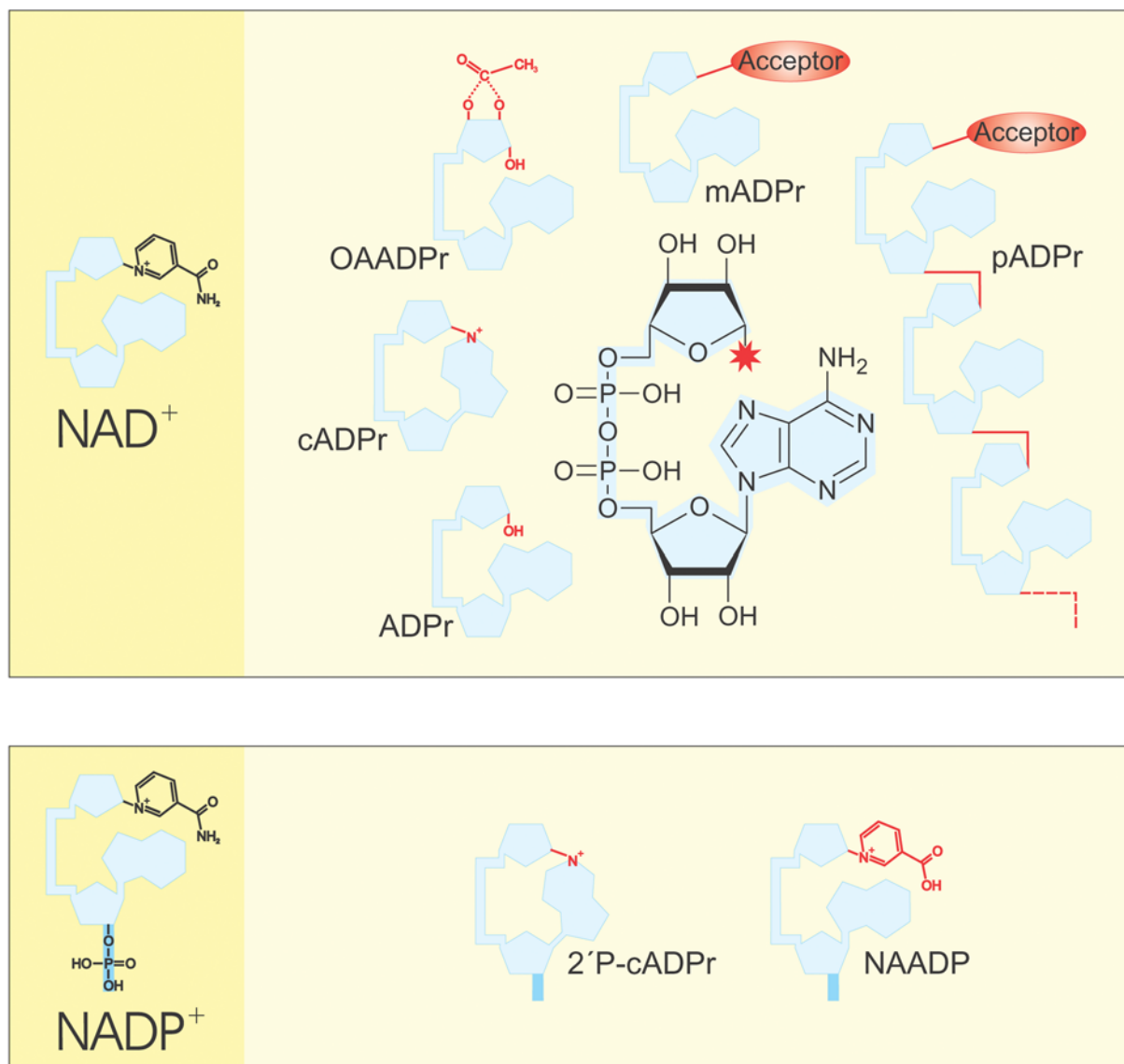
Figure 2   Signalling derivatives of NAD(P)

The ADP-ribosyl moiety commonly shared by all derivatives is illustrated in structural detail and adumbrated in light blue, the red asterisk indicates the site of ADP-ribosyl attachment to the acceptor. The individual portion of each derivative is presented in red and the 2′-phosphate group of NADP$^+$ in its derivatives is shown in dark blue. ADPr, ADP-ribose; mAPDr, mono-ADPr; pADPr, poly-ADPr; 2′P-cADPr, 2′-phosphate cADPr.

cytosolic [Ca^{2+}] elevation, presumably by activating the ryanodine receptor in the endoplasmic/sarcoplasmic reticulum [25]. In the presence of adenine, ADP-ribosyl cyclases can form other adducts of cADPr, which might also serve as Ca^{2+}-mobilizing molecules [26]. The mammalian ADP-ribosyl cyclases (CD38 and CD157), homologues of the enzyme originally identified in *Aplysia californica* [27], are ecto-enzymes which exhibit their catalytic activity at the cell surface [28]. Extracellular generated cADPr can possibly be taken up by certain cell types [29], but there is also evidence for the existence of alternative intracellular enzymatic activities [30].

Today, the most potent intracellular Ca^{2+}-mobilizing messenger known is NAADP (NA–adenine dinucleotide phosphate), a derivative of NADP$^+$. It was shown to trigger calcium release from lysosomal Ca^{2+} stores, which are independent of the stores activated by cADPr or inositol 1,4,5-trisphosphate [31]. Astonishingly, the only enzymatic activity shown to be capable

of generating NAADP is associated with ADP-ribosyl cyclases, which, in this case, exchange the Nam moiety of NADP$^+$ with NA. Whether alternative pathways of NAADP generation exist, for example by phosphorylation of NAAD, have yet to be solved [32]. On the other hand, a 2′-specific Ca^{2+}-dependent phosphatase has been characterized that degrades NAADP and thereby inactivates this messenger [33].

It is interesting to note that none of the conversions of NAD(P)$^+$ and their consequences described above has been found in yeast, although even some prokaryotes express ADP-ribosylating or ADP-ribosyl cyclase activities. Nevertheless, yeast was the first organism in which yet another NAD$^+$-mediated regulatory mechanism was detected that includes protein deacetylation and the generation of a potential messenger molecule, that is, the formation of OAADPr [34]. The corresponding enzyme activity was originally discovered for the Sir2 (silent information regulator-2) protein of *Saccharomyces cerevisiae*, which was

identified as an NAD^+-dependent histone deacetylase (reviewed in [35]). In the meantime, mammalian Sir2 homologues, sirtuins, have become of major interest owing, in part, to the remarkable effect of Sir2 on lifespan regulation by gene silencing [35]. The closest human relative to yeast Sir2, hSIRT1, exhibits the same enzymatic activity [35]. Besides histones, it also deacetylates other target proteins, including p53. Although closely related, not all mammalian sirtuins are in fact protein deacetylases, but some represent mono-ADP-ribosyltransferases [14,36].

The reaction mechanism of Sir2 involves the cleavage of the Nam moiety and subsequent transfer of the originally protein-bound acetyl group on to ADP-ribose. Owing to intramolecular isomerization, either the 2′- or the 3′-C atom of the terminal ribose is acetylated (Figure 2) [37]. Not only the deacetylation of the target protein, but also the generation of OAADPr appears to have signalling characteristics. OAADPr has Ca^{2+}-mobilizing properties by activating the TRPM2 channel [38] and affects the structure of the Sir2–Sir3–Sir4 silencing complex [39].

Taken together, $NAD(P)^+$ is extensively used in signal transfer reactions, both by modification of target molecules or generation of small signalling molecules. Thereby, the pyridine nucleotides influence virtually all vital cellular functions. It is important to note that all of the conversions described above require the oxidized form of $NAD(P)^+$. The reduced form is not a substrate for these signalling reactions. However, as discussed below, the redox state of the dinucleotides is of great importance in the cellular environment. In fact, depending on the redox state of NAD, transcriptional activity can be reciprocally regulated by direct binding of either NADH or NAD^+ to transcriptional co-repressor C-terminal binding protein [40].

REDOX STATE AND CELLULAR NAD(P) CONCENTRATIONS

The term redox state is commonly used to describe the balance of NAD^+/NADH and $NADP^+$/NADPH in the cell. It is reflected by several sets of metabolites (lactate and pyruvate, β-hydroxybutyrate and acetoacetate), that is, by the interconvertible oxidized and reduced forms of specific redox couples [41,42]. The cellular redox state influences many metabolic, signalling and transcriptional processes in the cell. It always has to be maintained such that sufficient reducing equivalents are available, for example, to counteract the potential damage by free radical intermediates, namely ROS (reactive oxygen species) and RNIs (reactive nitrogen intermediates). The acute shift of the intracellular redox balance towards oxidation, known as oxidative stress, is due to increased production or insufficient degradation of ROS and RNI and represents a major damaging factor in inflammatory processes, ischaemia/reperfusion injury, other pathological states and ageing [43–48].

Although the critical role of NAD(P) and its redox state have long been appreciated, there are still considerable uncertainties regarding their cellular dynamics, that is, overall concentration, free and protein-bound state, subcellular compartmentation and the redox state.

The assessment of these parameters is hampered by technical difficulties relating to 'visualization' in living cells and even to the accurate determination of overall concentrations. For example, owing to the instability of reduced and oxidized pyridine nucleotides under acidic or alkaline conditions respectively, commonly used extraction procedures are accompanied by a loss or breakdown of the nucleotides. Moreover, redox shifts can be brought about rather quickly and changes in concentration of the oxidized form (which is most commonly measured in acidic extracts) might not be due to usage or breakdown, but simply due to alteration of the redox state just prior to extraction. Therefore,

when examining changes of pyridine nucleotide concentrations, both the oxidized and reduced nucleotides are to be taken into consideration.

In order to circumvent some of the methodological problems, the lactate/pyruvate ratio and the pyruvate/malate ratio were measured assuming a direct proportionality to NAD^+/NADH and $NADP^+$/NADPH ratios respectively (e.g. [41,42,49]). Based on the fluorescence properties of reduced pyridine nucleotides, advanced technologies now enable more exact estimates of the intracellular NAD(P) distribution. Two-photon-excitation microscopy and fluorescence lifetime imaging have been used to measure intracellular NAD(P)H concentrations to distinguish between protein-bound and free nucleotides in living cells [41,50]. Comfortingly, the concentration values obtained with advanced methods are in good accordance with the values obtained by 'classical methods' [51].

Estimates of the total cellular NAD vary, but are commonly in the submillimolar range. The intracellular concentrations of $NADP^+$ and NADPH are low in comparison with the non-phosphorylated forms, and under normal metabolic conditions the NADP pool is predominantly in the reduced state. Thereby, a reserve for the regeneration of anti-oxidative defence systems is normally available. In contrast, the redox ratio of NAD^+/NADH is rather high (~ 3–10; [40]). In addition, it is most likely that the majority of the cellular NAD and NADP pools are not free, but are protein-bound [40,52]. Therefore, estimates of actual substrate availability, for example, for $NAD(P)^+$-mediated signalling processes are to be viewed with caution, at least, when based upon pyridine nucleotide determinations in cellular extracts.

It is interesting to note that the NAD concentration does not change when enzymes of its biosynthesis, e.g. NMNAT or NamPRT, are overexpressed [53,54], suggesting that the pyridine nucleotides can be turned over at a considerable rate. On the other hand, conditions such as the overactivation of PARP-1 by extensive DNA damage can lead to a rapid decline of the cellular NAD^+ content by up to 80 % [55] and, if at all, cells re-establish the original concentration only within hours or days. A major contributing factor to this severity is the ensuing oxidative stress imposed by most DNA-damaging agents, which aggravates ATP depletion and thereby limits the capacity of NAD^+ re-synthesis. The loss of NAD^+ by PARsylation further compromises the capacity to restore energy transduction by glycolysis [56]. In these scenarios, NADPH also has a critical role, as it provides the reducing power to oxidative defence systems. Although this function has long been appreciated, only over the past few years have the processes of its generation and maintenance been analysed in detail, in particular with regard to the molecular mechanisms, subcellular organization and regulation. The exploration of NADP synthesis and the mechanisms of maintaining its reduced state has provided important insights into the molecular details of its protective role; methods have been established to shift the activities of key enzymes regulating the concentration or redox state of NADP. Several new studies indicate the feasibility to increase the cellular content or reduced state of NADP thereby improving the cellular resistance towards oxidative stress. Below, we will summarize the major cellular systems that keep NADP in its reduced state. Then, we will discuss the roles and properties of NADKs, the key enzymes of NADP synthesis that have become the focus of intense investigation over the past few years.

THE MAJOR SYSTEMS OF NADPH GENERATION

Until recently, the major source of NADPH generation was thought to be the pentose phosphate pathway located in the

cytosol, in particular the first and rate-limiting step catalysed by G6PD (glucose-6-phosphate dehydrogenase). Perhaps to the disappointment of some textbook editors, other, at least as important, enzyme activities have been described more recently (although the original notion might still hold true for erythrocytes). Among them, the NADP$^+$-specific forms of IDP (isocitrate dehydrogenase), ME (malic enzyme), ALDH (aldehyde dehydrogenase) as well as NADK have received major attention. Furthermore, a direct *trans*-hydrogenation between NAD and NADP takes place in mitochondria, although evidence for such a system in *S. cerevisiae* is lacking [57].

Critical NADP-dependent dehydrogenases

G6PD is constitutively expressed in all organisms and cell types. It had been regarded as a housekeeping enzyme, but research in several laboratories demonstrated that this enzyme is highly regulated [58–60]. G6PD deficiency, a rather common genetic abnormality in humans, is the cause of haemolytic anaemia [61,62], while cancer cells may exhibit significantly increased activity [63].

Yeast cells disrupted for the G6PD gene, *ZWF1*, grow normally [64] and mouse ES (embryonic stem) cells are viable after knockout of the gene [65]. Although G6PD is obviously not essential for a single cell, mouse embryos lacking G6PD fail to develop normally [66]. Moreover, ΔG6PD yeast cells show enhanced sensitivity to oxidizing agents owing to depletion of NADPH [64]. Similarly, mouse ES cells deleted for the G6PD gene are highly sensitive to agents perturbing the intracellular redox status [65,67]. These cells were able to maintain a high NADPH/NADP$^+$ ratio. However, upon treatment with diamide, an oxidant of thiol groups, the NADPH and GSH contents decreased rapidly [67] and led to apoptotic cell death [68]. Transient up-regulation of G6PD activity and mRNA levels has been observed in various human cell lines following treatment with hydrogen peroxide or diamide [69], which is presumably triggered by the oxidation of NADPH to NADP$^+$ [67]. Accordingly, overexpression of G6PD increased the resistance to hydrogen peroxide [70,71], whereas inhibition of G6PD potentiated cell death [71]. It is interesting to note that G6PD activity is also subject to physiological regulation. For example, stimulation of cell growth is accompanied by an increase in G6PD activity [58,60].

Not only the surprisingly mild phenotype of G6PD-deficient cells, but also the fact that NADPH is produced by a cytosolic enzyme that is unavailable to mitochondria, the major site of superoxide production, prompted investigations of alternative routes of NADPH generation. Thus, other NADP-specific dehydrogenases, including IDP, ME and ALDH, have been identified as significant contributors to the reduced state of NADP. At least as important are several NADKs which can phosphorylate NADH and thereby generate NADPH directly (Figure 3). Although different organisms rely on the same enzyme activities to regenerate NADPH, there are differences, particularly with regard to subcellular localization and subsets of isoforms. Therefore, we will discuss the major NADP$^+$-reducing enzymes separately for yeast and mammalian cells.

NADP-dependent dehydrogenases in yeast

Yeast. The isocitrate dehydrogenase family [isocitrate + NAD(P)$^+$ + H$^+$ ⇌ α-oxoglutarate + CO$_2$ + NAD(P)H] consists of a mitochondrial NAD$^+$-dependent isoform involved in the citric acid cycle and NADP$^+$-dependent isoforms. Three IDP homologues, Idp1p, Idp2p and Idp3p, were identified in yeast
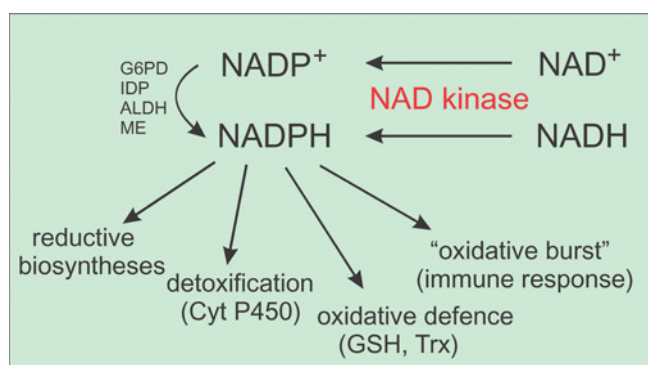
Figure 3　Generation and utilization of NADPH in eukaryotic cells

Cyt P450, cytochrome P450; Trx, thioredoxin.

and localized to mitochondria [72], cytosol [73] and peroxisomes [74] respectively. Both mitochondrial and cytosolic isoforms are not essential, even in the context of *ZWF1* (encoding G6PD) deletion, since the triple mutant ΔIDP1ΔIDP2ΔZWF1 is viable [75]. Disruption of the peroxisomal IDP impaired growth on unsaturated fatty acids as the carbon source [74], indicating involvement of this enzyme in their metabolic utilization by providing NADPH in the peroxisomes.

Disruption of the gene encoding ME [malate + NAD(P)$^+$ ⇌ pyruvate + CO$_2$ + NAD(P)H], a mitochondrial protein, did not result in an observable growth phenotype [76], nor did it worsen the growth phenotypes of *Idp* and *Zwf1* mutant strains [75]. The fact that the presumed major generators of NADPH, G6PD and IDP, were not essential under many growth conditions [75] prompted the search for alternative sources of NADPH. An isoform of the ALDH family [RCHO + NAD(P)$^+$ + H$_2$O ⇌ RCOOH + NAD(P)H + H$^+$], Ald6p, a cytosolic enzyme, was identified as a suppressor of the *ZWF1*-deletion phenotype. Initially, disruption of both *ZWF1* and *ALD6* was thought to be lethal, whereas *ALD6* deletion alone displayed no distinct phenotype [77]. However, the double mutant strain in this study was grown under conditions that also suppressed Idp2p expression. Indeed, it was later shown that co-disruption of only *ALD6* and *ZWF1* is not lethal [78]. Therefore, although contributing significantly to the reduction of NADP$^+$, none of the NADP-specific dehydrogenases appears to be essential.

NADP-dependent dehydrogenases in mammals

In contrast with yeast, mammals possess two NADP-dependent isocitrate dehydrogenases, a mitochondrial (IDPm) and a cytosolic (IDPc) isoform [79], although the latter has also been ascribed to peroxisomes [80]. Both isoforms are major sources of NADPH supply in mammals. IDPm activity is transiently increased after treatment of cells with hydrogen peroxide [81] and both IDP activities are induced by γ-irradiation [82]. IDPc activity and protein content were also demonstrated to increase during differentiation of adipocytes indicating a role in supplying NADPH for fat and cholesterol biosyntheses [83]. Furthermore, overexpression of either IDP isoform improved the cells' ability to cope with oxidative stress, whereas decreased expression aggravated oxidative damage [81,84,85]. It is interesting to note that deletion of G6PD in mouse cells did not cause an up-regulation of IDPc activity [67]. However, this could be due to the fact that IDPc is, perhaps, generally more active in NADPH production than G6PD. For example, in rat liver, the contribution by IDPc exceeds that of G6PD 16–18-fold [42].

Two NADP$^+$-dependent ME isoforms have been described in mammalian tissues and were localized to the cytosol and the mitochondria [86,87]. Both activities are not essential for cellular NADPH maintenance. However, cytosolic ME activity is increased in rat liver cirrhogenesis [88] and affected in acute hepatic injury [89], presumably by providing NADPH for detoxification reactions. Like IDPc, cytosolic ME is also not up-regulated in mouse cells deleted for G6PD [67]. The mitochondrial isoform plays an important role in the pyruvate-recycling pathway and in maintaining the intramitochondrial GSH in the brain [90]. In pancreatic islets, the mitochondrial pyruvate–malate shuttle could contribute far more to NADPH generation than the cytosolic pentose phosphate pathway [91].

The ALDH family consists of multiple forms in mammals being important for detoxification via NAD(P)-dependent conversion of endogenous and exogenous aldehyde substrates. ALDHs are up-regulated in response to oxidative stress, and several mutations in *ALDH* genes have been linked to diseases such as cancer and Alzheimer's disease [92]. The most abundant isoform 3A1 in mammalian cornea [93], a cytosolic protein, may play a role in cellular defence mechanisms that protect the corneal epithelium from oxidative damage [94]. However, whether mammalian ALDHs have a general role in the maintenance of reduced NADP has to be explored further.

The enzyme pyridine nucleotide transhydrogenase [H$^+_{out}$ + NADH + NADP$^+$ $\rightleftharpoons$ H$^+_{in}$ + NAD$^+$ + NADPH] is located in the membrane of many bacteria and in the inner membrane of animal mitochondria, where the catalytic site is exposed to the matrix side. It transports protons across the membrane in concert with hydride exchange between NAD and NADP, a reaction that couples the protonmotive force to the concentration of reducing equivalents [95]. Under physiological conditions the enzyme maintains mitochondrial NADP in the reduced state. Thereby, the transhydrogenase secures a high GSH/GSSG ratio in the mitochondria and modulates protein activities by regulating NADPH-dependent protein thiols [96]. Although knockout of the transhydrogenase gene in *Escherichia coli*, *Caenorhabditis elegans* and mouse was not lethal, these studies supported the important role of the transhydrogenase in defence against ROS (reviewed in [97]).

Taken together, none of the NADP$^+$-reducing enzymes is essential by itself for maintaining the cellular redox balance. Besides G6PD, there are other key contributors to NADPH generation in the majority of cells. Still, so far it has remained unclear exactly how the NADPH pools in different organelles, cells and tissues are generated and maintained. It is plausible that cells do not rely on a single NADPH-regenerating system given the permanent threat of oxidative damage. Despite the important new insights obtained over the past few years, several fundamental questions have still to be answered. These relate above all to the regulation of both the identified dehydrogenases, for example by post-translational modifications, and the availability of substrates according to the physiological demands. In any case, the key step regulating the capacity of cellular NADPH generation lies with the synthesis and availability of NADP by NADKs (Figure 3).

NADK

The biosynthesis of NADP requires the phosphorylation of NAD catalysed by NADK (ATP:NAD 2′-phosphotransferase). This enzyme is essential as has been demonstrated both in prokaryotes such as *E. coli*, *Mycobacterium tuberculosis*, *Bacillus subtilis* and *Salmonella enterica* [98–101] and in the yeast *S. cerevisiae* [102,103]. Apparently, mice lacking NADK are also not viable

(unpublished work cited in [103]). NADK activity has been known for decades. NADP formation was first observed in yeast homogenates [104,105] and the enzyme purified 10 years later [106]. Meanwhile, enzymes from animals [107–109], yeast [110,111] and plants [112] have been characterized. However, conclusive structural information has only recently become available following the identification of amino acid sequences of *Micrococcus flavus* and *M. tuberculosis* NADKs [113]. Based on this information, NADKs from a variety of organisms were soon identified and then studied in great detail (reviewed in [114]). At least for mammalian tissues a major obstacle for the isolation of the NADKs had been their relatively low abundance. NADKs are ubiquitous enzymes which apparently constitute a protein family distantly related to other kinases of metabolic intermediates [115]. In both yeast and plants, three NADK isoforms were identified with distinct subcellular locations. Strikingly, so far only a single isoform has been found in mammals.

Structural properties of NADKs

Almost all known NADKs are oligomeric proteins consisting of two to eight identical subunits of 30–60 kDa (Table 1). The comparison of amino acid sequences of several known prokaryotic and eukaryotic NADKs revealed a general structural organization consisting of a conserved catalytic domain (PFAM 01513) within the C-terminus and variable N-terminal parts. The catalytic domain includes two highly conserved motifs, an L/VGGDG motif and a glycine-rich motif, which are involved in substrate binding (Figure 4). The importance of these motifs for the catalytic activity of NADKs was confirmed by site-directed mutagenesis of several amino acid residues within the first and second nucleotide-binding motifs [115–117] and was supported further by the three-dimensional structures of prokaryotic NADKs [116,118–120]. The overall structure, as determined for *M. tuberculosis* NADK [116,118], 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 [121], 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 [118]. The structure of *Archaeoglobus fulgidus* NADK [119] is similarly comprised of two major domains. The substrates NAD$^+$ and ATP, and the product NADP$^+$ are all bound to a cleft between the N- and C-domains and intersubunit contacts. Accordingly, NADKs are homo-oligomers whose subunit interactions are required for ligand binding. No other larger putative structural motifs have been discerned, except for a calmodulin-binding domain within the long N-terminal part of *Arabidopsis thaliana* NADK-2 [122].

Structural analyses of the NADK from *A. fulgidus* complexed with substrates indicated a critical role for the GGDG motif [119]. The GGDG motif has been shown to be a highly conserved motif in a superfamily including NADK, diacylglyceride kinase, sphingosine kinase and 6-phosphofructokinase [115]. Moreover, structural studies of prokaryotic NADKs at higher resolution suggest a particular mechanism of phosphate transfer based on substrate-assisted catalysis (G. Poncet-Montange and G. Labesse, Centre de Biochimie Structurale INSERM U554-CNRS UMR 5048-UM1, Montpellier, France, personal communication).

Substrate specificity and catalytic properties of NADKs

Table 1 summarizes physical and kinetic properties of NADKs from various organisms. NADKs from both prokaryotes and eukaryotes utilize nucleoside triphosphates, preferentially ATP, as phosphoryl donor for catalysis. Interestingly, some bacterial enzymes, for example from *M. tuberculosis*, *Micrococcus flavus*

Table 1   Physical and kinetic properties of prokaryotic and eukaryotic NADKs

Activities using either NAD$^+$ or NADH as substrate are presented. The values are given as μmol of product (NADP$^+$ or NADPH respectively) formed per min per mg of protein. $-$, not detectable; poly(P) designates polyphosphate as the substrate instead of ATP.

Organism	Subunit mass and number	Activity NAD$^+$/NADH	K_m NAD$^+$	K_m NADH	K_m ATP	Reference
Homo sapiens						
hNADK	49 kDa tetramer	6.7/$-$	0.54	$-$	3.3	[127]
S. cerevisiae						
ScNADK-1[b] (Utr1p)	60 kDa hexamer	16.2–51.5/$\sim$2	0.5	3.9	0.6	[102,126,129,152]
ScNADK-2[b] (Yef1p)	56 kDa octamer	3.3–9.5/$\sim$1	1.9	2	0.17	[102,126,129]
ScNADK-3[b] (Pos5p)	46 kDa	0.2/0.4, but see [151]	[a]	[a]	[a]	[151,155]
A. thaliana						
AtNADK-1	58 kDa	<0.2/<0.1	0.52	[a]	0.73	[122,125]
AtNADK-2	109 kDa	0.2/$-$	0.43	$-$	0.74	[122]
AtNADK-3	35 kDa dimer	41.2/23.2	2.39	0.042	NAD$^+$: 0.19 NADH: 0.06	[160]
E. coli						
YfjB	30 kDa hexamer	13/$-$	2	$-$	2.5	[126,128]
M. tuberculosis						
Ppnk	33 kDa tetramer	0.5–4.3/0.7 (poly(P): 1.7/0.2)	0.9–3.3 (poly(P): 1.2–2.9)	[a]	1.8–2.5 (poly(P): 1.3–1.6)	[113,117,126]
Micrococcus flavus						
Mfnk	34 kDa dimer	18.3/8.7 (poly(P): 7.4/3.0)	0.53–0.83 (poly(P): 0.26–0.58)	0.45 (poly(P): 0.35)	NAD$^+$: 0.23 NADH: 0.32 (poly(P): NAD$^+$: 0.33 NADH: 0.58)	[113,126]

[a] Not reported.
[b] Proposed name.

and *B. subtilis*, can utilize both inorganic poly(P) (polyphosphate) and nucleoside triphosphates as phosphoryl donors (reviewed in [114]). Poly(P) is considered to be an 'ancient' energy carrier preceding ATP [123]. Even glucose 6-phosphate-dependent bacterial NADKs have been described [124], but were not investigated further. How do poly(P)/ATP–NADKs distinguish between the phosphoryl donors poly(P) and ATP and which amino acid residues determine the difference? This important question has yet to be answered and may represent a key to the design of new antibacterial drugs. In particular the mycobacterial NADK could be a promising target for the treatment of tuberculosis since its NADK is essential and exhibits higher affinity towards poly(P) than ATP [117].

Relatively high K_m values for ATP have been reported for *E. coli*, and human NADKs (Table 1, and also for *Salmonella enterica*), 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 not NADH, thus displaying high selectivity. Others exhibit a far less stringent substrate specificity and use both the oxidized and reduced nucleotide (see Table 1). Differences in substrate selectivity as reported for plant NADKs [122,125] may have arisen from the assay conditions. Moreover, native enzymes in their cellular context may interact with other factors that might influence substrate specificity. Recently, it was suggested that an arginine residue in the conserved domain of NADKs plays a major role in conveying a strict substrate preference towards NAD$^+$ [126]. Replacement of this arginine by glycine or polar amino acid residues converted highly selective prokaryotic NADKs to relaxed ones. It should be noted, however, that, compared with the original activity, the converted *E. coli* NADK used the substrate NADH far less efficiently. Conversely, *Micrococcus flavus* relaxed NADK did not become more selective by a reciprocal single amino acid replacement, but exhibited a significant loss of activity

regardless of the substrate used. Therefore, this amino acid position appears to be important for catalysis, but is not the only determinant of substrate specificity. The amino acid sequence alignment of NADK homologues (cf. Figure 4) revealed that primarily polar amino acid residues are found in eukaryotic homologues at the investigated position. This would imply relaxed substrate specificity in contrast with prokaryotic NADKs. However, although the human enzyme harbours glutamine, a polar amino acid, in this position, NADH phosphorylation was hardly detectable (N. Pollak and M. Ziegler, unpublished work).
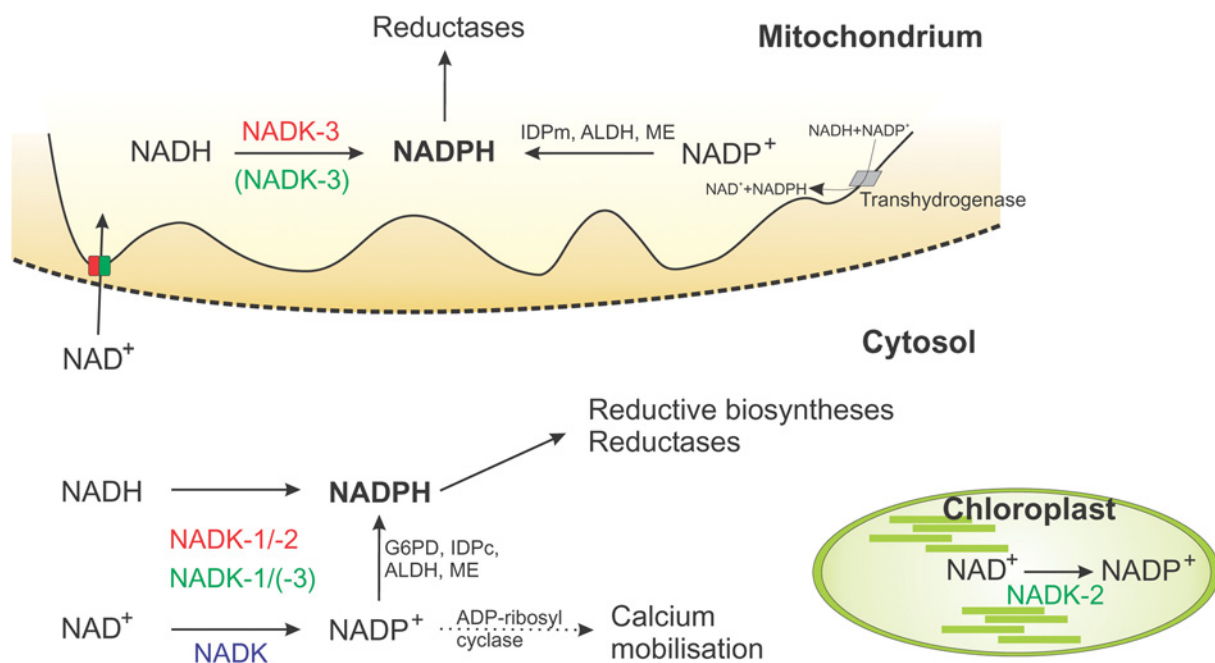
The discovery of NAADP suggested the possibility of NADKs involvement in the synthesis of a second messenger. As can be inferred from Figure 1, NAAD is a physiological intermediate whose phosphorylation would result in the generation of NAADP. Recently, Bieganowski et al. [102] reported NAAD kinase activity for recombinant yeast NADKs, which constituted about 1 % of the corresponding NADK activities. The physiological significance of this observation remains unclear, since no NAADP-responsive system in yeast has been described, while phosphorylation of NAAD by human NADK was not detected [127]. Still, other cellular factors could influence the affinity of human NADK towards NAAD.

Modulators of NADK activity

The catalytic activity of several NADKs is inhibited by the reduced pyridine nucleotides [99,117,128,129], by high concentrations of NADP$^+$ [117,130] or by HgCl$_2$ [128–130]. Interestingly, NADPH, NADH and NADP$^+$ inhibit *S. cerevisiae* NADKs to different extents suggesting a possible regulation by these compounds [129]. NADK of *Salmonella 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 [99]. QA, a precursor of *de novo* NAD$^+$ synthesis was shown to inhibit NADK of *Salmonella*

```
Human      98  ----------LTWNKSPKSVLVIKKMRDASLLQPFKELCTHLMEEN--MIVYVEKKVLED
AtNADK-2  661  FTHPSTQQQMLLWKTTPKTVLLLKKLGQELMEEAKEAASFLYHQEN--VNVLVEPEVHDV
ScNADK-1  130  ----------LMIVTKLNDVSLYFLTRELVEWVLVHFPRVTVYVDS--ELKNSKKFAAGE
ScNADK-2  109  ----------LIIICNINDVSFLMREVVEWILRNFHSITVYVQD--IFKKSTQFAVGD
AtNADK-1  204  ----QISLTWESDPQTVLIITKPNSTSVRVLSVDMVRWLRTQK--GLNIYVEPRVKE
E.coli      1  ------------MNNHFKCIGIVGHPRHPTALTTHEMLYRWLCTKG--YEVIVEQQIAHE
ScNADK-3   61  ----------LIWQNPLQNVYITKKPWTPSTREAMVEFITHLHESYPEVNVIVQPDVAEE
Ppnk        1  --------------MTAHRSVLLVVHTGRDEATETARRVEKVLGDNK--IALRVLSAEAVD
AtNADK-3    1  ---------------MAIRKLLLLKPIDPYPFLQTEGASLIKNPQVL-QYLESRCKVHKN

Human     146  PAIASDESFGAVKKK-FCTFREDYDDISNQIDFIICLGGDGTLLYASSLFQG-SVPPVMA
AtNADK-2  719  FARIPG--FGFVQT----FYIQDTSDLHERVDFVACLGGDGVILHASNLFKG-AVPPVVS
ScNADK-1  178  LCEDSKCRESRIKY----WTKDFIREHDVFFDLVVTLGGDGTVLFVSSIFQR-HVPPVMS
ScNADK-2  157  LCKDSNCSKNRVKY----WSKEFVKKHDSFFDLMITLGGDGTVIFASSIFTK-DVPPIVP
AtNADK-1  255  ELLSESSSFNFVQTW-E--DDKEISLLHTKVDLLITLGGDGTVLWAASMFKG-PVPPIVP
E.coli     47  LQLKNVKTGTLAEIG-----------QLADLAVVVGGDGNMLGAARTLAR-YDIKVIG
ScNADK-3  111  ISQDFKSPLENDPNRPHILYTGPEQDIVNRTDLLVTLGGDGTILHGVSMFGNTQVPPVLA
Ppnk       46  RGSLHLAPDDMRAMGVEIEVVDADQHAADGCELVIVLGGDGTFLRAAELARN-ASIPVLG
AtNADK-3   46  AIKFCQEILSKKPVEWKPISRNDLSHPIRDVDMVIVGGDGTLLHASHFIDD--SVPVLG

Human     204  FHLGSLGFLTPFESFENFQSQVTQVIEG-NAAVVLRSRLKVRVVKELRGKKTAVHNGLGEN
AtNADK-2  772  FNLGSLGFLTSHPFEDFRQDLKRVIHGNNTLDGVYITLRMRLRQEIYRKGKAMPG-----
ScNADK-1  233  FSLGSLGFLTNFKFEHFREDIPRIMNH-----KIKTNLRLRLECTIYRRHRPEVDPNTG-
ScNADK-2  212  FALGSLGFLTNFEFQNFKETLKHILTD-----EVRINLRMRLQCKLYRRNKPEIDAATG-
AtNADK-1  311  FSMGSLGFMTPFHSEQYRDCLEAILKG-----PISITLRHRLQCHIIRDKATHEYEPEE-
E.coli     93  INRGNLGFLTDLPDNAQQQLADVIEG-----HYISEKRFLLEAQVCQQDCQKRIS----
ScNADK-3  171  FALGTLGFLSPFDFKEHKKVFQEVISS-----RAKCLHRTRLEQHLKKDSNSSIVT---
Ppnk      105  VNLGRIGFLAEAEAEAIDAVLEHVVAQ-----DYRVEDRLTLDVVVRQGGRIVNRG----
AtNADK-3  104  VNSDPT---QAHEVEELSDQFDASRSTGHLCAATVENFEQVLDDILFGRVVPAKVSRIS-

Human     263  GSQAAGLDMDVGKQAMQYQVLNEVVIDRGPSSYLSNVDVYLDGH------LITTVQGDGV
AtNADK-2  827  ---------------KVFDVLNEIVVDRGSNPYLSKIECYEHDR------LITKVQGDGV
ScNADK-1  287  ------KKICVVEKLSTHHILNEVTIDRGPSPFLSMLELYGDGS------LMTVAQADGL
ScNADK-2  266  ------RKICYIDFISEHHVLNEVTIDRGPAPCLSLLELYGNDS------LMTKVQGDGL
AtNADK-1  365  ----------------TMLVLNEVTIDRGISSYLTNLECYCDNS------FVTCVQGDGL
E.coli    144  ------------------TAINEVLHPGKVAHMIEFEVYIDEI------FAFSQRSDGL
ScNADK-3  223  ------------------HAMNDIFLHRGNSPHLINLDIFIDGE------FLTRTTADGV
Ppnk      156  ------------------WALNEVSLEKGPRLGVLGVVVEIDGR------PVSAFGCDGV
AtNADK-3  160  --------LKLNSETLLSHALNDILIAQPCPAAVSRFSFKLKNKDGASSPKTVNCRSSGL

Human     317  IVSTPTGSTAYAAAAGASMIHPNVPAIMITPICPHSLSFRPIVVPAGVELKIMLSPEA--
AtNADK-2  866  IVATPTGSTAYSTAAGGSMVHPNVPCMLFTPICPHSLSFRPVILPDSAKLELKIPDDA--
ScNADK-1  335  IAATPTGSTAYSLSAGGSLVCPTVNAIALTPICPHALSFRPIILPESINLKVKVSMKS--
ScNADK-2  314  IVATPTGSTAYSLSAGGSLISPSVNAIAVTPICPHTLSFRPIILPDSMELKVRVDMNS--
AtNADK-1  403  ILSTTSGSTAYSLAAGGSMVHPQVPGILFTPICPHSLSFRPLILPEHVTVRVQVPFNS--
E.coli    180  IISTPTGSTAYSLSAGGPIITPSLDAITLVPMFPHTLSARPLVINSSSTIRLRFSHR---
ScNADK-3  259  ALATPTGSTAYSLSAGGSIVSPLVPAILMTPICPRSLSFRPLILPHSSHIRIKIGSKLNQ
Ppnk      192  LVSTPTGSTAYAFSAGGPVIWPDLEAILVVPNNAHALFGRPMVTSPEATIALEIEADG--
AtNADK-3  212  RICTAAGSTAAMQSAGGFVMPMLSRDLQFMVREPISPGSTASLMHSTFKPDQFMDVNWY-

Human     375  ---RNTAWVSFDGRKRQE-IRHGDSISITTSCYPLPSICVRDPVSDWFESLAQCLHWNVR
AtNADK-2  924  ---RSNAWVSFDGKRRQQ-ISRGDSVRIYMSQHPLPTVNKSDQTGDWFRSLIRCLNWNER
ScNADK-1  393  ---RAPAWAAFDGKDRIE-LQKGDFITICASPYAFPTVEAS--PDEFINSLSRQLNWNVR
ScNADK-2  372  ---RGTSWVNFDGKDRVE-LKQGDYVVITASPYSVPTIESS--ASEFFESLSKNLNWNDR
AtNADK-1  461  ---RSSAWVSFDGKDRKQ-TEAGDALVCSMAPWPVSTACQVESTNDELRSLHDGLHWNLR
E.coli    237  ---RNDLEISCDSQIALP-IQEGEDVLIRRCDYHLNLIHPK--DYSYFNTLSTKLGWSKK
ScNADK-3  319  KPVNSVVKLSVDGIPQQD-LDVGDEIYVIN---EVGTIYIDGTQLPTTRKTENDFNNSKK
Ppnk      250  ----HDALVFCDGRREML-IPAGSRLEVTRCVTSVKWARLD--SAPETDRLVRKFRLPVT
AtNADK-3  271  ---SDHGTLYIDGCQVQHSVQLGDTLEISSDAPVLNVFLSHG-----ISQLRSRY-----
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Figure 4   Partial multiple sequence 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/Utr1p, NADK-2/Yef1p and NADK-3/Pos5p (P21373, NP_010873 and NP_015136); *E. coli* YfjB (NP_417105); and *M. tuberculosis* Ppnk (BAB21478) were compared using Clustal W [170]. Identical and similar residues are highlighted in black and grey respectively. The two conserved NADK motifs are boxed in red.

enterica serotype Typhimurium [131] and to activate *B. subtilis* NADK [130], but had no effect on human NADK (N. Pollak and M. Ziegler, unpublished work).

Regulation of NADK activity by calcium and calmodulin

Although the molecular characterization of NADKs was only achieved recently, it had been known for almost three decades that at least the enzymes from plants, sea urchin eggs and human neutrophils are stimulated by calcium/calmodulin [132–134]. In fact, NADK was the first enzyme identified in plants to be regulated by the ubiquitous calcium-sensing protein calmodulin [135,136]. NADK activity was actually used as a tool to study calmodulins in plants, e.g. to determine their distribution in maize [137]. Plants possess both calmodulin-dependent and -independent NADK isoforms that differ in their subcellular localization [138–140]. In eggs from the sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus*, calcium-mediated

Figure 5   Subcellular distribution of NADP metabolism in eukaryotic cells

NADK isoforms described in mammals, yeast and *Arabidopsis* are highlighted in blue, red and green respectively. Transhydrogenase and ADP-ribosyl cyclase have not been detected in yeast.

activation of NADK occurs early after fertilization and can be prevented by the calcium/calmodulin antagonists, EGTA/trifluoperazine [133]. Enhanced NADK activity results in an increase of the NADPH/NADP ratio, which is mediated by enzymes of the pentose phosphate pathway [141]. Accordingly, the need for elevated NADK activity during fertilization has been explained by the requirement for NADPH of ribonucleotide reductase [142] and the effect on eukaryotic initiation factor eIF-2B [143]. Moreover, increased NADPH levels lead to increased hydrogen peroxide production via NADPH oxidase [144,145], which is important for the post-fertilization hardening of the fertilization envelope [146]. In human neutrophils, the activation of NADK by calcium and calmodulin, and the ensuing increase of the NADP content [134] are probably related to cellular activation in response to bacterial challenges, which includes superoxide production by NADPH oxidase. A similar 'oxidative burst' was reported for thyroid cells [147] and for the calcium-mediated defence response in plants [148]. So far, the molecular mechanism of the calcium/calmodulin-dependent activation of NADKs has remained unknown. Compared with a partially purified calcium- and calmodulin-dependent enzyme from *A. thaliana*, the activity of two recombinant isoforms, AtNADK-1 and AtNADK-2, was not influenced by calcium/calmodulin, although AtNADK-2 exhibited some affinity for calmodulin [122]. Calmodulin had also no detectable effect on the activity of human recombinant NADK [127]. These observations suggest that the activation of NADK by calcium/calmodulin is indirect and requires additional factors which could mediate, for example, post-translational modifications. Moreover, the mechanism could differ depending on the cell type or species. Since higher plants express calmodulin-dependent and -independent NADKs as well as various calmodulin isoforms [149], different calmodulin-mediated pathways could exist to regulate individual NADK activities. In contrast, only a single NADK isoform is known in mammals along with an invariant calmodulin. There have been no reports regarding a potential influence of calcium and/or calmodulin on the recombinant

yeast NADK isoforms. Interestingly, ScNADK-1/Utr1p has been functionally linked to Ald6p due to the involvement of both enzymes in salt stress, and since mutants lacking either protein show similar transcriptional responses [150]. Salt stress in yeast leads to an influx of calcium, which in turn might activate calmodulin-mediated modulation of ScNADK-1/Utr1p activity.

Properties and subcellular distribution of eukaryotic NADKs

The complexity of eukaryotic cells requires elaborate regulatory mechanisms to adjust to alterations in the environment. Owing to the essential functions of NADP, some of these mechanisms should be reflected in the modulation of NADK activities. Although so far very little is known about the transcriptional regulation of NADKs, recent studies have provided important insights into the alterations of NADK activities in response to changes in growth conditions. In addition, since NADP is membrane-impermeable, the pathways supplying different organelles with the pyridine nucleotide are of particular interest. Strikingly, while in yeast and plants three compartment-specific isoforms have been identified, only a single NADK isoform has so far been detected in mammals. Below, we will summarize the current knowledge about NADKs in yeast, plants and mammals. To avoid confusion and to emphasize the actual enzymatic activity, we propose to adopt the terminology established for the plant enzymes also for yeast NADKs based on sequence similarity and subcellular localization. Thus, below we will use the abbreviations ScNADK-1, ScNADK-2, and ScNADK-3 for the yeast NADKs Utr1p, Yef1p and Pos5p respectively.

NADKs in yeast

Of the three NADK isoforms in *S. cerevisiae* (see Table 1 and Figure 5), NADK-3 localizes to the mitochondrial matrix and prefers NADH as substrate [151]. NADK-1 [152] and NADK-2 [129] are presumably cytosolic proteins and exhibit relaxed substrate specificity with some preference towards NAD+.

According to a global analysis of protein expression in yeast only ~300 NADK-2 molecules per cell are present compared with ~5000 molecules of NADK-1 and NADK-3 [153]. Disruption of both NADK-3 and NADK-1 is synthetic lethal, with or without the deletion of NADK-2 [102,103]. The double mutant can, however, be rescued by overexpression of any of the yeast NADK isoforms [102] or human NADK [103]. Therefore, yeast requires a certain level of NADK activity for survival, which is in close agreement with the reportedly essential functions of NADKs in prokaryotes [98–101].

Yeast cells deleted for the mitochondrial NADK-3 are highly sensitive towards oxidative stress [154] and grow poorly on glycerol or in medium lacking arginine [151]. Deletion strains accumulate iron in the mitochondria and are defective in mitochondrial Fe–S cluster-containing enzymes [151] leading to up-regulation of genes involved in iron transport [103]. NADK-3 mutants also exhibit increased frameshift mutations in the mitochondrial DNA and increased petite colony formation [155]. In contrast, deletion of NADK-1 or NADK-2 does not result in severe growth defects or in hypersensitivity to elevated oxygen levels [151].

These observations establish NADK-3, the mitochondrial isoform, as the most critical generator of NADPH in yeast. In the mitochondrion, NADPH is needed for arginine synthesis (when absent from the growth medium), but its major physiological function relates to the protection against oxidative damage by feeding mitochondrial reducing systems. The role of NADK-1 is presumably to provide NADP for cytosolic NADP-dependent dehydrogenases and detoxifying systems. Given the low expression level and the absence of a phenotype of the deletion mutant, the role of NADK-2 remains rather unclear.

It has been demonstrated for yeast and plant cells that NAD^+ can be exchanged between the cytosol and mitochondria (Figure 5; [156,157]), whereas the cytosolic and mitochondrial NADP pools appear to be maintained independently. The occurrence of mitochondrial and cytosolic NADK isoforms is therefore plausible, which is also indicated by the severe phenotype of mutants lacking NADK-1 and -3. In contrast with these observations, Shi et al. [129] reported viable yeast cells upon disruption of all three yeast NADK genes, which would imply the existence of a hitherto unidentified NADK isoform or even an alternative pathway of NADP generation.

NADKs in plants

NADK activity in plants has been found in the cytoplasm [140], mitochondria [138] and chloroplasts [139,158] (Figure 5). An NADK isoform in the chloroplasts is not unexpected because of the importance of NADP in photosynthesis. Three *A. thaliana* NADK isoenzymes have been identified, and the recombinant proteins were characterized with regard to functional properties (Table 1) and subcellular localization (Figure 5). AtNADK-1 lacks any potential targeting sequence and is most probably cytosolic. AtNADK-2 and AtNADK-3 contain a putative chloroplast transit peptide and a mitochondrial targeting sequence respectively [159,160]. A green fluorescent protein–NADK-2 construct co-localized with chlorophyll [159]. The subcellular location of NADK-3 is less clear. The protein has been suggested to be cytosolic, because immunoblot analyses did not reveal a corresponding reactive band in mitochondrial fractions [160]. Still, the NADK-3 sequence is similar to the yeast mitochondrial NADK-3 and also prefers NADH to NAD^+ as its substrate.

Consistent with the important role of yeast NADKs in oxidative defence, irradiation or treatment with hydrogen peroxide of plant cell suspensions induces NADK-1 on both the mRNA and protein levels. Moreover, mutant lines exhibit increased sensitivity to oxidative stress [125]. Knockout of NADK-2 in *A. thaliana* delays growth and development, resulting in reduced leaf size and seed production. Furthermore, these mutant plants have lower levels of chlorophylls *a* and *b*, and are more sensitive to various stress conditions [159].

Two important aspects of NADP generation in plants have so far remained unresolved. First, the individual contributions of each of the three isoforms to the NADPH pools in different subcellular compartments have not been established, except, perhaps, for NADK-2. Secondly, the 'model organism' for the calcium/calmodulin-dependent stimulation of NADK activity has still not revealed the secret regarding the mechanism of this potentially critical regulation.

NADKs in mammals

Considering the eminent roles of NADKs in yeast and plants one would predict that in mammalian cells these enzymes are also essential. However, so far this assumption has not been experimentally addressed, except for the fact that mouse embryonic lethality upon NADK loss was reported as an unpublished observation by Shianna et al. [103]. Still, Akella and Harris [161] investigated NADK activity during development of rat conceptus and their results show dramatic spatio-temporal variations, reflecting the importance of NADK in maintaining the cellular redox state during organogenesis.

Perhaps the most astounding fact to mention here again is the absence of any evidence that would indicate the existence of more than one NADK isoform in mammals. A single human NADK cDNA has been cloned, overexpressed and the protein characterized [127]. An orthologous gene in rodents has been predicted from its sequence similarity. The deduced amino acid sequences exhibit high similarity to the NADKs of *A. thaliana* and *S. cerevisiae* (Figure 4). Similar to the situation for the human NADK, so far no additional isoform has been discerned.

Given the vital roles of at least two NADK isoforms in yeast, one would certainly expect more than one mammalian NADK isoform. That is, even a lethal knockout of the known gene in mice does not necessarily indicate that it is the only one. Nevertheless, at least according to the currently available information, an additional NADK isoform in mammals should have a primary structure substantially different from virtually all those that have been identified so far.

NADP DEGRADATION

NADP-degrading enzymes are supposed to act in concert with NADKs to maintain an adequate balance between NAD and NADP. Two enzyme activities are known that convert $NADP^+$, namely ADP-ribosyl cyclase and NADPase (NADP phosphatase), which dephosphorylates NADP. As described above, ADP-ribosyl cyclases have functions related to calcium signalling. NADPases, however, are not well studied. Their activity was observed in rat liver Golgi apparatus and mitochondria [162,163], in dormant seeds of *Avena sativa* L. [164] and to be necessary for NADP utilization in *Haemophilus influenzae* [165]. NADPase activity of the phytoflagellate *Euglena gracilis* ZC mutant shows circadian oscillation similar to NADK activity, suggesting that both enzymes represent clock 'gears' [166,167]. Two NADPase isozymes were purified from the *Arthrobacter* sp. strain KM [168]. Interestingly, the hyperthermophilic archaeon *Methanococcus jannaschii* possesses a protein, MJ0917, with both NADK and NADPase activities [169]. This novel bifunctional protein could be important for the regulation of the $NADP^+$ concentration and thereby maintain a defined $NAD^+/NADP^+$ ratio.

CONCLUSION

The pyridine nucleotides play vital roles in all organisms. While their roles in metabolic redox reactions have been well characterized, their function in signalling pathways has only been recognized recently. NAD$^+$ appears to be vital for various regulatory systems throughout the cell. On the other hand, NADP$^+$ derivatives are important constituents of cytosolic calcium signalling pathways. In addition, NADPH holds a key position in the oxidative defence systems. The synthesis of NADP and the enzymes that maintain its reduced state are of critical importance not only under conditions of oxidative stress, but also to counteract oxidative damage under normal physiological conditions. Therefore, further understanding of the pathways and molecular mechanisms of NADPH generation will also provide new tools to cope with the many pathological conditions that involve oxidative damage.

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