CHAPTER 12

NAD:
Metabolism and Regulatory Functions

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Abstract

Poly-ADP-ribosylation has turned out to be a major NAD-consuming process in most eukaryotic cells. Although PARP1 exhibits by far the highest capacity to synthesise poly-ADP-ribose, it is active only in situations that are accompanied by DNA damage. It would appear therefore that, under normal physiological conditions, biosynthesis of NAD should not be of critical importance as it is well-known that there is a mulitude of redox reactions using NAD as cofactor, but they are not accompanied by a net loss of the pyridine nucleotide. However, as will be discussed in this chapter, it has now become clear that besides poly-ADP-ribosylation, there are several important regulatory pathways using NAD as substrate. Since they all involve the cleavage of the glycosidic bond between nicotinamide and the ADP-ribose moiety, continuous biosynthesis of NAD is vital to all cells. Accordingly, over the past few years substantial progress has been made in the molecular characterisation of NAD biosynthetic enzymes. In this chapter the newly recognised NAD-mediated regulatory pathways and the advances in the understanding of NAD biosynthesis will be covered. It will also be highlighted that the relationship between PARP1 and NAD synthesis deserves particular attention under conditions of cellular stress involving DNA damage.

Energetic Functions of NAD(P)

Being the major redox carriers in metabolic reactions the pyridine nucleotides (NAD and NADP) are essential molecules in all organisms. According to established nomenclature, $NAD(P)^+$ designates the oxidized and NAD(P)H the reduced forms, whereas NAD(P) includes both. Although most dehydrogenases accept both NAD and NADP, there is a preference for NAD^+ in oxidative, catabolic routes. On the other hand, NADPH is used for reductive syntheses catalysed by substrate-specific reductases. In catabolic reactions NAD^+ receives two electrons and a proton from a substrate molecule. A second proton of the substrate is released. The reactions of NAD-linked dehydrogenases can, therefore, be regarded as hydride (H^-) transfer reactions. It is interesting to note that in these reactions NAD is commonly designated as a coenzyme, although it effectively serves as a cosubstrate. Strictly speaking, a coenzyme would usually return to its original structure at the end of a catalytic cycle. In a dehydrogenase reaction, however, NAD^+ is converted to NADH which then leaves the enzyme. Nevertheless, NADH is readily reoxidized to NAD^+ by a subsequent metabolic reaction and thereby available for another catalytic round of the original dehydrogenase reaction. An example of NAD^+ behaving unambiguously as a coenzyme is represented by the reaction of UDP-glucose epimerase, the isomerisation of UDP-glucose to UDP-galactose. In this reaction, enzyme-bound NAD^+ is transiently reduced to NADH by the sugar moiety of UDP-glucose.[1]

Poly(ADP-Ribosyl)ation, edited by Alexander Bürkle.

The major site of NADH reoxidation is complex I of the respiratory chain. As opposed to the dehydrogenase reactions, two electrons and a proton are transferred onto protein-bound FMN, but not to metabolic intermediates. Eventually, the reducing power of NADH is converted into high-energy phosphoryl bonds of ATP.

Signalling Functions of Pyridine Nucleotides

Besides their well-established roles in energy transduction, NAD and NADP also hold a key position in a variety of regulatory pathway.[2] Similarly to such a dual function of ATP, $NAD(P)^+$ is used for covalent protein modification and the generation of biologically active messenger molecules (Table 1). The reactions of NAD^+-mediated protein modification include mono- and poly-ADP-ribosylation as well as NAD^+-dependent protein deacetylation. Moreover, both NAD^+ and $NADP^+$ serve as precursors of intracellular calcium mobilising agents including cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP; note that according to the nomenclature the correct abbreviation would have to be $NAADP^+$. However, it is barely used). Importantly, for these functions only the oxidised pyridine nucleotides (NAD^+ and $NADP^+$) can be used, because the respective reactions involve the cleavage of the glycosidic bond between the nicotinamide and ADP-ribose moieties of $NAD(P)^+$. The nicotinamide is released in these reactions. It is important to realise that the redox reactions of NAD(P) are not accompanied by any net consumption of the nucleotides. Conversely, the reactions involved in regulatory processes do lead to a net loss of NAD. This is of particular interest with regard to poly-ADP-ribosylation as illustrated in several other chapters of this book.

ADP-Ribosylation

The transfer of the ADP-ribose moiety from NAD^+ onto a biological macromolecule (in almost all cases an amino acid side chain of a protein) is referred to as ADP-ribosylation. Subsequently, another ADP-ribose unit can be attached to the protein-bound ADP-ribose. Further elongation of the ADP-ribose chain will result in poly-ADP-ribosylation. This process will not be further addressed in this chapter. However, mono-ADP-ribosylation, i.e., attachment of a single ADP-ribose unit to a protein, has also been established as a specific protein modification with important regulatory functions.

Originally, mono-ADP-ribosylation was discovered as a catalytic activity of several bacterial toxins.[3] It is now clear that mono-ADP-ribosylation occurs as an endogenous process in prokaryotes as well as eukaryotes.[4] Recently, Glowacki et al[5] have presented a comprehensive analysis of human and mouse mono-ADP-ribosyltransferases. A well established example for the regulatory role of mono-ADP-ribosylation is the modulation of the catalytic activity of dinitrogenase reductase of the photosynthetic bacterium *Rhodospirillum rubrum*.[6] Induced by an external stimulus, such as darkness or a source of fixed nitrogen, the enzyme becomes ADP-ribosylated in an arginine residue and thereby inhibited. When the stimulus is removed, the ADP-ribose is cleaved by an ADP-ribosylarginine hydrolase resulting in the reactivation of the reductase. Therefore, nitrogen fixation in this organism is controlled by an ADP-ribosylation cycle including an ADP-ribosyltransferase and a specific hydrolase which attach or remove the modification, respectively.

In mammalians, mono-ADP-ribosylation appears to take place primarily on the surface of immune cells. The corresponding transferases are either anchored in the plasma membrane via glycosylphosphatidylinositol (GPI) or secreted.[7] For example, in the lung, the antimicrobial function of α-defensin-1, which is secreted by immune cells, is diminished by mono-ADP-ribosylation.[8]

Intracellular mono-ADP-ribosylation has also been demonstrated to have important functions. The mitochondrial enzyme glutamate dehydrogenase is regulated in a fashion similar to that described above for dinitrogenase reductase.[9] Moreover, mono-ADP-ribosylation was also shown to regulate the structure of the Golgi complex through the modification of the β-subunit of a cytosolic G-protein.[10] Despite of these interesting observations, the general role of mono-ADP-ribosylation is still far from being understood.

Table 1. Functions of the major energy carriers in cellular signalling

	ATP	NAD(P)
Covalent protein modification	Phosphorylation Adenylation	Poly-ADP-ribosylation Mono-ADP-ribosylation Protein deacetylation
Synthesis of messenger molecules	cAMP	Cyclic ADP-ribose NAADP O-acetyl-ADP-ribose

Calcium-Mobilising Derivatives of NAD(P)$^+$: Cyclic ADP-Ribose and NAADP

NAD^+ can be converted to cyclic ADP-ribose (Fig. 1A) by the bifunctional ADP-ribosyl cyclases/NAD glycohydrolases. These enzymes are widely distributed among pro- and eukaryotes.[11] The reaction mechanism includes the formation of an enzyme-bound highly reactive ADP-ribose-intermediate (oxocarbenium cation). Therefore, besides the reaction with the adenine ring (resulting in the formation of cADPR), water or pyridine bases (such as nicotinic acid) can react with this intermediate (reviewed in ref. 12). As a result, NAD^+ is hydrolysed to ADP-ribose or the corresponding NAD^+ analogue (nicotinic acid adenine dinucleotide, $NAAD^+$) is formed. ADP-ribosyl cyclases/NAD glycohydrolases also accept $NADP^+$ as substrate. Consequently, they can generate the 2'-phosphorylated forms, cyclic ADP-ribose phosphate (cADPRP), ADP-ribose phosphate, and NAADP. The latter is generated by exchanging the nicotinamide moiety of NADP for nicotinic acid.[11]

Importantly, the cyclic products and NAADP (Fig. 1B) have been established as highly potent intracellular calcium-mobilising agents. Their role for the regulation of fundamental cellular processes, via activation of intracellular calcium stores, has been demonstrated in a variety of model systems (reviewed in refs. 11,13-15). It is interesting to note that the calcium

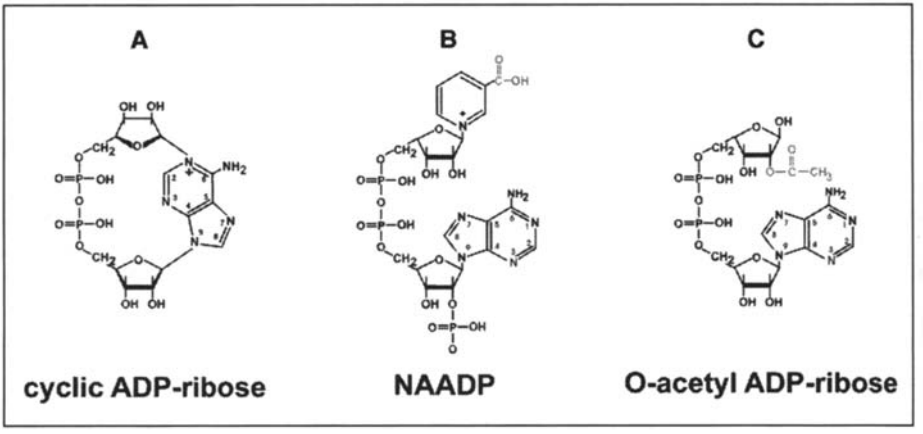

Figure 1. Derivatives of NAD and NADP with messenger functions. While cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) have been established as intracellular calcium mobilising molecules, the potential role of O-acetyl ADP-ribose as a signalling molecule has yet to be identified.

release mediated by cADPR shares a number of features with that of the well-known inositol-1,4,5-trisphosphate (InsP3): Both messengers release calcium from the endoplasmic reticulum. Moreover, an initial, preceding rise of the cytosolic calcium concentration is required to activate the receptors.[16] This mechanism has been termed calcium-induced calcium release. NAADP, however, appears to act quite differently. It releases calcium presumably from lysosomes[17] and does not require an elevated cytosolic calcium concentration.[18] Therefore, it is a likely possibility that NAADP serves as a trigger of intracellular calcium signalling which can then be propagated by the cADPR- and InsP3-dependent mechanisms.[19]

NAD-Dependent Protein Deacetylation

Only very recently, it was discovered that a class of histone deacetylases, the sirtuins, is dependent on NAD^+.[20] Sirtuins deacetylate proteins and concomitantly cleave NAD^+. The reaction mechanism appears to be unique for ADP-ribosyl transfers.[21,22]

Acetylated chromatin is transcriptionally active. Histone deacetylation in a particular region of a chromosome leads therefore to specific gene silencing. Originally, the yeast silent information regulator 2 protein, Sir2p, was found to act as a histone deacetylase which requires NAD^+. Strikingly, the activity of this protein has been associated with the regulation of lifespan. For example, overexpression of the protein results in a substantial extension of lifespan owing to specific transcriptional inactivation.[23] There are several members of the sirtuin family in human cells which have been localised to the nucleus, the cytosol and the mitochondria. Accordingly, their targets are not only histones, but also other acetylated proteins including p53[24,25] and α-tubulin.[26]

Besides the deacetylation of the target protein, the by-product generated by the reaction of sirtuins is of considerable interest. The acetyl group becomes eventually attached to the 2' or 3'-hydroxyl of the terminal ribose of ADP-ribose forming O-acetyl-ADP-ribose,[21,22] (Fig. 1C). It is suspected that this molecule may have messenger functions, too.[20]

NAD(P) Biosynthesis

Considering the absence of degradation in the redox reactions, the need for continuous NAD(P) synthesis had been considered minimal over many years. Nevertheless, the routes of NAD(P) generation were established long ago (reviewed in refs. 2,27). However, until recently, a molecular characterisation of the enzymes of NAD(P) biosynthesis was lacking. Owing to the discovery of a variety of NAD-mediated regulatory pathways involving the cleavage of NAD, the molecular analysis of NAD(P) biosynthesis has now become a topic of major interest. In particular, as detailed in several chapters of this book, poly-ADP-ribosylation may consume large amounts of NAD, primarily in situations of cellular stress. In some instances the cellular NAD may be almost depleted. Therefore, only if there is a highly efficient mechanism of NAD resynthesis, the cells will be able to survive such conditions. In addition, it has been demonstrated that manipulation of biosynthetic pathways of NAD may influence fundamental cellular processes such as gene regulation and ageing.[20,28]

Biosynthetic Pathways in Mammalians

Nicotinamide serves as the major precursor of NAD in mammalians. The reaction with phosphoribosyl pyrophosphate (PRPP) leads to the formation of nicotinamide mononucleotide, NMN. Finally, this mononucleotide is linked to the adenylate moiety of ATP forming NAD (Fig. 2). This reaction is catalysed by nicotinamide mononucleotide adenylyltransferases, NMNATs.

As an alternative to nicotinamide, quinolinic acid (a degradation product of tryptophan) may be used to form nicotinic acid mononucleotide (NaMN). Quinolinic acid contains two carboxyl groups one of which is cleaved off during the reaction. All known NMNATs may use NaMN to form a dinucleotide and the subsequent reaction with ATP then yields nicotinic acid adenine dinucleotide, NAAD. This intermediate is the substrate of NAD synthase, an enzyme

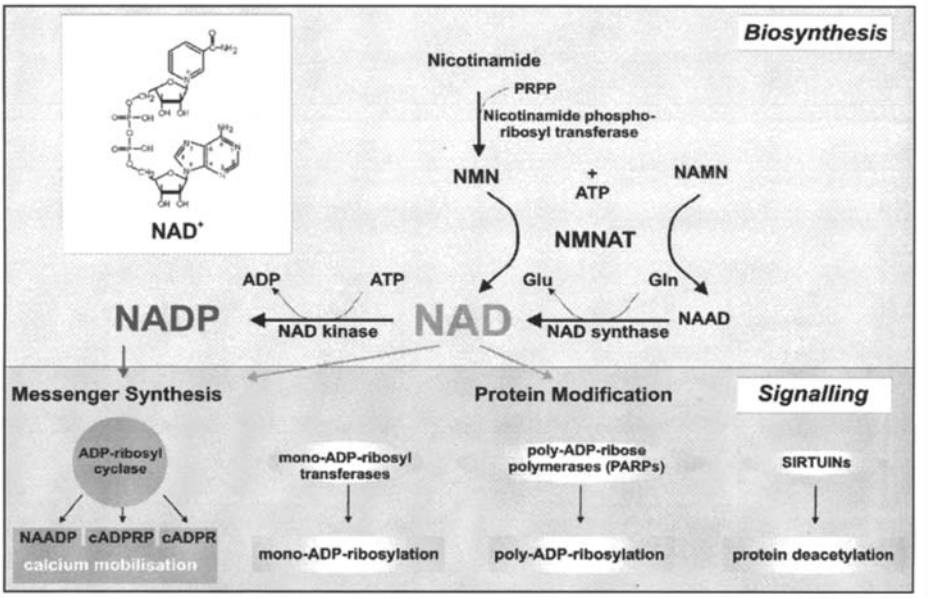

Figure 2. NAD biosynthesis and its role for regulatory pathways in mammalian cells. Note added in proof: A recent study demonstrates an alternative biosynthetic route of NAD, not indicated in Figure 2: a specific kinase has been found which converts nicotinamide riboside to NMN (Bieganowski P, Brenner C; Cell 2004; 117:495-502). Nicotinamide riboside could be an important nutritional factor. Also, nicotinamide riboside kinase could be involved in the conversion of the anticancer drug tiazofurin to its active form.

which amidates NAAD to NAD using glutamine (instead of free ammonium, as found in prokaryotes) as source of ammonium, Figure 2.

The phosphorylation of NAD at the 2' position yields NADP and is catalysed by NAD kinase. Plant isoforms of this enzymes have been demonstrated to be regulated by calmodulin.[29,30] However, the recombinant human enzyme was not directly affected by calcium or calmodulin.[31] Still, this enzyme is presumably tightly regulated considering the essential roles of NADPH for reductive syntheses and especially for oxidative defence systems.

Regulatory Function of NAD Metabolism

A major influence of NAD metabolism on lifespan regulation was originally found in yeast. As described above, histone deacetylation by Sir2p results in gene silencing and thereby in extended lifespan. It has been found that nicotinamide is a potent endogenous inhibitor of Sir2 enzymes (sirtuins). Therefore, speeding up the reaction metabolising nicotinamide will diminish the cellular concentration of this Sir2p inhibitor. Even the acceleration of a subsequent reaction in the pathway (e.g., overexpression of NMNAT) could enhance nicotinamide utilisation by shifting the equilibrium and thereby increase Sir2p activity. Indeed, overexpression of enzymes of the NAD biosynthetic or salvage pathways has been demonstrated to extend lifespan, presumably by releasing the inhibition of Sir2p.[23,32,33]

However, it should be borne in mind that other enzymes using NAD in regulatory reactions, including PARPs and ADP-ribosyl cyclase, are also inhibited by nicotinamide. In fact, nicotinamide has been used as PARP inhibitor in a variety of experimental settings. Perhaps, some of these studies need to be reevaluated with regard to effects of nicotinamide on other important regulatory mechanisms such as NAD-dependent protein deacetylation.

Subcellular Compartmentation of NAD and Its Metabolism

The cellular NAD concentration has been measured many times in a variety of tissues and cells and can generally be stated to be in the low (sub)millimolar range. It has to be noted, however, that most of the dinucleotide is protein-bound in vivo. Additional variablity has been observed with regard to the ratio of NAD^+/NADH,[34] even at resting state. Clearly, this ratio also depends on the metabolic conditions.

Compartmentation of NAD In Mammalian Cells

A further complication regarding the assessment of the intracellular NAD concentration is brought about by the subcellular compartmentation. It has been estimated that up to 70% of the total cellular NAD can be sequestered within mitochondria.[35] Apparently, the relative amount of mitochondria within a tissue correlates with the share of mitochondrial (versus cytosolic) NAD. As a result, the mitochondrial fraction of NAD is very high in heart and other mitochondria-rich tissues (brain, liver). The other cellular pool of NAD is considered to be cytosolic, freely exchangeable with the nucleus. Since there appears to be no physiological pathway for the exchange between cytosolic and mitochondrial NAD (except in plants, see ref. 36), these two pools have to be regarded as independent of one another. However, the redox ratio is transmitted between these two compartments by means of transport systems for metabolites that are substrates of NAD-dependent dehydrogenases present in both compartments. For example, the well known malate-aspartate shuttle system carries redox equivalents (but not NADH itself) from the cytosol into mitochondria.

Compartmentation of NAD Metabolism

Given two major independent NAD pools, cytosolic and mitochondrial, it would be reasonable to expect a corresponding localisation of the biosynthetic enzymes. Surprisingly, some of the key enzymes of NAD biosynthesis are localised within the nucleus. In yeast, both nicotinate phosphoribosyltransferase and an isoform of NMNAT have been localised to the nucleus. The predominant human isoform of NMNAT (hNMNAT1) is also located within the nucleus.[37] It is still puzzling why presumed simple metabolic enzymes are present within the nucleus. However, the discovery of nuclear NAD-dependent signalling events, such as poly-ADP-ribosylation and histone deacetylation by sirtuins, provides at least a rationale for the constant need for NAD in this compartment. Despite the fact that nuclear NAD is supposed to freely permeate into the cytosol, a second human isoform of NMNAT exists[38] and appears to be cytosolic.[39]

On the other hand, the pathway of mitochondrial NAD generation is still obscure. NMNAT activity in mitochondria has been reported[40] and a third human NMNAT isoform could possibly be localised within these organelles.[39] However, it is unknown how NMN would enter the mitochondria or be formed therein. Fortunately, there is at least no question as to how the second substrate of NMNAT, namely ATP, is generated within mitochondria.

Interplay between PARP1 and NAD Metabolism

It is now well established that PARP1 possesses the highest potential activity to consume NAD within a cell. Since the activity of this enzyme is induced by DNA damage, agents causing such damage also trigger a net loss of cellular NAD. In fact, conditions leading to overactivation of PARP1 are inevitably accompanied by dramatic NAD loss. Since NAD is essential for all cells, the resynthesis of NAD is an imperative prerequisite for cell survival in these situations, besides the repair of damaged DNA. There are indications that NMNAT1 and PARP1 may interact and thereby influence each other's catalytic activities.[41,37] However, a more detailed analysis is required to understand the potential functional importance of this interaction.

The observed massive NAD consumption following extensive DNA damage has promoted the view that PARP1 may serve as a suicide device to exclude cell survival by eliminating a vital energy carrier (the "suicide hypothesis", see ref. 42 for a review). In fact such an interpretation seemed even more appealing when it turned out that NMNAT1 is also located within the

nucleus. It has been reasoned that net loss of NAD within the nucleus would trigger NMNAT1 activity to restore the pool. NMNAT1 also being localised within the nucleus would provide the product, NAD^+, directly to PARP1 and thereby rapidly exhaust all resources to regenerate NAD. Moreover, since this enzyme requires ATP, this mechanism would also cause ATP depletion. Although such a scenario is in accordance with many experimental observations, other interpretations are possible and, perhaps, more adequate.

Several experimental findings are indeed hard to accomodate within this model. For example, it has been reported that ATP depletion preceded NAD depletion in brain slices of mice challenged with genotoxic stress.[43] Moreover, the cellular concentration of NMN is likely to be well below millimolar. Therefore, the capacity of NMNAT1 to decrease the ATP concentration to significant extent is rather limited (the physiological concentration of ATP being about 3-10 mM). Importantly, as described above, a major proportion of the cellular NAD pool is localised within the mitochondria and, therefore, under physiological conditions unlikely to be accessible to nuclear PARP1. Consequently, a decrease of the cellular NAD concentration to less than about 40-50% strongly suggests that the mitochondrial NAD pool is also affected. Since there is no known physiological pathway mediating NAD release from mitochondria, it would appear that mitochondrial derangement has to precede further NAD loss catalysed by PARP1. It is possible, therefore, that under conditions leading to cellular NAD depletion as a result of PARP1 activity, a major cause of ATP loss consists in the loss of the mitochondrial membrane potential and the ensuing stop or even reversal of the ATP synthase activity.[35] However, both NAD and ATP concentrations are largely maintained in the presence of PARP1 inhibitors. Possibly, when no NAD loss occurs the glycolytic pathway of ATP generation is sufficient to keep the concentration at almost physiological levels. Indeed, treatment of cultured cells with inhibitors of the respiratory chain or oligomycin does usually not cause a substantial decrease of the cellular ATP concentration as long as glucose is available.

Concluding Remarks

In mammalian cells, multiple NAD consuming processes exist which are involved in the regulation of major cellular events (Fig. 2). Moreover, each of the principal NAD-mediated signalling pathways (mono- and poly-ADP-ribosylation, protein deacetylation, calcium messenger synthesis) is catalysed by several isozymes, which have been detected in various subcellular compartments. The wide array of regulatory functions of pyridine nucleotides suggests that they are involved in all vital cellular functions, not only as energy carriers, but also as essential components of signalling mechanisms. Therefore, the biosynthetic routes and their intimate relationship to these processes have also gained considerable interest over the past few years.

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