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Enzymology of mammalian NAD metabolism in health and disease

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1. ABSTRACT

Mounting evidence attests to the paramount importance of the non-redox NAD functions. Indeed, NAD homeostasis is related to the free radicals-mediated production of reactive oxygen species responsible for irreversible cellular damage in infectious disease, diabetes, inflammatory syndromes, neurodegeneration and cancer. Because the cellular redox status depends on both the absolute concentration of pyridine dinucleotides and their respective ratios of oxidized and reduced forms (*i.e.*, NAD/NADH and NADP/NADPH), it is conceivable that an altered regulation of the synthesis and degradation of NAD impairs the cell redox state and likely contributes to the mechanisms underlying the pathogenesis of the above mentioned diseases. Taking into account the recent appearance in the literature of comprehensive reviews covering different aspects of the significance of NAD metabolism, with particular attention to the enzymes involved in NAD cleavage, this monograph includes the most recent results on NAD biosynthesis in mammals and humans. Due to recent findings on nicotinamide riboside as a nutrient, its inclusion under "niacins" is proposed. Here, the enzymes involved in the *de novo* and reutilization pathways are overviewed.

2. INTRODUCTION

The importance of NAD metabolism in health and disease stems from the pioneer finding that niacins are effective drugs for pellagrins (1). In 1937, indeed, Elvehjem and co-workers showed that nicotinic acid (Na) and nicotinamide (Nam) are active in prevention and cure of black tongue in dogs, as well as in the treatment of human pellagra (2). Although the disappearance of pellagra in developed Countries shortly defueled interest on this pleiotropic syndrome, the recent observation that several disorders mimic pellagra in its typical multiplicity of symptoms (four Ds, *i.e.*, dermatitis, diarrhea, dementia, and death), re-boosted interest on the elucidation of the mechanisms underlying the action of NAD and its metabolites (3-7).

Intriguingly, almost contemporaneous to the discovery of the pharmacological action of niacins was the establishment of the NAD chemical structure following the early recognition of its central function as a redox coenzyme in the cellular metabolism (8, 9). Since then, NAD has become the subject of intensive studies focused on the identification of the enzymatic pathways involved on both its synthesis and degradation. The NAD

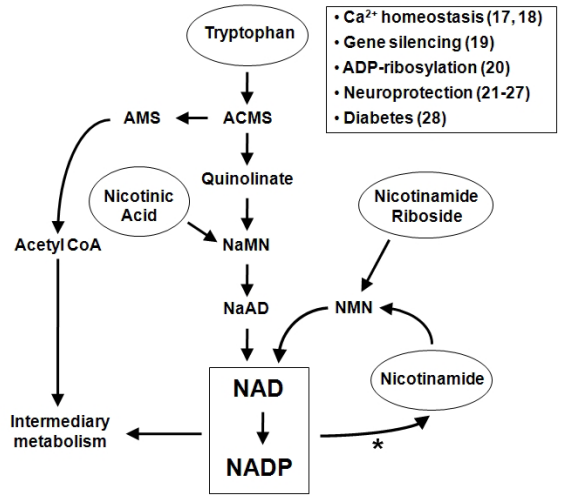

Figure 1. Mammalian NAD biosynthesis. The box summarizes major physiological and pathological processes implicating NAD metabolism. The asterisk, indicating the route from NAD/NADP to nicotinamide, includes the reactions catalyzed by SIRTs , PARPs and other ADP-ribosyltransferases. Na and N indicate nicotinic acid and nicotinamide, respectively. ACMS and AMS refer to 2-amino 3-carboxymuconate 6-semialdehyde and 2-aminomuconate 6-semialdehyde, respectively.

pyrophosphorylase reaction, early discovered by Kornberg in 1948, was indeed found to coincide with the key reaction for NAD biosynthesis, catalyzed by the nuclear enzyme nicotinamide mononucleotide adenylyltransferase (10). Another milestone is the discovery in the 60's of a crucial NAD-consuming activity specifically involved in transferring ADP-ribosyl moieties to protein acceptors (11, 12). In the same years, the term "pyridine nucleotide cycle" was introduced to designate the enzymatic steps involved in the synthesis and breakdown of NAD, although the significance of NAD consumption remained unclear (13). In 1974, the observation that NAD turnover was strongly suppressed in enucleated yeast cells, where the NAD half life increased up to three times (14), first demonstrated that nucleus is the compartment mainly responsible for both breakdown and resynthesis of NAD, and also suggested that nuclear events are linked to NAD homeostasis. Since then, studies on the enzymology of NAD homeostasis have boosted in parallel with those on the effect of NAD-related metabolites in cellular physiology, highlighting multiple signalling roles played by the coenzyme itself and also by some of its derivatives. Due to its key position in the control of fundamental cellular processes, the study of NAD metabolism has become attractive for novel strategies of rational drug design. In microbial pathogens, indeed, the NAD biosynthetic pathway represents a very generous source of enzymes as targets for developing novel antibiotics. In mammals, alteration of NAD metabolism is associated with several physiopathological conditions such as neurodegenerative disorders, autoimmune diseases, cancer, heart diseases, and ageing.

While it seemed of fundamental importance to study the individual enzymes involved in NAD biosynthesis and degradation, the biological significance of NAD turnover taking place within the cell has remained elusive for long time. As a matter of fact, the first homogeneous enzyme preparation within the whole NAD biosynthetic pathway is dated 1986 (15), while the first crystal 3D structure has been solved only in 2000 (16). Recent recognition that NAD metabolic enzymes may represent targets for drug design catalysed a burst in the study of the coenzyme homeostasis. As a consequence, a large number of papers on the enzymology of pyridine nucleotide metabolism appeared in the literature, as well as a significant number of comprehensive reviews covering different aspects of the significance of NAD metabolism, with particular attention to the enzymes involved in NAD cleavage. Therefore, this review will be essentially limited to the enzymology of NAD biosynthesis in health and disease. Figure 1 shows a schematic overview of mammalian NAD metabolism depicting those routes whose occurrence has been experimentally confirmed.

3. THE KYNURENINE PATHWAY AND *DE NOVO* NAD BIOSYNTHESIS

In mammals, NAD can be synthesized *de novo* starting from dietary tryptophan through the kynurenine pathway, leading to the formation of quinolinic acid (Qa), the pyridine coenzyme precursor (Figure 2). In recent years the kynurenine pathway has become the subject of deep investigation due to the finding that some intermediates of the pathway are endowed with neuroactive properties (29-31). Among them are: Qa, a neurotoxic molecule acting both as a selective *N*-methyl-D-aspartate (NMDA) receptor agonist and free radicals generator; kynurenic acid, a non selective antagonist of excitatory amino acid receptors, that can antagonize some of the effects of Qa; 3-hydroxykynurenine and 3-hydroxyanthranilic acid, that generate toxic free radicals. It is well established that fluctuations in the endogenous levels of these metabolites are associated to neuronal injury during several major inflammatory brain diseases, including AIDS dementia complex, cerebral malaria, amyotrophic lateral sclerosis, multiple sclerosis, neonatal asphyxia, Hungtington disease, schizophrenia (32). The inflammatory state in fact induces the expression of indoleamine-2,3-dioxygenase (IDO), the first and rate-limiting enzyme of the pathway in extrahepatic tissues, via a cytokine (mainly interferon-γ)-dependent mechanism. IDO induction results in enhanced production of the neurotoxic tryptophan metabolites, whose accumulation in the brain has been implicated in the pathogenesis of the above listed diseases. As a consequence, the kynurenine pathway is considered an attractive targets' source for new drugs development (32, 33). Indeed, increasingly specific and potent inhibitors of the individual enzymes of the pathway are being designed and characterized (34).

Moreover, the kynurenine pathway is regarded as a defense mechanism induced by IFN-γ during immune response. In fact, an upregulated IDO expression in the infected tissues causes deprivation of tryptophan from the infected area, that together with the proapoptotic effect of some of the released tryptophan metabolites, exerts

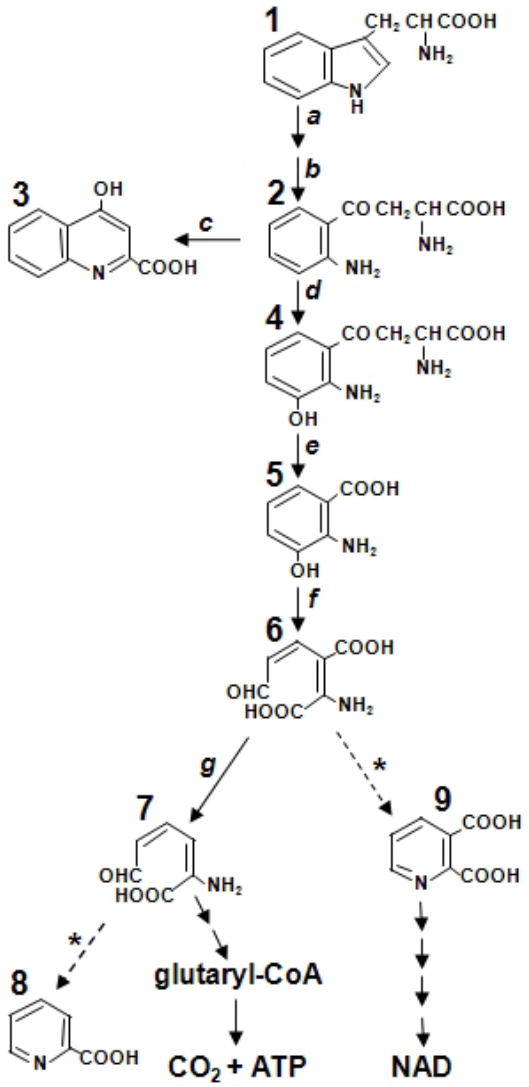

Figure 2. Schematic overview of tryptophan catabolism through the "kynurenine pathway". Digits indicate the intermediates of the pathway: tryptophan (1); L-kynurenine (2); kynrenic acid (3); 3-hydroxykynurenine (4); 3-hydroxyanthranilate (5); 2-amino 3-carboxymuconate 6-semialdehyde (ACMS) (6); 2-aminomuconate 6-semialdehyde (AMS) (7); picolinate (8); quinolinate (9). Italicized fonts refer to the enzymes: tryptophan/indoleamine 2,3-dioxygenase (a); kynurenine formamidase (b); kynurenine aminotransferase (c); kynurenine 3-hydroxylase (d); kynureninase (e); 3-hydroxyanthranilate 3,4-dioxygenase (f); 2-amino 3-carboxymuconate 6-semialdehyde decarboxylase (g). Dotted arrows marked with asterisks indicate the two non-enzymatic reactions of the pathway leading to quinolinate or picolinate formation.

antimicrobial activity (35, 36). A large and systemic induction of IDO can also occur, as shown in rat during endotoxin shock (37), or upon injection of interleukin-12 (38), or during malaria infection (39), suggesting that IDO might also play a role in the host response to systemic infections. Cytokine-induction of IDO in dendritic cells and macrophages depletes tryptophan from the microenvironment, such that tryptophan starvation selectively affects surrounding T-cells, and either inhibits their replication or induces apoptosis. As a result, the antigen-dependent T-cell activation is impaired. Indeed, it is well established the involvement of IDO induction in the development of T-cell-mediated immune tolerance (35). Expression of IDO is in fact associated to inhibition of T-cell mediated rejection of allogenic fetuses (40) and allografted pancreas islets in mice (41), as well as inhibition of the T-cell mediated experimental asthma (42). On the other hand, IDO induction in tumour cells, causing suppression of T-cell immunity in the tumour microenvironment, is responsible for the tumour escape from the immune surveillance (43-46). Likewise, it has been reported that the immunosuppressive role of the kynurenine pathway might contribute to development of immunodeficiency (47). As a consequence, the pharmacological modulation of the kynurenine pathway is considered as a promising tool for developing new therapies in a variety of human disorders. However, little information exists in the literature on the role played by the pathway in cellular NAD homeostasis and, to our knowledge, no data are available on its specific contribution to the overall NAD synthesis. This section will therefore focus on the existing evidence on the effect of the modulation of the kynurenine pathway on NAD levels.

In the mouse monocyte/macrophage cell line RAW 264.7, IDO induction by IFN-γ results in a significant increase in the *de novo* NAD synthesis (48). The Authors suggest that in these cells the kynurenine pathway plays an important role in meeting the increased requirement of NAD synthesis during the immune response, mainly due to the activation of the enzyme poly ADP-ribose polymerase (PARP) which consumes most of the intracellular dinucleotide. That IDO induction is involved in NAD synthesis as a response to PARP-induced NAD depletion, has been also evidenced in astrocytes treated with hydrogen peroxide, where induction of IDO via IFN-γ boosts intracellular NAD concentrations and reduces cell death (49). Conversely, specific inhibition of IDO activity in the IFN-γ-stimulated astrocytes results in the concomitant reduction of cellular NAD levels (50). This finding should be carefully considered when inhibitors of the kynurenine pathway are pharmacologically used, since they might dangerously reduce NAD levels in astrocytes (50). Investigations performed on primary murine macrophages revealed that IDO induction via TNF results in a decrease of intracellular NAD levels (51). This apparent discrepancy likely arises from the different experimental conditions used, *e.g.*, the different control of the concomitant upregulation of NAD-consuming enzymes.

Very recently, an interesting review on pharmacological targeting of IDO-mediated tolerance for treating autoimmune disease emphasizes the idea that autoimmune diseases may in part be considered as "localized pellagra", manifesting symptoms particular to the inflamed target tissues (6). In many autoimmune

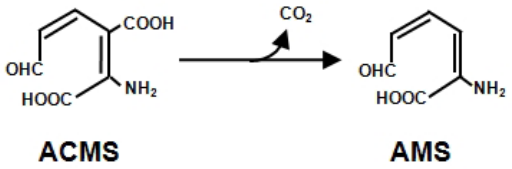

Figure 3. 2-Amino 3-carboxymuconate 6-semialdehyde decarboxylase-catalyzed reaction.

diseases, indeed, cytokines are chronically elevated, resulting in a persistent IDO induction (52, 53). As a consequence, also in this case, a systemic or local depletion of tryptophan occurs, impairing NAD synthesis in non immune cells, and causing symptoms resembling the classic ones occurring in pellagra (7). Significantly, there are many examples of NAD precursors preventing or ameliorating autoimmune diseases (6). Likewise, in HIV infection, IDO induction by specific viral antigens leads to a decrease of intracellular NAD levels (54, 55) and this HIV-induced pellagra-like cellular state can be reversed by Nam administration (56). In carcinoid syndrome patients, up to 99% of tryptophan can be catabolized to serotonin, thus significantly reducing the flow tryptophan-to-NAD (57, 58). Indeed, pellagra has been reported in some carcinoid patients (59, 60), and niacin supplementation not only resolves symptoms common to carcinoid and pellagra syndromes, such as skin lesions and diarrhea/steatorrhea, but also generally improves the health of these patients (60, 61).

Given the importance of the tryptophan-to-NAD flux in health and disease, we will describe in detail the enzymes 2-amino 3-carboxymuconate 6-semialdehyde (ACMS) decarboxylase and quinolinate phosphoribosyltransferase (QaPRT), catalyzing the two divergent reactions channelling ACMS to either NAD biosynthesis or acetyl-CoA formation (Figure 2), in our opinion representing the key regulators of *de novo* NAD biosynthesis.

3.1. ACMS decarboxylase diverts the kynurenine pathway from NAD synthesis

The enzyme ACMS decarboxylase (EC 4.1.1.45) catalyzes the ACMS decarboxylation to 2-aminomuconate 6-semialdehyde (Figure 3), thus diverting ACMS from NAD synthesis and channelling tryptophan towards complete oxidation and/or conversion to picolinate, which is the product of the spontaneous 2-aminomuconate 6-semialdehyde cyclization (Figure 2). The enzyme is widely distributed in both bacteria and eukaryotic organisms. It was first isolated in 1956 from pig liver (62) and since then it has been thoroughly characterized from several mammalian sources (63-65). Only recently the cDNA coding for human ACMS decarboxylase has been identified (66) and the human recombinant enzyme has been purified and characterized (67). It is a 336-residues protein with a molecular mass of about 40 kDa, which behaves as a monomer in solution. It shows a broad pH optimum ranging from 6.5 to 8.0. K_m value for ACMS is 6.5 μM and k_{cat} is 1.0 s^{-1} (67). ACMS decarboxylase is a member of the metal-dependent amidohydrolase protein superfamily (68) and catalyzes a novel type of metal-dependent nonoxidative decarboxylation reaction (69). The bacterial enzyme requires Zn^{2+} as the cofactor (70). The presence in the human enzyme of the residues involved in the metal binding in bacterial ACMS decarboxylase, together with site-directed mutagenesis experiments, confirmed the metal-ion dependence also for the human ACMS decarboxylase-catalyzed reaction (67). However, the nature of the native metallocofactor in the human enzyme remains to be determined, since the recombinant protein easily takes up different metal ions from the host environment during protein synthesis, and any attempt to obtain the metal-free protein has been unsuccessful (67).

In rat the ACMS decarboxylase gene is under nutritional control, being down-regulated by dietary polyunsaturated fatty acids, phtalate esters and peroxisome proliferators, and up-regulated by high protein diet (71-75). Changes in ACMS decarboxylase activity are readily reflected in serum and tissue Qa levels and in the rate of tryptophan-to-NAD conversion, clearly indicating the role of ACMS decarboxylase activity in the *de novo* NAD biosynthesis regulation. Recently, transcriptional regulation of the rat gene by Hepatocyte Nuclear Factor 4α (HNF4α) and Peroxisome Proliferator-activated Receptor α (PPRα) has been demonstrated (76).

In humans, the enzyme shows the highest expression in kidney (66, 67), where ACMS decarboxylase might play an important role in preventing the excessive formation and accumulation of toxic Qa. Indeed, experiments performed on patients with renal insufficiency, as well as on rats with induced renal failure, showed a decrease of kidney ACMS decarboxylase activity and significant elevation of Qa in serum, urine, cerebrospinal fluid, and peripheral tissues (77-80). In liver and brain, the enzyme is expressed at significantly lower levels than in kidney, suggesting that the tryptophan-to-NAD conversion might represent a relevant pathway in these organs. Indeed, most of the intracellular NAD in liver is synthesized from the amino acid rather than from dietary niacin and the liver dinucleotide is considered as the main vitamin source for extrahepatic tissues (81). In brain, the enzyme has been detected in primary neurons (82), that are indeed able to constitutively produce picolinic acid and do not show detectable amounts of Qa (82). Notably, ACMS decarboxylase is not present in SK-N-SH neuroblastoma cells, that unlike neurons show accumulation of Qa, but not production of picolinic acid (82). These results, for the first time, reveal the ability of ACMS decarboxylase to affect Qa and picolinate formation in humans, and highlight a possible role of the enzyme in neuroprotection. In fact, picolinic acid is endowed with neuroprotective properties, being able to protect, at nanomolar concentrations, against the Qa induced neurotoxicity (83, 84). Very recently, the presence of ACMS decarboxylase has also been demonstrated in human primary macrophages stimulated with INF-γ (82)

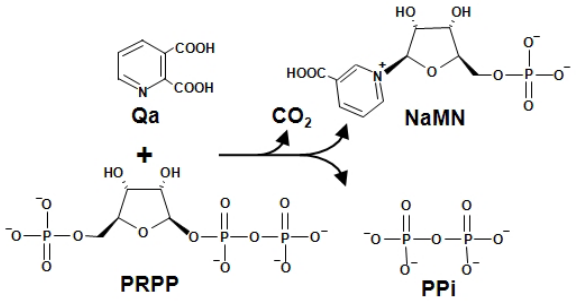

Figure 4. Quinolinate phosphoribosyltransferase-catalyzed reaction.

3.2. Quinolinate phosphoribosyltransferase commits the kynurenine pathway towards NAD synthesis

The enzyme responsible for tryptophan channelling towards NAD synthesis is QaPRT (EC 2.4.2.19), which catalyzes the transfer of the phosphoribosyl moiety of 5-phosphoribosyl 1-pyrophosphate (PRPP) to Qa, yielding NaMN, pyrophosphate and CO_2 (Figure 4). Human QaPRT cDNA encodes a 297-residues protein with a molecular mass of about 30 kDa (85). The enzyme has been purified and characterized from human brain and liver (86) and recently the 3D structure of the recombinant protein has been solved (87). Human QaPRT is an hexamer in solution and the monomer is structurally similar to that of the bacterial counterpart (87). It belongs to the family of type II phosphoribosyltransferases, comprising a characteristic seven-stranded α/β barrel structure. Kinetic analyses revealed a K_m of 5.6 μM for Qa and 23 μM for PRPP (86). The k_{cat} value has never been reported. The activity is dependent on Mg^{2+} ions and shows optimum value around pH 6.5. The enzyme is inhibited by micromolar concentrations of phthalic acid and by both the product pyrophosphate and the substrate PRPP (86, 87). The enzymatic activity has been detected in liver, brain and kidney (78, 86) and in erythrocytes and platelets (88).

In brain, the enzyme is present both in neurons and in glial cells (82, 89, 90). While in astrocytes its expression significantly increases upon IFN-γ treatment (90), comparable levels of QaPRT activity have been measured in stimulated and unstimulated neurons (82). In these cells the kynurenine pathway appears to be impaired: in fact astrocytes do not express the enzyme kynurenine hydroxylase and cannot produce Qa, and neurons show no detectable Qa production as well (90-92). The presence of QaPRT in these cells allows them to catabolise Qa that is produced by microglia and brain infiltrating macrophages (91), even though the mechanism of the molecule uptake is not known. In fact no specific receptor or membrane transport systems for Qa have been so far identified (90). Anyway, QaPRT in brain is not able to significantly lower the neurotoxic concentration of Qa produced by the immune cells (90). Even though a significant increase of the enzyme activity has been observed in the brain of Huntington disease patients (93), as well as in the cerebellum of patients with olivopontocerebellar atrophy (94), it is likely that the enzyme is not able to efficiently counteract the dangerous Qa raise in these disorders. It cannot be excluded the possibility that efficiency in Qa degradation might be responsible for the pathological accumulation of the molecule; in fact, it has been observed a local deficit of QaPRT activity in epileptic human brain, which might contribute to the establishment or maintenance of the epileptic focus (95).

In view of the fact that Qa levels in normal brain rarely exceed submicromolar concentrations (96, 97), it has been inferred that this molecule cannot contribute significantly to NAD regeneration under conditions of acute coenzyme depletion, due for example to PARP activation. Indeed, experimental evidence indicates that in glial cells Na is the preferred precursor for NAD biosynthesis through its phosphoribosylation (96).

In the cells of the immune system, where the whole kynurenine pathway is operative, and the synthesis of Qa has been demonstrated to occur and to be enhanced following various modes of immune stimulation (35, 98), expression of QaPRT seems to be induced by specific stimulatory signals, like IFN-γ (48, 82).

4. FROM NMN/NaMN TO NAD (P)

Newly synthesized or preformed NAD (P) precursors are conveyed to the final effective coenzyme forms by means of enzyme reactions, described below, that are common to both *de novo* and salvage biosynthetic routes.

4.1. NMN adenylyltransferase

In mammals and most living organisms, the deamidated pyridine mononucleotide NaMN formed by QaPRT is further used for the synthesis of NaAD through an adenylyl group transfer reaction catalyzed by the enzyme nicotinamide mononucleotide adenylyltransferase (NMNAT, EC 2.7.7.1). The same NMNAT-catalyzed reaction can also lead directly to NAD formation from NMN (Figure 5). The enzyme is also able to catalyze the synthesis of NAD analogues were the pyridine moiety is replaced by chemotherapic pro-drugs, like 3-acetylpyridine, after their intracellular phosphoribosylation to the corresponding mononucleotides. Indeed, injection of 3-acetylpyridine into mouse tumours resulted in a consistent reduction of the tumour size, correlating with the formation of the acetylpyridine analogue of NAD, and a concomitant reduction of NAD concentration (99, 100). Furthermore, the mechanism underlying the remarkable effect of 6-mercaptopurine in limiting tumour cell growth has been related to the ability of 6-mercaptopurine riboside triphosphate to competitively inhibit NMNAT, thereby reducing the level of NAD to below that required for survival (101, 102). Finally, NMNAT catalyzes the metabolic activation of antineoplastic compounds such as tiazofurin and benzamide riboside, and sufficient NMNAT activity is needed to achieve therapeutic efficacy and to overcome tiazofurin resistance observed in certain tumour cell lines (103-105). On the assumption that in cancer tissues NAD is reduced fairly close to its critical level, the

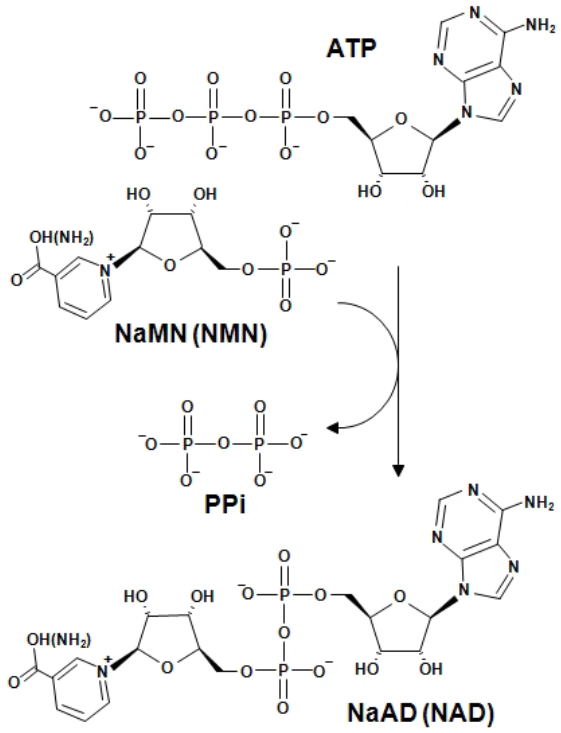

Figure 5. NMN adenylyltransferase-catalyzed reaction.

NAD biosynthetic key enzyme NMNAT has been already regarded as an interesting target for anticancer chemotherapy, aiming at lowering NAD to below vital concentration, although it may vary among different cancer tissues and immortalized cells (102, 106). However, despite a number of structural studies and experimental tests with natural and synthetic molecules, the search for effective NMNAT-targeted inhibitors has been fruitless up to date. Indeed, the polyphenolic plant metabolite gallotannin (107) appears a poorly selective inhibitor, while other synthetic geometric oligophosphate-containing NAD analogues, exerting their inhibitory effect in the sub-millimolar range, can be mainly regarded as lead compounds for further structure-based drug design to improve both their efficacy and stability (108).

Structural, kinetic and regulatory properties of NMNAT from different sources have been studied since many years assuming the existence of a single enzyme located within the cell nucleus (109-111). On the other hand, NMNAT isozymes, encoded by different genes and showing distinctive organelle and tissue distribution, have been reported in eukaryotes (112-116), except for plants (117). The presence of compartmented multiple isozymes first indicates that NAD biosynthesis is not exclusively a nuclear event. In addition, rather than simply reflecting functional redundance, it appears related to organelle- and tissue-specific functions, still largely unknown as far as the physiological and pathological implications are concerned. In humans, the three isozymes so far identified are indicated as NMNAT1, NMNAT2 and NMNAT3, where NMNAT1 is the first (10) and best characterized nuclear isozyme variant (118-120), while NMNAT2 and NMNAT3 are the isoforms localized in the Golgi complex and mitochondria, respectively (107, 120). Northern blot analyses in human tissues have revealed that NMNAT1 is ubiquitous and less abundant in brain where NMNAT2 is specifically expressed, while NMNAT3, in general as widely distributed as NMNAT1 though at lower level, is mostly present in lung, spleen, placenta, and kidney (107, 119-123). Yet, all three isozymes are expressed to a lesser extent in cancer cell lines (120, 121), consistent with early assumption of reduced cellular NAD pools upon cancerous transformation (102, 106, 124). The three human isozymes share with all NMNATs so far characterized the conserved motifs GXXXPX (T/H)XXH and SXXXXR (125), and catalytically essential residues have been identified by mutagenic techniques (126, 127). Despite their structural similarity (35-50 % identity along ~300 residues), they show different oligomeric states (120, 125), being NMNAT1 a homohexamer, NMNAT3 a homotetramer, and NMNAT2, whose 3D structure is not available yet, behaving as a monomer (119). Their individual properties, compared in previous work (107, 108), are summarized in Table 1.

Kinetic characterization performed using bacterially expressed human NMNATs, has revealed an ordered sequential Bi-Bi mechanism with different, isozyme-specific, substrate addition order (108). The reaction equilibrium constant fairly close to 1 also suggests the possible physiological occurrence of the reverse reaction, *i.e.* NAD pyrophosphorolysis (107, 118, 128). Compared to NMNAT2 and 3, NMNAT1 utilizes more efficiently NMN than NaMN, and shows the greatest catalytic efficiency (k_{cat}/K_m) (Table 1). All three isozymes can also synthesize NADH from NMNH *in vitro* with comparable efficiencies, thus suggesting a direct role in regulating the cellular redox state, *i.e.*, the ratio NAD/NADH (107, 108). NMNAT3 appears the most catalytically flexible toward alternative substrates, like GTP and ITP, and is also able to catalyze, like NMNAT1, the metabolic activation of the antineoplastic intermediate tiazofurin monophosphate (108). These findings led to the conclusions that NMNAT2, typically expressed in neuronal tissue (119, 123), can not represent the physiological target for tiazofurin-based chemotherapy, and that the mitochondrial location of NMNAT3 should be carefully considered in view of the possible synthesis in this organelle of NADH and NAD analogues, a largely unexplored issue (108, 129). Not less important, the investigation on isozyme-distinctive substrate specificity and metal ion requirement (107, 119, 120), led recently to develop a discrimination assay to quantify the individual isozyme's activities in tissue and cell extracts (108), providing a new tool for a direct functional investigation on the possible isozyme-specific roles in physiological and pathological contexts. Notably, when the discrimination assay was applied to different normal human tissues and related transformed cell lines, the three isozymes were not present simultaneously nor equally contributed to NAD formation. Their differential expression preliminarily evidenced a potentially interesting association of NMNAT2 to the hepatocarcinoma cell malignancy, an unexpected finding undoubtedly deserving further exploration (108).

Table 1. Comparative properties of the three human NMNAT isozymes

	NMNAT1			NMNAT2			NMNAT3		
Subunit	31.9 kDa (279 aa)			34.4 kDa (307 aa)			28.3 kDa (252 aa)		
Chromosome	1p36			1q25			3q23		
Subcellular local.	Nuclear			Golgian			Mitochondrial		
Native Structure	Hexameric			Monomeric			Tetrameric		
Tissue distribution	**High level**: heart, skeletal muscle, kidney, liver **Low level**: thymus, brain, spleen			**High level**: brain **Low level**: heart, skeletal muscle, lung			**High level**: lung, spleen **Low level**: placenta, kidney		
pI	9.0			6.6			9.28		
Optimal pH	6.0 - 8.0			7.0 - 8.5			6.0 - 9.0		
Optimal (Mg^{2+})	12 mM			0.3 mM			2 mM		
Metal requirement	Co^{2+}>Ni^{2+}>Mg^{2+}>Zn^{2+}>Mn^{2+}			Mn^{2+}>Mg^{2+}>Zn^{2+}>Ni^{2+}>Co^{2+}			Mg^{2+}>Cr^{2+}>Mn^{2+}>Co^{2+}>Ni^{2+}		
Kinetics[1]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
ATP	59	54	$9.2 \cdot 10^5$	89	8.8	$1.0 \cdot 10^5$	42	2.5	$5.8 \cdot 10^4$
NMN	22	54	$2.4 \cdot 10^6$	21	8.8	$4.1 \cdot 10^5$	66	2.5	$3.8 \cdot 10^4$
NaMN	68	43	$6.3 \cdot 10^5$	15	6.9	$4.8 \cdot 10^5$	111	3.8	$3.4 \cdot 10^4$
NMNH	294	19	$6.3 \cdot 10^4$	304	3.3	$1.1 \cdot 10^4$	130	7.2	$5.5 \cdot 10^4$
TrMP	370	15	$4.0 \cdot 10^4$	>100,000	~ 0.5	> 5.5	2,010	0.4	209

[1] Reported values are from ref (97). TrMP indicates tiazofurin monophosphate.

Since more than 20 years, the nuclear localization of both NMNAT1 and the most relevant NAD-consuming enzyme PARP suggested a functional link between them in regulating the cellular NAD turnover (130). Indeed, inhibitory effects on PARP activity by NMNAT1 have been subsequently described (111, 131), and assumed to counter NAD and ATP depletion occurring when DNA damages cause PARP over-activation. Very recently, it has been demonstrated that NMNAT1 associates with PARP1, which, following automodification, increases the extent of cellular poly ADP-ribosylation (132). Moreover, in the same paper it has been demonstrated that *in vitro* specific phosphorylation of NMNAT1 by protein kinase C precludes the NMNAT1-mediated activation of PARP1. However, the occurrence *in vivo* of such mechanism should be corroborated by further experiments.

In the early 2000's, the *NMNAT1* gene has been identified as the human homologue of *D4Cole1e*, the gene that contributes the great majority of amino acids to the chimeric protein responsible for delaying Wallerian degeneration in the Wld^s mouse (24, 122). Wallerian degeneration distal to an axon injury is an important and relevant experimental model of axon degeneration in neurological diseases. In fact, axon degeneration is, in addition to neuronal cell death, a major morphological change observed in peripheral neuropathies as well as in neurodegenerative disorders, like Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis (22, 23, 26, 27). The protective *Wld^s* gene indeed encodes an N-terminal fragment of the ubiquitination factor E4B (Ube4b) fused to a full-length NMNAT1, and it was shown to confer a phenotype of dose-dependent delay on Wallerian degeneration. Surprisingly, the Wld^s protein was located predominantly in the nucleus, indicating an indirect protective mechanism acting on distal axon compartment. Total enzyme activity, but not NAD content, was increased up to four-fold in the mutant Wld^s brain, suggesting that NAD homeostasis was maintained. Since then, mounting evidence on the importance of NAD biosynthesis in neuroprotection has steadily accumulated in the literature. In this respect, it should be pointed out that the results obtained in this area are often contradictory, mostly depending upon the biological system investigated. For example, in *ex vivo* experiments, it has been demonstrated that NMNAT1 overexpression delays axon degeneration through profoundly different mechanisms (27, 126). In one case, SIRT1, a mammalian ortholog of SIR2, is the downstream effector of the increased NMNAT activity leading to axonal protection, whereas others could not reproduce this result and, instead, concluded that NAD works directly within axons irrespective of SIRT1. On the other hand, a recent conflicting report shows that *in vivo* NMNAT1 alone cannot substitute for the whole Wld^s protein to delay Wallerian degeneration, indicating that the ability of the fused protein to protect axon is far greater than NMNAT1 itself (21). The above contradictory results need certainly to be reconciled before a clear picture on the molecular mechanism underlying Wallerian degeneration can be drawn and, mostly, on the primary role played by either NMNAT1 itself or its NAD product in delaying such process. Nonetheless, axon degeneration seems to be associated to NAD depletion (25, 27) and, in this respect, it can be included among diseases related to cell commitment into a pellagra-like state.

4.2. NAD synthetase

In those organisms and/or tissues where NaAD is formed as the product of NMNAT activity, a further amidation reaction catalyzed by NAD synthetase (NADS) is required to achieve the effective NAD form, representing the last step in NAD biosynthesis in both *de novo* and salvage pathways (Figure 6). Two classes of NADSs have been reported and classified under EC 6.3.1.5 and EC 6.3.5.1. The first one is strictly ammonia-dependent, while the other uses glutamine as the nitrogen donor. Enzymes belonging to the first class are typically found only in prokaryotes, *e.g., E. coli* (133), *B. subtilis* (134), *S. typhimurium* (135), and *B. anthracis* (136), while glutamine-dependent NADSs have been reported in eukaryotes (137, 138) and selected prokaryotes like *M. tuberculosis* (139). Structural alignments evidence that the two classes of NADSs share highly conserved C-terminal

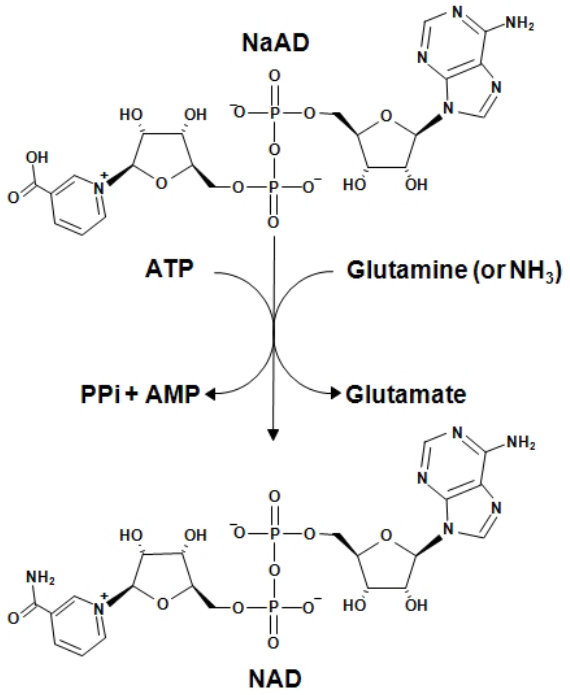

Figure 6. NAD synthetase-catalyzed reaction.

portions, comprising the NAD synthase domain and the P-loop motif, characteristic of the N-type family of ATP pyrophosphatases (140). In addition, the glutamine-dependent NADSs possess an N-terminal extension, corresponding to a Carbon-Nitrogen (CN) hydrolase domain, shared by enzymes of the nitrilase family and responsible for the ability to use glutamine (141). This domain serves as the glutamine aminotransferase domain and contains a catalytic triad consisting of conserved Glu and Lys residues together with a Cys nucleophile (142). The reaction catalyzed by glutamine-dependent NADS appears to proceed in two separate phases, consistent with the two-domain structure. The first reaction (Gln + H_2O = Glu + NH_3) takes place within the CN domain, while the second reaction (NaAD + ATP + NH_3 = NAD + AMP + PPi) takes place in the C-terminal NAD synthase domain *via* a transient NaAD-adenylylated intermediate (143). The two domains have been shown to be reciprocally regulated such as to avoid wasteful hydrolysis of glutamine and ATP, and the presence of a channel for translocation of ammonia to the NAD synthetase active site has been identified by modeling and structure-based sequence analysis (143). Consistent with such domain organization, glutamine-dependent enzymes can also use ammonia, although with K_m values generally less favorable. We have previously reviewed the literature on known NADSs from prokaryotic and eukaryotic sources, and their respective nitrogen donor preference (118).

In eukaryotes, glutamine-dependent NADS activity has long been known in human erythrocytes and yeast (137, 138). More recently, the human enzyme gene *NADsyn1* (yeast *qns1*), encoding a 706 amino acids protein, has been identified, and the protein has been characterized after overexpression in COS-7 cells (144). The recombinant enzyme in solution behaves as a 500-kDa homohexamer (144), consistent with previous observation from human erythrocytes and yeast enzyme preparations (137, 138). Kinetic characterization of human NADS, performed at pH 7.5, showed K_m values for NaAD, glutamine, and ATP of 0.49 mM, 1.44 mM, and 0.089 mM, respectively. Instead, the K_m for ammonium ion, measured at pH 8.8, resulted higher than 10 mM, as expected (144). Interestingly, yeast NADS shows comparable K_m values and is inactive toward NaMN (137), suggesting also failure of the human enzyme to amidate the mononucleotide substrate. The original report also identified a sequence coding for NADsyn2, a claimed human ammonia-dependent NADS, subsequently shown to be a bacterial enzyme arising from contaminating *Pseudomonas* (145).

Northern blot analyses in normal mouse tissues revealed that *NADsyn1* is expressed at higher level in small intestine, liver, kidney, and testis, and very weakly in skeletal muscle and heart (144). This profile appears not consistent with the above reported tissue distribution of human NMNAT, the enzyme forming the substrate for NADS from NaMN in the deamidated route for NAD synthesis. This discrepancy, however, might be due to the use of different RNA sources. In addition, NADS and NMNAT expression levels do not necessarily need to correlate; in fact, in those tissues where NMN is available, NAD synthesis can be accomplished by NMNAT without NADS action. Indeed, the elucidation of the distribution of the amidated and deamidated routes for NAD synthesis throughout human tissues in health and disease is needed in order to assess the tissue-specific essentiality of human NADS. To date, no evidence is present in the literature on the differential expression of NADS in pathological conditions. By contrast, most studies have been performed on the strictly ammonia-dependent, essential for cell viability, bacterial NADSs (124, 133, 134, 136), whose 3D structures have also been solved for structure-guided rational drug design (136, 146, 147).

4.3. NAD kinase

NAD kinase (NADK, EC 2.7.1.23) catalyzes the only known cellular reaction leading to the phosphorylation of NAD to NADP, using ATP as a phosphate donor (Figure 7). (Note that the cognate reaction specific for NADH and yielding directly the reduced product NADPH is referred as EC 2.7.1.86.)

NADP is involved in several crucial cellular functions (148). Its deamidated form (NaADP) plays a signaling function as a potent intracellular Ca^{2+}-mobilizer. Its reduced form (NADPH) acts as an electron donor in most reductive biosyntheses, as well as in the oxidative stress defense pathways, by regenerating the oxidized protectants glutathione and thioredoxin. On the other hand, NADPH can even contribute to the oxidative stress, by producing partially reduced oxygen species (ROS) when it is used by NADPH oxidases or by detoxifying enzymes like cytochromes P450 and catalase (148). Although redox reactions do not change total NADP level, cell redox state is characterized by balance between NADP/NADPH and

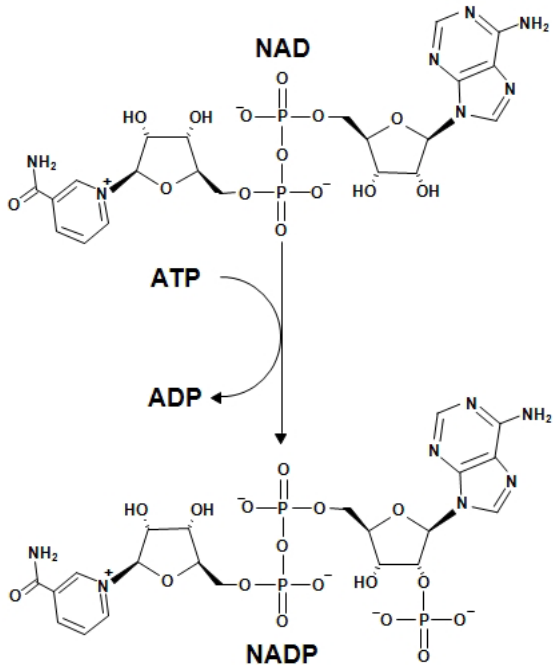

Figure 7. NAD kinase-catalyzed reaction.

NAD/NADH ratios. In principle NADK can alter this balance thus contributing to the regulation of NAD- and/or NADPH-dependent pathways. Accordingly, NADK has been recently found to be essential for cell survival in many organisms, ranging from bacteria, to yeast, to plants (149-154). By contrast, little evidence, except an unpublished report (153), is available on NADK essentiality in mammals, as well as on its involvement in physiological and pathological cell states presumably involving redox imbalance, like the inflammatory and immune processes.

Evidence for human NADK activity was first reported from placenta (155), and subsequently from white and red blood cells (156, 157). A partially purified preparation from human neutrophils described a dependency of maximum velocity, but not the NAD and ATP affinities, on the presence of both calmodulin and Ca^{2+} ions (158). As opposed to the three compartment-specific isoforms known in plants and yeasts (148), only one human NADK has been so far evidenced. It has been recently identified by sequence similarity with bacterial and yeast counterparts, and shown to be cytoplasmic (159). The gene, located on chromosome 1p36.21-36.33, is widely expressed in human tissues except the skeletal muscle, as revealed by Northern blot analyses (159). The recombinant enzyme comprises four identical 49 kDa-subunits and shows optimal catalysis at 55 °C, pH 7.0-8.0, and requires divalent cations in the following order of preference: Zn^{2+}, Mn^{2+} and Mg^{2+} (159). Recombinant NADK appears rather selective for NAD and ATP (K_m values of 0.54 and 3.3 mM, respectively), and it does not use the alternative substrate NaAD, thus not leading to direct synthesis of the potent Ca^{2+}-mobilizing dinucleotide NaADP. Subsequent study revealed that the same enzyme preparation can also use NADH as substrate, though less efficiently, thus possibly driving the NADPH formation within the cell (160). Most strikingly, unlike the partially purified enzyme from neutrophils, the recombinant NADK does not appear to be sensitive to Ca^{2+}/calmodulin stimulation (159), suggesting either the presence of multiple human NADK isoforms, similarly to sea urchin eggs (161, 162) and plants (163-166), or the existence of unknown factors mediating calmodulin-dependence of NADK activity. Further characterization of the human NADK revealed that its 200-fold overexpression in HEK293 cells resulted in only 5-fold increase of the cellular NADP pool, almost exclusively present in its reduced form. The relatively modest product accumulation could be partly explained by a postulated product inhibition of NADK activity (160). The NADPH accumulation, which was unexpected because the human enzyme preferentially phosphorylates NAD, was ascribed to the basal activity of pre-existing NADP-dependent dehydrogenases. Besides, the exceedingly high NADK activity in transformed cells, while not affecting cell viability, only moderately enhanced protection from oxidative stress (160). Conversely, stably deficient NADK (-) cells, showing about 30% NADK expression, activity and NADPH content, exhibited a decreased resistance to H_2O_2 treatment (160). Although in yeast and plants mitochondrial and cytoplasmic NADK isoforms exist, in humans a mitochondrial isozyme has not been so far identified. Therefore, how two distinct NADP pools, cytosolic and mitochondrial, may be maintained still remains an open question.

As for other NAD-biosynthetic enzymes, the structure of bacterial NADKs has been solved for the rational design of novel antibiotic drugs. The 3D structure of human NADK (s) is awaited to establish structure-based mechanistic differences with respect to the bacterial enzymes for the design of selective drugs. The metabolic effect of such compounds on the human organism might be influenced by the possible existence of multiple NADKs.

5. FORMATION AND UTILIZATION OF NIACINS

The term “niacins” include both Na and Nam. Because of the recent discovery of nicotinamide riboside (NR) as a nutrient in mammals and fungi (167), we propose to also consider NR as a “niacin”. In this section, the role played by each niacin in NAD synthesis will be described.

5.1. Nicotinic acid

In mammals, Na can arise from either dietary uptake or endogenous NAD splitting/reutilization pathways. In particular, owing to the reported lack of nicotinamide deamidase (EC 3.5.1.19) in mammals (168), this niacin can be formed in the cell by the action of NAD glycohydrolases (EC 3.2.2.5), as well as other hydrolytic activities, acting on NaAD and NaMN (118, 169). In this regard, it should be emphasized that, even though in mammalian genomes no homologs of nicotinamide deamidase gene have been identified, a nicotinamide deamidase activity from many mammalian sources has been described since long time (118). Furthermore, in our laboratory we have been able to detect a remarkable deamidase activity in human hepatic tissue (unpublished

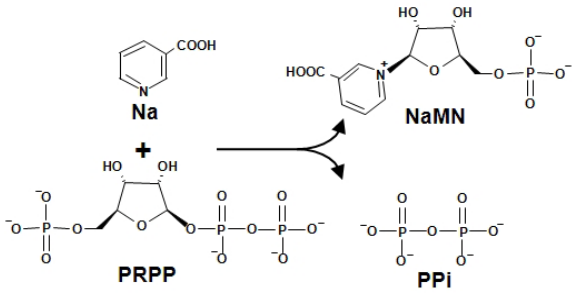

Figure 8. Nicotinate phosophoribosyltransferase-catalyzed reaction.

results). The presence of nicotinamide deamidase activity in animals could be ascribed either to an ortholog gene not yet identified, or to a residual activity possessed by another enzyme catalyzing esterolytic reactions. This hypothesis is consistent with the observation that the rabbit liver enzyme exhibits the capability to hydrolyze, in addition to nicotinamide, a large variety of amides and esters (170).

In addition to its pellagra-preventing action, Na has been used for decades as a lipid-lowering agent, although the biochemical mechanism is not fully understood. Only recently, a G-protein-coupled receptor, highly expressed in adipose tissue and capable to bind Na with high affinity, has been identified (171, 172). This finding represents a possible basis for the treatment of hyperlipidaemia and insulin-resistant states (173).

During the preparation of this manuscript, an interesting report has been published (174), describing the functional expression of a specific, high-affinity carrier involved in the uptake of Na in human liver cells. The Authors show that transport is pH-dependent and apparently regulated by an intracellular Ca^{2+}/calmodulin-mediated pathway.

5.1.1. Nicotinate phosphoribosyltransferase

Nicotinate phosphoribosyltransferase (NaPRT, EC 2.4.2.11) catalyzes the synthesis of NaMN from Na and PRPP (Figure 8). The mammalian enzyme was first described in human erythrocytes and subsequently purified to apparent homogeneity and characterized from the same source (175, 176). The protein shows a native molecular mass of 86,000 Da. The enzyme activity is optimal over a broad pH range, from pH 6.5 to pH 8.0. Like other mammalian NaPRT enzymes and in contrast with the enzyme from other sources, the activity of human NaPRT is stimulated by ATP, but its requirement is not obligatory. At saturating concentrations, ATP is hydrolyzed to ADP in a 1:1 molar ratio with respect to the formation of NaMN, whereas in the absence of ATP the enzyme is still active, though at a lower rate (175). In the absence of ATP, K_m values for Na and PRPP are 24 μM and 100 μM, respectively. In the presence of saturating ATP, K_m values are 0.5 μM and 5 μM, respectively, and the V_{max} value increases by a factor of three. Thus, ATP appears to act both as an energy-yielding co-substrate and as an allosteric modulator, and in this respect NaPRT represents a paradigm for a new energy-coupling mechanism of enzyme action (177). NaPRT activity has also been evaluated in crude extracts from human lymphocytes: the apparent K_m values for Na and PRPP were 0.165 mM and 0.33 mM, respectively (178). These values, obtained in the absence of added ATP, are much higher than those reported above for the purified erythrocyte enzyme. Very recently, an interesting paper describing the isolation and expression of human NaPRT gene appeared in the literature, providing direct evidence of a tight link between NaPRT and the increase in cellular NAD levels (179). In mouse, the enzyme is abundantly expressed in the small intestine, liver, and kidney. In human kidney epithelial cells the addition of Na, but not Nam, almost doubled cellular NAD content, and decreased cytotoxicity by H_2O_2. Both effects were reversed by knocking down the NaPRT expression. Kinetic analysis revealed that NaPRT, but not nicotinamide phosphoribosyltransferase (NamPRT), is insensitive to the physiological concentration of NAD. The Authors conclude that Na increases cellular NAD level through NaPRT function and, thus, protects the cells from oxidative insult, partly because of the lack of feedback inhibition of NaPRT by NAD. The ability of Na to increase cellular NAD contents may account for some of the clinically observed effects of the vitamin, and further implies its novel application for the treatment of such diseases, *e.g.,* photodamaged skin (180), associated with cellular NAD pool depletion.

5.2. Nicotinamide

In a previous review article we have indicated as “non redox NAD-dependent reactions” those routes implying NAD cleavage with release of Nam (181). It is noteworthy that a common distinctive feature of these reactions is the inhibition exerted by Nam towards all the enzymes involved. Therefore, it is conceivable to hypothesize that the fluctuations of Nam concentration regulate the metabolic fluxes occurring in these pathways, which are known to be strictly associated to fundamental events of the cellular life. The inhibitory role played by Nam constitutes the basis of the rationale underlying its adoption in therapeutic trials to prevent cancer recurrence and insulin-dependent diabetes (182, 183). The Nam concentrations in turn fluctuate in response to the activities of the enzymes involved in NAD recycling, suggesting that its level is strictly controlled and thus Nam might be regarded as a putative “signal molecule”. However, no specific transporter (s) nor receptor (s) for Nam have been so far characterized, although their existence has been demonstrated by radioactive uptake studies in human leukemic K-562 cells (184).

5.2.1. Nicotinamide phosphoribosyltransferase

NamPRT catalyzes the transfer of the phosphoribosyl moiety of PRPP to Nam, yielding NMN and PPi (Figure 9). It is a 473-residues protein with a molecular mass of about 52 kDa (185). It belongs to the family of type II phosphoribosyltransferases, together with the functionally related NaPRT and QaPRT, the other two phosphoribosyltransferases contributing to NAD biosynthesis. Indeed, the overall fold of mammalian NamPRT, solved in complex with NMN and the inhibitor FK866, shows similarity to the other two enzymes,

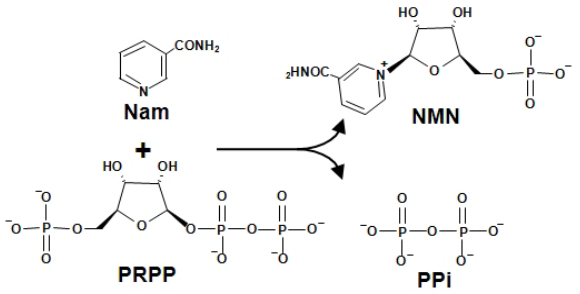

Figure 9. Nicotinamide phosphoribosyltransferase-catalyzed reaction.

although their amino acid sequences are highly divergent (186-188). Like NaPRT and QaPRT, NamPRT forms a dimer, with an extensive molecular interface where two active sites are located. Nevertheless, important conformational differences among the three phosphoribosyltransferases are crucial for determining the dimer organization, the composition of the active site defining the substrate specificity and the inhibitor sensitivity (186-188). The human enzyme has a K_m for Nam of 2 μM (187). Like NaPRT, it is able to hydrolyze ATP and activation by (auto)phosphorylation has been suggested (187, 188).

The human gene is localized on the long arm of chromosome 7 (7q22) and comprises 11 exons and 10 introns (189). The presence of two distinct promoters, together with the alternative use of two transcription initiation sites and two different polyadenylation signals results in the formation of three mRNA transcripts of 2.0, 2.4 and 4.0 kb (185, 189). The three transcripts are ubiquitously expressed, and particularly abundant in lymphocytes, bone marrow stromal cells, liver, muscle and fetal membranes (185, 189). The protein has been detected in the cell nucleus, cytoplasm and mitochondria (129, 190). It lacks a typical signal for secretion, but it can be secreted through a non-classical pathway (191, 192).

NamPRT represents the rate-limiting enzyme in the salvage pathway of NAD biosynthesis from Nam (57, 58), and is directly involved in the regulation of NAD levels in cells undergoing high NAD turnover (193). In tumour cells, for example, where NAD turnover is very elevated due to an intense ADP-ribosylating activity (194, 195), cell survival relies on upregulation of NamPRT, making it an interesting target for the development of novel anticancer agents (196). Indeed, the specific and strong inhibition of the enzyme by the molecule FK866 (K_i of 0.3 nM) is sufficient to lower NAD levels and induce apoptosis in various human carcinoma cells, as well as in various types of tumour xenografts *in vivo* (197, 198). Treatment with FK866, recently introduced in clinical trials, is effective in inducing tumour growth delay and enhancing radiation sensitivity in mammary carcinoma tumours (199). Recent studies have shown that the enzyme NamPRT regulates the transcriptional function of mammalian sirtuins, a conserved family of NAD-dependent deacetylases involved in a variety of biological processes, like stress and cytokine response, differentiation and metabolism (200). In particular, overexpression of the enzyme in mouse fibroblasts increases the total intracellular level of NAD and enhances the transcriptional regulator activity of SIRT1, a sirtuin regulating lipid and carbohydrate metabolism and mediating lifespan extension in caloric restricted animals (193). In human vascular smooth muscle cells and fibroblasts, the NamPRT-dependent activation of SIRT1 results in delayed senescence and substantial extension of cell lifespan, together with enhanced resistance to oxidative stress (201, 202). Upregulation of NamPRT has also been found to protect against programmed cell death due to PARP overexpression (203); notably such protection is mediated by the mitochondrial sirtuins SIRT3 and SIRT4 and occurs because NamPRT is able to prevent depletion of the mitochondrial NAD pool (129). Not surprisingly, NamPRT level is increased specifically in mitochondria by hypoxia, serum deprivation or fasting (129). Very recently, it has been shown that active NamPRT is naturally secreted by fully differentiated adipocytes (191). The Authors showed that its mediated NMN/NAD biosynthesis is essential for the maintenance of NAD levels in pancreatic islets and for normal glucose-stimulated insulin secretion (191). They found that NamPRT+/- female mice show decreased levels of pyridine nucleotides, impaired glucose tolerance and significantly reduced insulin secretion in response to glucose. The finding that such defects are all ameliorated by NMN supplementation, clearly indicates that NAD supply by NamPRT is critical for insulin secretion (191, 204). The effect of NAD on insulin secretion indeed could be either direct or secondary to the increased activity of SIRT1 in the glucose-stimulated insulin secretion (205, 206).

Overall, the above considered reports identify intracellular and extracellular NamPRT as the crucial enzyme in the maintenance of NAD levels. The current opinion is that such a key role might explain the plethora of intra- and extracellular biological effects exerted by the enzyme, that earned to NamPRT the name of "cytokine-like molecule" (207). Indeed, the human NamPRT gene was originally isolated as the gene coding for an unknown cytokine-like molecule, named pre-B cell colony-enhancing factor (PBEF), shown to enhance the maturation of B cell precursors in the presence of interleukin and stem cell factor (185). Only 8 years later the identity of PBEF as the enzyme NamPRT was established, based on its strong similarity with the bacterial counterpart (208). The 5'-flanking region of the NamPRT gene shares with other cytokines several hormonally and chemically responsive regulatory elements (189). The protein, secreted in response to both mechanical and inflammatory stimuli (185, 189, 192, 209-212), is able to significantly enhance expression of IL-6 and IL-8 in amniotic epithelium (213), to prolong neutrophil survival by inhibiting apoptosis (209) and to act as an adipokine (named visfatin), that exerts insulin-mimetic action (214). Notably, the latter effect has not been confirmed by a recent work, where the glucose intolerance observed in NamPRT+/-mice was demonstrated to be primarily due to impaired NMN biosynthesis (and not to the loss of the insulin-mimetic activity of NamPRT)

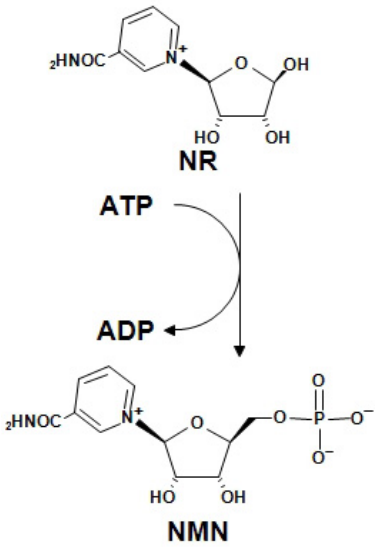

Figure 10. Nicotinamide riboside kinase-catalyzed reaction.

(191). Indeed, we believe that all the PBEF- and visfatin- related properties should be reconsidered taking into account the robust enzymatic activity associated with the secreted form of NamPRT, trying to link the observed metabolic effects with the enzyme action.

5.3. Nicotinamide riboside

Since our last review on NAD metabolism (181), different reports attest for the metabolic importance of NR as a nutritionally relevant NAD-metabolite. In particular, as extensively reviewed in (215), NR seems to play an interesting role in neuroprotection by delaying Wallerian degeneration. Furthermore, in general the use of NR in therapy to elevate NAD could be preferred to the other niacins since it does not inhibit sirtuins like Nam, and does not cause undesirable side-effects like Na. In mammalian cells, NR can be formed from NMN in the reaction catalyzed by NMN 5'-nucleotidase (EC 3.1.3.5) (118), and reutilized after its conversion to NMN via a reaction catalyzed by nicotinamide riboside kinase (EC 2.7.1.22) (Figure 10).

5.3.1. Nicotinamide riboside kinase

The enzyme has been first purified to near homogeneity from human placenta (216). The final preparation consists of a monomeric protein, whose molecular weight was calculated to be 29,000 under denaturating conditions, and 32,000 under native conditions. The pI value is 5.6, and the pH optimum is in the range of pH 6.5 to 6.9. The human enzyme exhibits broad substrate specificity, being able to phosphorylate, in addition to NR, guanosine and, more importantly, tiazofurin and 3-deazaguanosine, known antineoplastic agents. The K_m values for these substrates are 9.6 μM, 115 μM, 90 μM and 16.5 μM, respectively.

Since the last three years, owing to the increasing interest in searching for new therapies based on fostering the NAD levels, nicotinamide riboside kinases genes, *NRK1* and *NRK2*, have been identified in humans (167). The catalytic analyses of the recombinant enzymes, Nrk1 and Nrk2, show that they are capable to synthesize with a good efficiency NMN, when they utilize ATP as the phosphate donor, whereas Nrk1 exhibits a catalytic efficiency 100 times higher than Nrk2 when ATP is replaced by GTP (217). Furthermore, Nrk2 phosphorylates the known antineoplastic agent tiazofurin three times more efficiently than Nrk1. The isozyme 1 3D structure has been solved and the crystal structure of the binary complex with NMN and the ternary complex with ADP and tiazofurin is in keeping with the observation that Nrk is active towards other phosphor-acceptors like those above reported (216-218). In addition, co-crystal structures with substrates suggested that the enzyme also could phosphorylate nicotinic acid riboside (NaR). Indeed, it has been demonstrated that the two human variants possess the capability to phosphorylate NaR and NR (217). Further studies are needed in order to establish the nutritional role as a "niacin" of this metabolite.

6. CONCLUDING REMARKS

The present review is the result of a growing interest in the involvement of NAD metabolism in mammalian physiopathological phenomena. Indeed, in recent years several comprehensive monographs on the topic have been published, summarizing the most advanced results on the enzymology of NAD synthesis and utilization, in different organisms, ranging from yeast to man. The most recent data highlighted the discovery of novel pathways to NAD synthesis, and presented the functional and structural characterization of the enzymes involved. The crucial role of NAD in metabolism and signaling appears to fuel two main lines of research on its enzymology, focusing on either microbial pathogens or humans, aiming at the discovery of new druggable enzyme targets, involved in health maintenance, as well as in a variety of different pathologies, spanning from infectious disease, to diabetes, inflammatory syndromes, neurodegeneration, and cancer. We are convinced that, in the near future, new exciting discoveries will arise from the ongoing studies on pyridine nucleotide metabolism, that also will hopefully reconcile the above described conflicting findings into a more clear overall picture. This will be of help in the design of strategies for the treatment of apparently unrelated syndromes sharing pellagra-like symptoms.

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