[Frontiers in Bioscience 14, 410-431, January 1, 2009]

The NMN/NaMN adenylyltransferase (NMNAT) protein family

Corinna Lau, Marc Niere, Mathias Ziegler

Department of Molecular Biology, University of Bergen, Thormøhlensgate 55, N-5008 Bergen, Norway

TABLE OF CONTENTS

1. ABSTRACT

NAD biosynthesis has become of considerable interest owing to the important signaling functions of the pyridine nucleotides which have been recognized over the past years. The formation of the dinucleotides from ATP and the mononucleotide of niacin (either nicotinamide or nicotinic acid) constitute the critical step in NAD generation which is catalyzed by NMN/NaMN adenylyltransferases, NMNATs. Recent research has established the molecular, catalytic and structural properties of NMNATs from many organisms. Detailed studies, particularly of the human NMNATs, have revealed distinct isoform-specific characteristics relating to enzyme kinetics and substrate specificity, oligomeric assembly as well as subcellular and tissue distribution. Moreover, direct functional relationships between NMNATs and major NAD-mediated signaling processes have been discovered suggesting that at least some of these proteins might play more than just an enzymatic role. Several investigations have also pointed to a critical role of NMNATs in pathological states such as cancer and neurodegeneration. This article intends to provide a comprehensive overview of the family of NMNATs and highlights some of the recently identified functional roles of these enzymes.

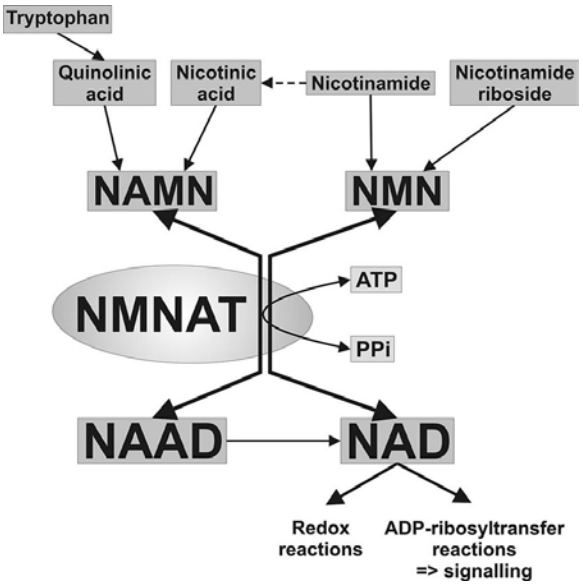

Figure 1. The central role of NMNAT in NAD biosynthesis. NMNAT catalyzes the synthesis of both NAD and its deamidated form, NAAD, from the respective mononucleotides and ATP. The precursors of the mononucleotides originate from dietary sources or NAD degradation. (NaMN, nicotinic acid mononucleotide).

2. INTRODUCTION

The key step in the biosynthesis of pyridine nucleotides (Figure 1) is the formation of a dinucleotide catalyzed by NMN/NaMN adenylyltransferases (NMNATs, also referred to as pyridine mononucleotide adenylyltransferases, PNATs; (1)). The reaction includes the transfer of the adenylyl moiety from ATP onto NMN or NaMN to give rise to NAD or NaAD under release of pyrophosphate. While the formation of the mononucleotides of nicotinamide (NMN) or nicotinic acid (NaMN) may occur by several independent routes, NMNAT activity is the only one known to form the respective dinucleotides from these precursors and is therefore essential (see Figure 1). Consequently, NMNATs have significant impact on the cellular NAD homeostasis. The reaction catalyzed by NMNATs is reversible, and the enzyme was originally discovered through its NAD pyrophosphorylase activity (2, 3). The equilibrium of the reaction actually indicates a preference for the reverse reaction. Nevertheless, NAD synthesis is considered the physiological activity of NMNAT and likely to be regulated, among others, by the presence of substrates. Although its activity has been known for decades, only over the past decade has structural information become available. Clearly, broad interest in the biosynthesis of NAD has developed over the past few years due to the emerging multitude of signaling reactions which, require pyridine nucleotides as substrates (4). Although NMNAT activity is well conserved among all species, the corresponding enzymes and isoforms exhibit a broad range of catalytic and structural properties. This may not only reflect species- or cell-specific needs, but also indicate that some isoforms may have acquired additional functions.

Pioneering work regarding the structure and catalytic properties of bacterial, yeast and human NMNATs has been conducted in Magni's group (5, 6, 7). More recently, a number of laboratories have made major contributions to the field by genetic, enzymatic, structural and functional analyses of these enzymes from various organisms. The growing interest has led to impressive progress over the last few years. This review attempts to summarize the current state of knowledge about this important enzyme family.

3. STRUCTURE, PHYSICOCHEMICAL AND CATALYTIC PROPERTIES OF NMNATs

3.1 Overview

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), nicotinic acid/nicotinamide mononucleotide adenylyltransferases, NMNATs, are classified as nucleotidyltransferases (EC 2.7.7). The family of NMNATs, EC 2.7.7.1, was originally established in 1961 as a result of the work by Atkinson *et al.* (8). NaMNATs were recognized and designated as EC 2.7.7.18 in 1964, after Imsande *et al.* had described NAD biosynthesis in *Escherichia coli* (9).

NMNATs are composed of relatively small globular proteins consisting of about 160 to 400 amino acids, amounting to subunit a molecular mass ranging between 20 and 50 kDa (Table 1). Most identified NMNATs assemble into homo-oligomers of 2-6 identical polypeptide chains. While prokaryotic NMNAT activity is mostly preserved by active site conservation, the eukaryotic homologs exhibit 30 to 50 % protein sequence identity in pairwise alignments. In several eukaryotic organisms, distinct isoforms have been identified which display up to 98 % identity among their respective orthologs. Most NMNATs have theoretical isoelectric points (pI) between pH 5 and 6.5. Exceptions can be found among archaeal and mammalian NMNATs.

Apart from the thermophilic archaeons, adenylyltransfer onto N(a)MN preferentially occurs at optimum temperature of 30 to 37 °C. The reaction seems to be relatively unaffected by the surrounding pH. NMNATs commonly display a pH optimum within the range of pH 6 to 9. Nevertheless, isozyme specific differences were found in yeast, for example. All NMNAT activities depend on the presence of bivalent metal ions at low millimolar concentrations. As for other nucleotide binding enzymes, magnesium appears to be the preferred metal ion. However, some specific preferences have been discovered with isolated enzymes.

An important consideration relates to the species-specific preference regarding the precursor of NAD^+ synthesis, namely nicotinamide and nicotinic acid. Although most NMNATs are capable of catalyzing the adenylyltransfer onto both nicotinamide mononucleotide (NMN) and nicotinic acid mononucleotide (NaMN), differential regulation or structural constraints have been suggested to account for high selectivity in some instances.

Table 1. Biochemical properties and database references of selected NMNATs

	hNMNAT1	hNMNAT2	hNMNAT3	scNMA1	scNMA2	mjNMNAT	ecNMNAT	ecNaMNAT	AtNMNAT	
NCBI Entrez Gene ID	**64802**	**23057**	349565 [1]	**851039**	**852893**	**1451406**	**948911**	**945248**	**835675**	
Ensembl: ENSG00000	**173614**	**157064**	**163864**							
Chromosome	1p36.2[90]	1q25[47,91]	3q23[116] [1]; 8	XII[34]	VII[35]				5[117]	
Locus tag	RP11-807G9.1-001	RP11-181K3.3-004	n.a.	YLR328W	YGR010W	MJ0541	b4390	b0639	AT5G55810	
Gene name			FKSG76			nadM	nadR	nadD	AT5G55810.1	
mRNA levels in tissue	**high**	Skeletal muscle, heart, liver, kidney[44]	Brain[62, 47]	Lung, spleen[1]						Vegetative tissue[40]
	low	Brain[90] cancer cells[44]	Heart, muscle[62]	Placenta, kidney, cancer cells[1]						
NCBI Protein Ref. Seq.	NP_073624	NP_055854 [3]	AAK52726 [2]	NP_013432	NP_011524	NP_247520	NP_418807	NP_415172	NP_200392	
UniProtKB accession number	Q9HAN9	Q9BZQ4-1 [3]	Q96T66-1	Q06178	P53204	Q57961	P27278	P0A752	(Q8VZB8 [4])	
Molecular mass [5]	**Theoretical (Dalton)**	31 932	34 439	28 322	45 859	44 909	19 610	47 346	24 528	26 923
	Apparent (kilo Dalton)	33[46]			48[34]	46[35]	19.6[29] 21.5[24]		24.5[10]	
Tertiary structure	Hexamer [41-43] tetramer[44]	Monomer [47]	Tetramer [1]	Tetramer [34]	Tetramer [35]	Hexamer [25,24]	Tetramer [13]	Monomer [17]		
Crystal structure	[41-43]		[1]			[12]		[10,17]		
Specificity	NMN[49]	NMN/ NaMN[49]	NMN/ NaMN[11]	NMN[6]	NaMN[35]	NMN[25]	NMN[13]	NaMN[10]	NMN/ NaMN[40]	
K_M in µM	**ATP**	36-58 [44,49,71]	89[49]; 204[71]	29[71] 42[49]	104[35]	1400[35]		1.7[13]		
	NMN	22-34 [44,49,71]	21-32 [10,49,30]	66[49] 209[10]	170[35]	130[35]		700[13]		
	NaMN	68[49] 116[44]	15[49]	111[49]						
	NMNH	294[49]	304[49]	130[49]						
	NAD	59[71]	70[71]	130[71]	73[35]	23[35]				
	PPi	937[71]	1119[71]	390[71]	83[35]	5000[35]				
Alternative substrates	dATP[44] TrMP[43,49]		ITP, GTP[71,49]	dATP[35]	dATP, ITP[35]		Nicotin-amide riboside[20] [6]			
Product inhibition [49]	**Competitive**	NAD, NaAD vs. ATP	NAD, NaAD vs. ATP	NAD, NaAD vs. NMN						
	Non-competitive	PPi vs. NMN and ATP	None	None						
NAD synthesis	**Inhibitors** [7]	Nap4AD[49] NADP, gallo-tannin[71] heavy metals[45]	Np4AD[49] ADP, AMP, NADP, gallo-tannin[71]	Nap4AD[49] N-2'-MeAD, Na-2'-MeAD[80] gallo-tannin[71]	Hg^{2+} [34]					
	Activators		EGCG[71]							
Intracellular localisation	Nucleus[45] putative NLS: PGRKRKW	Golgi apparatus[71] cytoplasm[1] nucleus[62]	Mito-chondria[71] cytoplasm[1]	Nucleus (putatively) [35]	Nucleus[6]					
Tissues of confirmed protein expression	Placenta[44, 46]	Endocrine pancreas[62]							e.g. Roots, cotyledon, gameto-phyte[40]	
Protein sequence identity [8] **%**	**hNMNAT1**	**hNMNAT2**	**hNMNAT3**	**scNMA1**	**scNMA2**	**mjNMNAT**	**ecNMNAT**	**ecNaMNAT**	**atNMNAT**	
	hNMNAT2	36; 37[1]								
	hNMNAT3	49; 50[1]	39; 34[1]							
	scNMA1	35; 40[44]	27	35						
	scNMA2	37; 42[35]	28; 40[35]	33	77; 72[35]					
	mjNMNAT	11	3	21	14	13				
	ecNMNAT	4	4	3	5	4	13			
	ecNaMNAT	17	12	23	12	12	21	11		
	atNMNAT	39	39	38	39	39	8	4	19	

[1] This annotated nmnat3 gene is localized to chromosome 3. The 1886 bp transcript (NCBI RefSeq NM_178177) has not been reported to be specifically detected or cloned. The encoded protein (RefSeq NP_835471; UniProtKB accession number Q96T66-2) lacks the N-terminal 37 aa, including the essential active site GxFxPx[H/T]xxH motif, which is essential for NMNAT activity (see text). According to ENSEMBL (ENSG00000163864), the gene product might exist in splice variants. [2] The corresponding NCBI Nucleotide RefSeq is AF345564. The coding sequence of the 1223 bp transcript has been cloned, and the gene product exhibits NMNAT specific activity (1, 49, 71). In AF345564, $_{502}$A G T_{507} (Ser169) corresponds to $_{369}$G G T_{371} (Gly123) in NM_178177. A polymorphism in AF345564 has been noticed with regard to $_{505}$A G T_{507} (Ser169). The obtained coding sequence from human heart cDNA exhibits $_{505}$G G T_{507} instead, encoding for a glycine at position 169 (not published). [3] This annotation refers to hNMNAT2 transcript variant 1. A second, uncharacterized variant is annotated as NCBI Protein RefSeq NP_733820 and UniProtKB acc. no. Q9BZQ4-2. [4] Corresponds to the expired NCBI Protein RefSeq entry NP_200392.2. This was replaced by NP200392.3, which possesses 80 additional N-terminal amino acids and, thereby, the essential active site GxFxPx[H/T]xxH motif. [5] The numbers refer to the native proteins. Calculation of theoretical molecular mass was done by ExPASy Server tool Compute pI/Mw applying the average resolution mode. [6] NadR gene products are multifunctional enzymes which catalize both NMN-synthesis from NR and ATP and NAD-synthesis from NMN and ATP (see text). [7] No specific and efficient inhibitor exists for NMNAT activity. The here listed compounds alter NMNAT activity in vitro but mostly at comparably high concentrations (see text for further information). [8] According to pairwise alignment extracted from *ClustalW* V1.83 (115, http://www.ebi.ac.uk/clustalw/), unless according to cited reference. [(...)] Reference; n.a. not annotated

For some eubacteria, homologs being exclusively specific for either NMN or NaMN have been described. Similarly, pyrophosphorylysis has been shown to be generally non-selective for NAD or NaAD. In the following chapters, we will focus on well-characterized NMNATs, primarily on the three human isoforms1, the NMNATs from the archaeon *Methanocaldococcus jannaschii*2, the eubacterium *Escherichia coli*3, *Saccharomyces cerevisiae*4, and *Arabidopsis thaliana*5 (see Table 1).

3.2. Physicochemical properties of NMNATs

3.2.1. Bacterial NMNATs - NadD, NadR, and NadM

The first bacterial NMNAT was discovered in *Escherichia coli* and exhibited NaMN-specific activity (9). Since then, several bacterial NMNAT activities have been identified and investigated. The literature distinguishes between eubacterial NadD, eubacterial NadR and archaeal NadM activities.

The NadD enzymes which have been enzymatically characterized so far preferentially utilize NaMN as substrate (10, 11). *Escherichia coli* NadD, NadD_ECOLI, is a monomer of 25 kDa. It catalyzes the adenylyltransfer onto NaMN 20 times faster than onto NMN (12, 10).

Escherichia coli NadR (NadR_ECOLI) assembles into a tetramer of 45 kDa subunits and is considered highly specific for NMN, although the K_M of 700 µM NMN greatly exceeds the value reported for other NMNATs. The NMNAT activity of NadR_ECOLI has a distinct alkaline pH optimum at pH 8.6. Compared to magnesium, nickel and cobalt ions serve as more efficient cofactors *in vitro* (13, and references therein). NadR proteins were shown to possess multiple functions. In addition to NMNAT activity, they exhibit nicotinamide riboside kinase (NRK) activity (13, 14). NRKs catalyze the phosphorylation of nicotinamide riboside (NR) to synthesize NMN and, thereby, enable NAD synthesis via a Preiss-Handler independent route (15). NRK activity is facilitated by a Walker type ATP binding motif at the C-terminus of NadR enzymes (14) which is present in addition to the NMNAT-specific N-terminal ATP recognition and binding motif (13). The C-terminus of NadR proteins has also been associated with the regulation of NMN transport across the plasma membrane. NadRs act in concert with the transmembrane protein PnuC in response to changes in intracellular pyridine nucleotide levels (16-18). Therefore, NadR enzymes synthesize NMN and NAD and participate in pyridine nucleotide import. Moreover, they may exert NAD-dependent transcriptional repressor activity on NAD biosynthetic genes. This is mediated by an N-terminal helix-turn-helix motif for DNA binding (19-22). The proposed model suggests NadRs to function as repressor at high NAD levels, while it facilitates NAD synthesis at lower levels. However, *Haemophilus influenzae* NadR, NadR_HAEIN, does not exhibit DNA binding activity, which is presumably due to the lack of any other gene implicated in NAD biosynthesis or the salvage pathway (23).

NadM activities are designated archaeal NMNATs and tend to prefer NMN as substrate. The enzymes from *Methanocaldococcus jannaschii* (NadM_METJA) (24, 25), *Methanobacterium thermoautotrophicus* (NadM_METTH) (26, 27) and *Sulfolobus solfataricus* (NadM_SULSO) (28, 24) have been identified and characterized. Both NadM_METJA and NadM_METTH crystallize as hexameric proteins in presence of their substrates (25, 26). According to gel filtration experiments, NadM_METJA exhibits a native molecular mass of 72 kDa, which is, however, not consistent with hexamer formation (24). The NMNAT activity of this thermophilic archaeon follows non-linear kinetics and shows negative cooperativity with regard to both substrates, NMN and ATP, when measured at 60 °C. Nickel and cobalt ions are the most efficient cofactors (24, 29). Structural analyses indicate that NadM_METJA is not selective for NMN or NaMN (25).

The slr0787 protein of the cyanobacterium *Synechocystis sp.* was annotated as NadM_SYNY3 based on the sequence similarity to the archaeal nadM gene products and its NMN-specific NMNAT activity (30). Additionally, NadM_SYNY3 harbors a highly conserved sequence that classifies it as NUDIX hydrolase. Genes encoding NUDIX (**nu**cleoside **di**phosphate linked to another moiety, **X**) hydrolases are present in nearly every organism and exist in up to 30 copies depending on the genome size. NUDIX hydrolases catalyze the cleavage of a variety of organic pyrophosphates with different substrate specificities. Thereby, they are assumed to either fulfill a 'housecleaning' function or play a role in metabolic reprogramming (31). The NUDIX hydrolase activity of NadM_SYNY3 appears to be specific for ADP-ribose and 2'-phospho-ADP-ribose (30)

In summary, bacterial NMNATs are the most diverse group of NMNAT. Their primary structures are not highly conserved both among them as well as compared to their eukaryotic counterparts. However, they show high similarity concerning the ATP binding site and the quaternary structure (see Figure 2).

3.2.2. Yeast NMNATs - scNMA1 and scNMA2

In 1950, Arthur Kornberg for the first time characterized an NMNAT activity in yeast autolysates (32). In 1986, Natalini *et al.* were the first to purify an NMNAT activity from *Saccharomyces cerevisiae* which exhibited a native molecular mass of approximately 200 kDa (33). Eventually, two distinct isoforms with 72% sequence identity were identified and characterized, scNMA1 and scNMA2 (6, 34, 35). According to gel-filtration chromatography, both proteins are likely to be homo-tetramers based on the subunit molecular masses of approximately 48 kDa and 46 kDa, respectively (Table 1). While scNMA1 is considered to catalyze NAD and NaAD synthesis with similar efficiency, scNMA2 appears to prefer NaMN. Besides, scNMA2 exhibits unusually high K_M values for ATP and PP_i, which amount to 13 and 60 times the values for scNMA1, respectively. Therefore, scNMA2 activity has been suggested to be important at

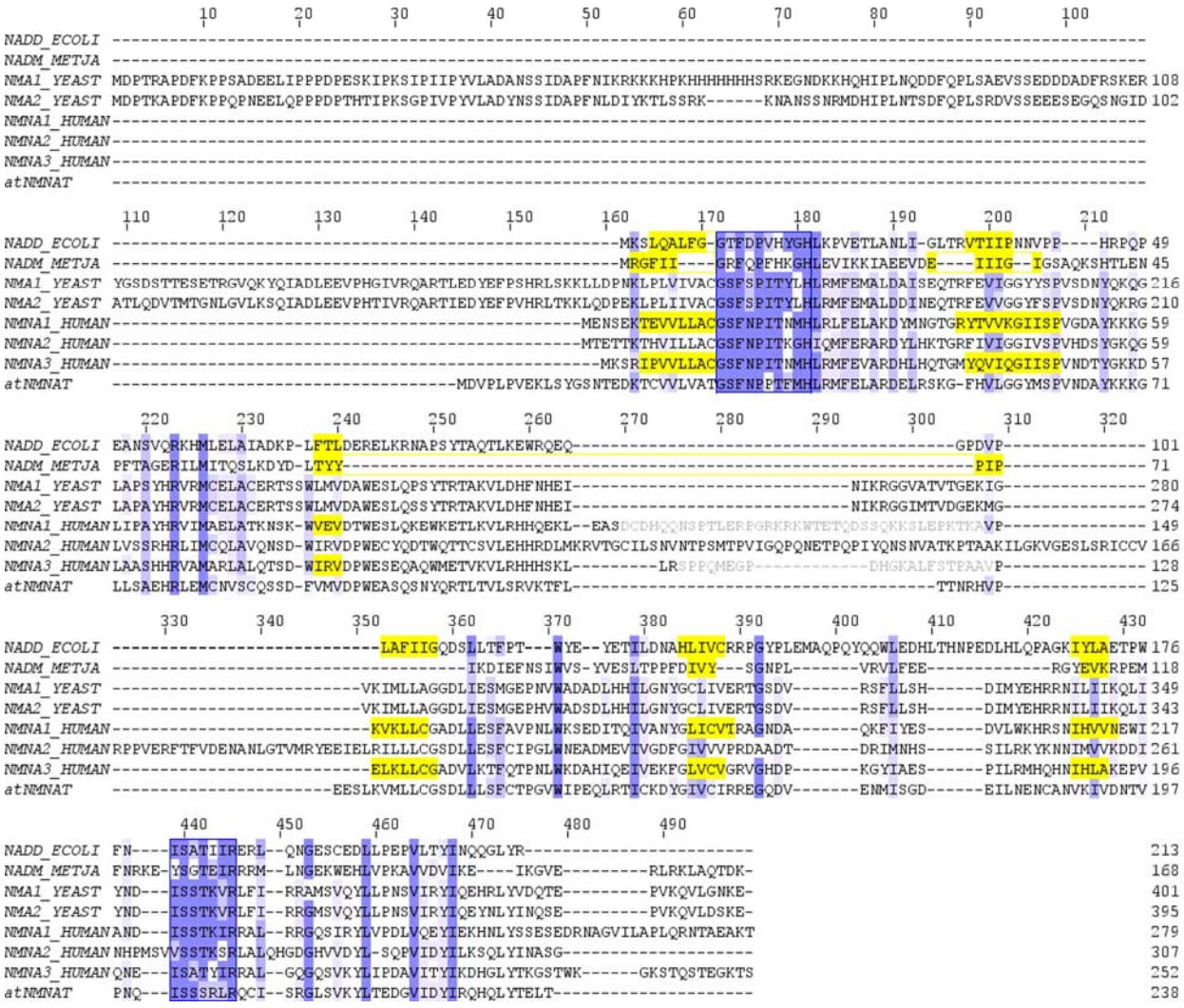

Figure 2. Multiple sequence alignment generated by MUSCLE (118) and subsequent editing using Jalview (119). The intensity of blue color increases according to the significance of the BLOSUM62 matrix score. Boxes are independently scored. Residues of the structurally resolved β-sheets of the conserved dinucleotide binding motif (UniProt ID - PDB ID: NADD_ECOLI - 1k4k, NADM_METJA - 1f9a, hNMNAT1 - 1kku, hNMNAT3 - 1nur) are highlighted in yellow. Gray residues are missing in the respective PDB files.

increased metabolic flux when scNMA1 would already be saturated (35).

3.2.3. Plant NMNAT

NAD(H) and NADP(H) pools and redox shuttles have been intensely studied in plants (reviewed in 36-38), while little is known about the enzymes involved in maintaining NAD(H) homeostasis. In *Arabidopsis thaliana*, a nicotinamidase activity (39) might be required to compensate for the apparent lack of an NMN synthesizing nicotinamide phosphoribosyltransferase (NamPRT) (36). As in bacteria and yeast, plant NAD synthesis apparently occurs via deamidation of nicotinamide, NaMN synthesis, adenylyltransfer onto NaMN, and finally amidation of NaAD to NAD (36, see also Figure 1). The first plant NMNAT was cloned from *A. thaliana* (Table 1) and investigated in more detail by Hashida *et al.* (40). The cDNA encodes a protein of 27 kDa. AtNMNAT produces both NAD and NaAD at similar efficiencies. Plants depleted in the atNMNAT encoding gene could not be bred at all. Moreover, atNMNAT$^{-/+}$ heterozygous plants displayed impairments in gametophysis. Therefore, the enzyme seems to be essential in NAD synthesis during plant growth (40). This also points to the existence of a single plant NMNAT gene, as has already been suggested (36).

3.2.4. Vertebrate NMNATs – human NMNAT1, 2, and 3

Among the NMNATs of higher eukaryotes, the human enzymes have been most comprehensively studied. Of the three isoforms known in humans, hNMNAT1 appears to be the predominant one with regard to both cellular activity and tissue distribution. Crystallization of the recombinant hNMNAT1 by three independent groups

revealed the enzyme to be a homo-hexameric protein (41-43) composed of 32 kDa subunits (44, 45). In contrast, the activity purified from human placenta by Emanuelli *et al.* exhibited a native molecular mass of only about four times the mass of a subunit (46). Thus, the protein might exist in dynamic oligomerization states (43, 44).

Soon after the molecular characterization of hNMNAT1, two additional human isoforms were identified, hNMNAT2 and hNMNAT3 (1, 47). Human NMNAT2 is a 34 kDa protein and supposed to exist as monomer, similar to NadD_ECOLI (47). However, the sequence identity between both enzymes is comparably low (Table 1). Human NMNAT2 exhibits 98 % sequence identity to putative homologs in mouse and rat[6].

The third isoform, human NMNAT3, was found to assemble into a homo-tetramer of 29 kDa subunits (1). Similar to hNMNAT1 but in contrast to hNMNAT2, isoform 3 is a positively charged protein with a theoretical isoelectric point at a basic pH (1). Compared to the average amino acid composition of all entries at the UniProtKB/Swiss-Prot protein knowledgebase (48, http://www.uniprot.org), human NMNATs display a relatively high content of lysine, tryptophan and histidine residues. Moreover, hNMNAT2 harbors nine cysteine residues, which is almost twice the average.

The hNMNATs differ in their dependency on bivalent metal ions. While hNMNAT1 requires at least 10 mM magnesium to attain maximum activity, hNMNAT2 is already fully active in presence of 0.3 mM (47, 49). Similar to the yeast enzymes, hNMNATs accept also zinc, copper and nickel for NAD synthesis *in vitro*, however, to different extents (35, 34, 44, 49). These findings have recently been applied to explore isoform-specific, endogenous activities in human tissue and cell extracts (49).

Apart from the human enzymes, mouse NMNAT1 is of particular interest with regard to its controversial implication in the phenotype of the Wallerian Degeneration (slow) mice, C57BL/Wld^S (see paragraph *4.4.*). High throughput cDNA sequencing revealed the possible existence of up to three murine NMNAT transcripts. A putative NMNAT encoding complete transcript has also been amplified from the Zebrafish Gene Collection (ZGC, http://zgc.nci.nih.gov/)[7] and the deduced amino acid sequence suggests similarity to hNMNAT2. *In situ* hybridization studies confirmed spatially regulated gene expression during zebrafish embryogenesis (50)[8].

3.3. NMNAT protein structure and substrate binding

3.3.1. NMNATs - globular α/β-proteins

NMNATs are globular proteins and belong to the α/β class[9]. The N-terminus of NMNATs is consistently characterized by an immediate β-strand (β) followed by alternating α-helices (α) and further four to six β-strands (Figures 1, 3B and C). As shown in Figure 2, both yeast enzymes possess N-terminal extensions of approximately 150 amino acids, which are not found in NMNATs from other organisms. A cluster of seven histidine residues in scNMA1 distinguishes these otherwise nearly identical extensions and was suggested to be involved in metal ion binding (34). Except for NadRs and NadM_SYNY3, the C-termini of NMNATs contain primarily α-helices and loops. The very C-terminus (approximately 15 residues) is diverse in protein sequences. While, for example, residues 267–274 in human NMNAT1 form a structurally unique α-helix (43), the primary structures of hNMNAT2, atNMNAT and NadD_ECOLI lack this region (Figure 2).

A striking structural variability can be observed within the linker region between β3 and β4, following a conserved α-helix, αC (see Figure 2). As can be inferred from Figure 2, this linker region consists of up to 85 amino acids (hNMNAT2, Lys107 to Glu192) depending on the species. Human NMNATs exhibit the most extended insertions. Interestingly, crystallographic analyses on recombinant hNMNAT1 and hNMNAT3 could not resolve the fold of this region, which appears to be highly flexible (1, 41, 42, 43). Human NMNAT1 harbors a nuclear localization sequence (NLS) $_{123}$PGRKRKW$_{129}$ in this region (45) as well as a protein kinase C-specific phosphorylation site at position Ser136 (53). Therefore, this particularly variable region could bear regulatory functions, at least in the mammalian NMNATs, promoting isoform specific targeting, posttranslational modifications or intermolecular interactions.

3.3.2 The dinucleotide binding Rossmann fold represents the core structure of NMNATs

From the first available structures of archaeal NMNATs it became evident that NMNATs belong to the nucleotidyltransferase superfamily of dinucleotide-binding fold containing α/β-phosphodiesterases (25, 26, 54, 55). A comparison of the nucleotide-binding sites and the topological organization within the subunits of archaeal NMNATs revealed striking structural similarity to *E. coli* phosphopantetheine adenylyltransferase, PPAT. Despite differences in assembly, both, NMNATs and PPATs appear as functional hexamers of common electrostatic-field distribution (26, 25) and promote adenylyltransfer through stabilizing a transition-state intermediate (25, 55). Archaeal NMNATs exhibit a dinucleotide-binding fold consisting of a highly twisted five-stranded parallel β-sheet, which is flanked by seven α-helices and connecting loops (25, 26). *E. coli* NaMNAT forms a seven-stranded β-sheet (12), whereas the crystal structures of human NMNATs reveal the presence of a six-stranded β-sheet instead (1, 41-43) (see also Figure 3C). All tertiary structures resemble the six-stranded NAD(P) dinucleotide binding motif (Figure 3A), which is characterized by two βαβαβ mononucleotide-binding motifs known as Rossmann fold (56). The folds are structurally related by a pseudo twofold rotation where β1 aligns to β4. A glycine-rich GxGxxG loop connects the C-terminus of β1 with the N-terminus of αA (Figures 3B and C). During NAD(P) binding, a structurally conserved water molecule mediates hydrogen bonding between the αA proximity of the loop and the dinucleotide pyrophosphate (57, 58). In contrast, during adenylyltransfer the pyrophosphate binds the loop at its beginning, that is, close to β1. This deviation from the standard Rossmann fold is considered a specific feature

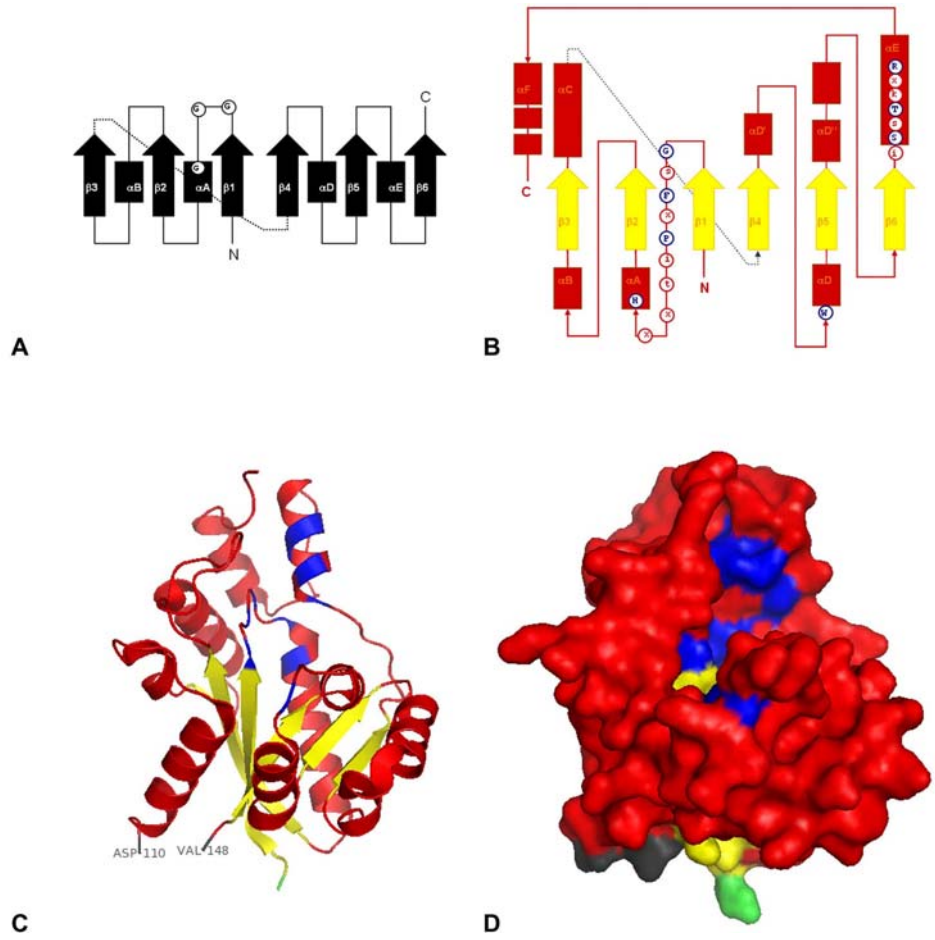

Figure 3. Human NMNAT1 active site fold and 3-D-structure. In A and B boxes represent α-helices, arrows denote β-sheets, circles highlight conserved residues implicated in catalysis. The linker between strands β3 and β4 is shown as dotted line. A, classical dinucleotide binding Rossmann fold. Circles represent the glycine-rich loop, GxGxxG, modified according to Wierenga *et al.* (57) and Bottoms *et al.* (58). B, secondary structure of the hNMNAT1 apo-enzyme. Circles represent the motifs GxFxPx[H/T]xxH and ISSTxxR as well as the pyridine ring stacking residue Trp169, as indicated. Conserved residues are highlighted in blue; modified according to the PDBsum wiring diagram [http://www.ebi.ac.uk/pdbsum] for PDB ID: 1kku (41), subunit A (http://www.pdb.org). C and D, crystal structures of hNMNAT1 (1kku, subunit A) in stereo ribbon (C) and surface (D) view produced with PyMOL (120). The N-terminus is shown in green, the central six-stranded β-sheet in yellow and the dinucleotide binding site containing conserved residues (as shown in B) in blue. Asp110 and Val148 are the residues which adjoin the unresolved loop harboring the NLS.

among all α/β-phosphodiesterase types of nucleotidyltransferases and attributed to the catalytic utilization of the dinucleotide instead of its binding as cofactor (26, 58).

3.3.3. ATP binding is highly conserved

The conserved active site motif [H/T]xGH mediates ATP recognition and binding by NMNATs (59) (Figures 2 and 3B). The glycine residue in this motif is the second within an alternative pyrophosphate binding loop (GxxxxxxxG), which is found in archaeal NMNATs (58), as well as in NadD_ECOLI and hNMNAT2. In other eukaryotic NMNATs this glycine is substituted by a non-polar residue (Figure 2). The proposed ATP binding site motif specific for NMNATs is represented by GxFxPx[H/T]xxH, based on sequence alignments of human, yeast, nematode, and bacterial NMNATs (44). In addition, mammalian, fly, yeast and plant NMNATs share a phenylalanine four residues downstream of the NMNAT motif. The substitution of Phe28 in mouse NMNAT1 by the polar amino acid serine abolished enzymatic activity (60). Interestingly, in all prokaryotic NMNAT homologs, *Caenorhabditis elegans* NMNATs and even members of other nucleotidylyltransferase families (i.e. PPAT) the corresponding residue is an aliphatic non-polar amino acid (1, 23) (Figure 2).

During adenylyltransfer, the ATP α-β-phosphate bond is highly bent within the enzyme's active site. Structural magnesium ions found in close proximity to the ATP α-phosphate presumably polarize the active site and, thereby, promote the nucleophilic attack on the α-

Table 2. Overview of all known NMNAT structures submitted to the protein database of the research collaboratory for structural bioinformatics

Organism name UniProtKB entry	**apo-enzyme**	**Co-crystallization with**					
Homo sapiens NMNA1_HUMAN	[41]	NMN[42]			NAD^+[43]	NaAD[43]	β-CH_2TAD[43]
	1KKU	1GZU[1]			1KQN	1KQO	1KR2
Homo sapiens NMNA3_HUMAN	[1]	NMN[1]	AMP-CPP[1]	NMN•AMP-CPP[1]	NAD^+[1]	NaAD[1]	
	1NUR	1NUP	1NUT	1NUS	1NUU	1NUQ	
Escherichia coli NadD_ECOLI	[12]					NaAD[12]	
	1K4K					1K4M	
Bacillus subtilis NadD_BACSU	[11]					NaAD[11]	
	1KAM					1KAQ	
Staphylococcus aureus NadD[2]						NaAD[66]	NaAD, Ca^{2+}[66]
						2H29	2H2a
Pseudomonas aeruginosa NadD_PSEAE	[65]	NaMN[65]	ATP[65]				
	1YUL	1YUM	1YUN				
Haemophilus influenzae NadR_HAEIN					NAD^+[23]		
					1LW7		
Methanocaldococcus jannaschii NadM_METJA			ATP[25]				
			1F9A				
Methanobacterium thermoautotrophicum NadM_METTH		NMN, SO_4^{2-} [26]			NAD^+, SO_4^{2-} [26]		
		1HYB			1EJ2		
Methanobacterium thermoautotrophicum NadM_METTH mutants					NAD^+[27]		
His19Ala					1M8F		
Arg11Ala					1M8G		
Arg11Lys					1M8J		
Arg136Ala					1M8K		

phosphorous (25). The role of magnesium ions in catalysis rather than substrate binding was also supported by the finding that enzyme-substrate complexes could also be co-crystallized in their absence (1).

Within the active site, the pyrophosphate is surrounded by the histidines of the GxFxPx[H/T]xxH motif and other positively charged residues to facilitate nucleotidylation of the second substrate (61). In mjNMNAT, His19 interacts with the ATP α-phosphate and its orientation is fixed by hydrogen bonds to the ring of Pro14 (25). In human isoforms, this histidine residue corresponds to His24 in hNMNAT1 and hNMNAT2 or His22 in hNMNAT3 and binds the β-phosphate instead (1) (see Figure 2). Its key role in catalysis is indicated by the finding that replacement by the non-polar amino acid alanine completely abolished enzymatic activity in both archaeal and mammalian NMNATs (26, 62).

The conserved ISSTxxR motif found at the C-terminus of all NMNATs is suspected to facilitate substrate binding rather than actual catalysis (27). It mediates interactions with the γ-phosphate of ATP and, possibly, the leaving PP_i through the positive charge of the arginine residue (1, 25, 26, 63).

3.3.4. Structural water molecules facilitate NMN binding and dual substrate specificity

Pyridine mononucleotide binding is facilitated by a number of conserved amino acids. Although recent kinetic studies suggest defined, but different, substrate binding orders for hNMNAT1 and hNMNAT3 (49, see paragraph *3.5.2.*), both proteins have been co-crystallized with NMN or NAD^+ (or NaAD) only (see Table 2), which revealed similar interactions (1, 42).

The pyridine ring of nicotinamide stacks against Trp169 in hNMNAT1 and interacts with Trp92 in a face-to-edge fashion (42). Trp169 is conserved among all NMNATs and is crucial for NMNAT activity, since its substitution by alanine causes complete inactivation (53, 64). Trp92 is conserved among the human NMNATs and replaced by a tyrosine in other homologs (see Figure 2). Both Glu94 and Trp92 interact with the ribose oxygens. The ribose phosphate interacts with Ser16 and Lys57 (42, 43). A similar arrangement has been observed for hNMNAT3. Here, the phosphate is additionally bound to Phe14 through hydrogen bonds mediated by a structural water molecule (1). Structural water molecules (ω) play a key role in the binding specificity for NMN or its deamidated form (NaMN) or their corresponding dinucleotides, NAD^+ or NaAD. In hNMNAT1, ω1 mediates hydrogen bonds between Leu96 and the carboxamide oxygen, which additionally interacts with Thr95 (43). According to the alignment in Figure 2, Thr95 appears to be conserved, while Leu96 is not. Leu96 is even replaced by a cysteine residue in hNMNAT2. A crucial role for ω1 was further suggested by its presence in the hNMNAT3-NMN complex (1). Another water molecule, ω2, stabilizes the carboxamide nitrogen within the active site. In hNMNAT1, it mediates interactions with Lys170 and Asp173, which also holds a third water molecule (ω3) in place. The negative charge of Asp173 and the presence of ω1-4 are considered pivotal to accommodate substrates

of different electrostatic properties by allowing changes within the electrostatic distribution rather than conformational changes (43). Interestingly, while this aspartate is found in human NMNAT1 and 2, as well as scNMA1 and 2, hNMNAT3 contains a positively charged histidine instead (see Figure 2). Nevertheless, Zhang *et al.* observed that His152 in hNMNAT3 communicates in the same way as Asp173 in hNMNAT1 (except for the hydrogen bond to ω3), thereby facilitating the dual specificity (1). An exchange of hNMNAT1 Asp173 to histidine affects neither the K_M value for NMN nor its NAD^+ synthesis activity [Lau *et al.* unpublished observations]. Further implications of this substitution on binding specificity towards pyridine nucleotide substrates have not been reported so far. On the other hand, hNMNAT1 specifically enables dual specificity with the help of a fourth water molecule, ω4. In complex with NAD^+, ω4 is held in place by Glu94 and Asn273. Upon NaAD binding, ω4 is displaced further into the active site and forms hydrogen bonds to Leu168 and Asn273 instead. However, Asn273 is part of a C-terminal α-helix, which is unique for hNMNAT1 (43). Certain mobility within the active site during catalysis is also reflected by the existence of relaxed and bent substrate conformations (1) or variable hydrogen bonds to the substrate when bound to different subunits (42).

Prokaryotic NMNATs are less relaxed with regard to pyridine nucleotide selectivity due to major structural differences. Zhang *et al.* compared the rather distinct N(a)AD binding mechanisms of the NaAD-specific ecNaMNAT and the NAD-specific archaeal NMNATs in detail (12). Essentially, ecNaMNAT forms an anion binding pocket which stabilizes the positive dipole of the carboxyl carbon of the deamidated substrate. The specificity of NMNATs from archaeons is mediated by the amide portion, which is held in place by an isoleucine and a non-charged, polar residue downstream of the third β-strand (26).

3.3.5. Homo-oligomeric assembly of NMNATs

The oligomeric assembly of NMNATs has initially been assessed by gel filtration chromatography. In the meantime, nine crystal structures have been generated and analyzed including seven bacterial and two human enzymes (1, 11, 12, 23, 25, 41-43, 65, 66) (see Table 2). Except NadD_ECOLI and, perhaps, human NMNAT2, which might be monomeric (47, 12), all identified NMNATs represent functional homo-oligomers composed of dimers, tetramers or hexamers (Table 1). So far, only eubacterial NaMNATs from *Pseudomonas aeruginosa* (pa), *Staphylococcus aureus* (sa) and *Bacillus subtilis* (bs) have been found to crystallize as dimers, mostly upon purine nucleotide binding (11, 65, 66). Interestingly, the interface stabilizing the dimer in these structures was also found in those NMNATs which are consisting of four and six subunits (for visualization see Figure 4). Dimerization occurs in a pseudo-twofold symmetry which is centered at an anti-parallel β-sheet interaction of the two monomers (11) located just upstream of the ATP binding ISSTxxR motif (see paragraph *3.3.3.* and Figure 2). Most prominent are the interactions which are mediated by Pro150 (bsNaMNAT) and a hydrohobic residue at position 152, which seems to be conserved among eubacteria (11). The equivalent residues in NadD_ECOLI are Pro175 and Phe177. As can be seen in Figure 2, in vertebrates and plants, this pattern is absent, apart from the common proximate Gln178 (NadD_ECOLI). In hNMNAT1, an additional interaction takes place between residues Phe217 and Ile221 of both adjacent subunits, thereby supporting a strong hydrophobic interaction (41). Interestingly, a single nucleotide polymorphism within the gene encoding hNMNAT1 gives rise to either phenylalanine or leucine at position 217, but does not notably change subunit interactions (42). Rather conserved among all NMNATs appear the intra-dimer interaction (dimer interface) provided by a loop C-terminally to β5 of both subunits as well as interactions between the first α-helix of one subunit and the loop between αE and αF of the other (e.g., hNMNAT1, residues 26-33 and 234-238 in subunit A and E, respectively) (1, 11, 41, 42). Importantly, the interface between monomers A and E in the hexameric hNMNAT1 is slightly shifted for instance with respect to A and C in the tetrameric hNMNAT3, which is schematically displayed in Figure 4 (compare Figure 4B to C).

In hNMNAT3, the four subunits are overall related by D_2 symmetry (1), a dihedral symmetry in which a twofold rotational axis is intersected by a twofold axis at right angle (Figure 4B). The oligomeric assembly is promoted by two largely polar interfaces (1), the dimer interface, and a unique dimer-dimer interface. The latter comprises interactions across the vertical twofold axis (Figure 4B). Besides hNMNAT3, the yeast homologs are also suspected to be tetrameric (35). Among the prokaryotic enzymes, paNMNAT forms a tetramer in complex with NaMN. However, it adopts other oligomeric states in presence of different substrates (65). In contrast, bsNMNAT seems to be tetrameric as apo-enzyme and forms a dimer in complex with NaAD (11).

Hexameric NMNATs have been crystallized with asymmetric units composed of one, three or six monomers, depending on the type of crystal. Based on the similarity to the dimer interface in di- and tetrameric NMNATs the complexes were referred to as trimer-of-dimers, of which the structural symmetry can be characterized by a twofold and a threefold axis (25, 41, 42). As illustrated in Figure 4C, the twofold axis relates monomers of the upper part of the trimer to those of the lower. In cäse of hNMNAT1, it is maintained by hydrophobic and electrostatic interactions (41-43). The dimer interface in hNMNAT1 connects for example subunit A of the upper trimer with E of the lower. Additionally, subunit A is associated with subunit D of the lower trimer through hydrogen bonds between Trp198 (A) and Trp216 (D). The same interactions take place between the corresponding subunits of the upper and lower trimer (see Figure 4C). The interactions across the twofold axis in hNMNAT1 differ considerably from those found in the hexameric NMNATs from archaea (42). This is due to the additional α-helices D' and D'' (see Figure 3B), which force the trimers to rotate with respect to each other, as well as to the lack of β6 in archaeons.

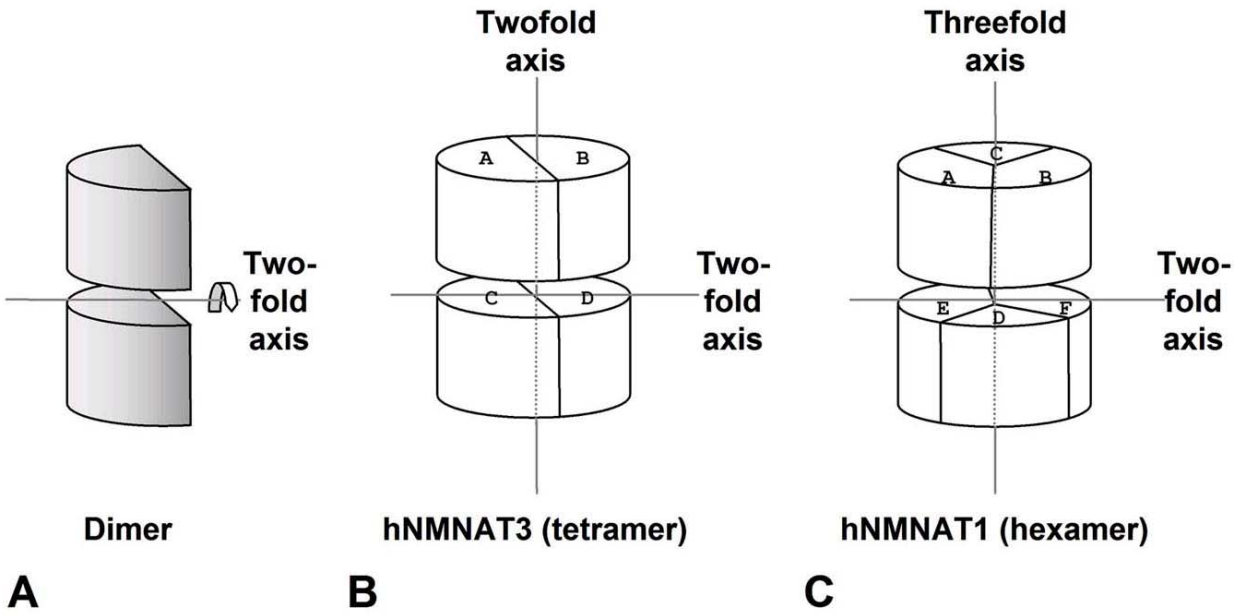

Figure 4. Rotational symmetry in NMNAT quaternary structures. A, analyses of NMNAT oligomeric structures imply that the minimum biological unit is a dimer (see *3.3.5.*). The subunits interact through the dimer interface. B, some bacterial and presumably the yeast enzymes assemble into tetramers. Human NMNAT3, as an example, forms a dimer-of-dimers. C, NMNAT hexamers form trimer-of-dimers. In contrast to archaeal NMNATs, the upper trimer of hNMNAT1 is rotated with respect to the lower one.

Subunits that are packed around the threefold axis of hexameric NMNATs (subunits A, B, C of the upper or D, E, F of the lower trimer; Figure 4C) are related by an interface with fewer interactions. This may account for the formation of a loose trimer (41). Most prominent are two hydrogen bonds provided by Tyr198, which facilitate the interaction with Arg228 and Arg231 in the neighboring monomer. Upon NMN binding, Tyr198 loses contact to Arg228, which is the ISSTxxR active site arginine (42). Interestingly, Tyr198 is not conserved. Only in NadM_METJA the equivalent residue is also aromatic.
Arg228 and Arg231 are also part of a positively charged surface which is built up by the four monomers of two adjacent dimers and only appears upon hexameric assembly. Therefore, low pH or neutralizing molecules are suspected to facilitate hexamerization of hNMNAT1 (43). In fact, recombinant hNMNAT1 requires a solvent of at least 0.3 M salt concentration to fold or assemble properly. A positively charged surface can also be observed in the archaeal NMNAT hexamer (25). In contrast to hNMNAT1 but similar to bacterial PPATs, the distribution of the charge forms a pronounced solvent channel crossing the hexamer along the threefold axis. Thereby, it faces the catalytic sites and possibly guides the ATP there. However, negatively charged residues and a constriction of the channel at its center may prevent substrate diffusion from the upper trimer part to the lower, and vice versa (25). Morris and Izard reported the channel to be closed upon substrate binding by PPAT (69).

As mentioned before, human NMNAT2 and NadD_ECOLI are the only members of the NMNAT family that appear to exist as monomers. The crystal structure of the NadD_ECOLI apo-enzyme reveals three highly flexible regions, which become ordered upon substrate binding. One flexible region is found at the surface of the enzyme and resides C-terminally to β5. A proximal insertion bearing the additional β-strand of NadD_ECOLI displaces conserved residues, which seem to be involved in dimerization of other eubacterial enzymes (11). Recombinant hNMNAT2 eluted as monomer from a gel-filtration column (47). However, whether it also functions as monomer still needs to be explored. Flanking regions of the ISSTxxR motif, which mediate dimerization (see above), are slightly enlarged in hNMNAT2 (Figure 2) and might cause different surface properties. This and the low stability of the purified protein may also indicate the possibility of interactions with other cellular components *in vivo*. In fact, the enzyme's stability is greatly enhanced by zwitterionic detergents, high salt and reducing conditions (47). However, this could also be due to the specific and rather hydrophobic insertion between β3 and β4.

The mechanism of oligomer formation among NMNATs is not yet understood. It has been attributed to the electrostatic properties of the surrounding which could be affected by substrate binding (41, 42, 65) or local enzyme concentrations (23, 43), as it has also been discussed for the related PPATs (69). The effect of substrate binding may not always be important, because several enzyme-substrate complexes crystallized with the same quaternary structure as the apo-enzyme (i.e. hNMNAT3 and NadM_METTH). Nevertheless, as demonstrated above, the ISSTxxR ATP binding motif is found just downstream of sites that facilitate interactions across the threefold axis in hNMNAT1. The dihedral

symmetry of tetrameric and hexameric NMNATs could possibly allow for allosteric control. However, only for NadR allosteric regulation with respect to NAD binding has been reported (16, 18).

Even though NMNATs assemble with similar interfaces, the observed differences in electrostatic distribution and catalytic properties may imply that functional diversity is not a result of oligomer formation. Variations in the quaternary structures within protein families can evolve, for example, by sequence drifting (70). In NMNATs, the irregular length of the loop between β3 and β4 (Figure 1 and paragraph *3.3.1.*) might contribute to this and influence affinities or specificities between subunits and, thereby, affect the preferred oligomeric state, as suggested by Zhou *et al.* (43).

3.4. Catalytic properties and substrate specificities of the human NMNATs

The presence of three NMNAT isoforms in humans raised the question about their individual physiological roles. NMNAT holds a central position in NAD metabolism (Figure 1). As discussed in later sections, major reasons for multiple isoforms may be to execute specific functions in different subcellular compartments and to regulate NAD turnover rates in individual tissues. However, the existence of several NMNAT activities might also reflect a mechanism to fine-tune NAD synthesis by isoforms with different catalytic properties. Using proteins isolated from tissue or expressed in prokaryotes from recombinant DNA, human NMNAT isoforms have been subjected to in-depth biochemical analyses to determine their kinetic properties and substrate specificities (summarized in Table 1).

Synthesis of NAD^+ by NMNAT requires binding of two substrates, NMN and ATP. The K_M values for both substrates do not exceed 200 μM for any of the hNMNAT isoforms. Human NMNAT1 is the most efficient isoform regarding both the forward and the reverse reaction (71). The affinities to ATP and NMN are lowest for hNMNAT2 and hNMNAT3, respectively (Table 1). The calculated equilibrium constant, K_{eq}, (K_{eq} = ([NAD^+] [PP_i]) / ([NMN] [ATP]), is about 0.3, indicating a preference for pyrophosphorylysis over adenylyltransfer (32, 71). In addition, pyrophosphorylysis of pyridine dinucleotides other than NAD^+ catalyzed by hNMNATs also seems to be carried out more efficiently than the synthesis (71). Nevertheless, NAD synthesis is considered the predominant reaction *in vivo*, and PP_i is thought to limit the reverse reaction. The relatively low affinities of hNMNATs towards PP_i support this assumption (see Table 1) (71).

3.4.1. Pyridine nucleotide substrates

Similar to NMNATs from other sources, all human NMNAT isoforms do not exclusively exhibit NAD^+ synthesizing activity but also accept the deamidated mononucleotide NaMN to produce NaAD. Interestingly, the affinity of hNMNAT2 for NaMN exceeds five- to sixfold that measured for the other two isoforms (Table 1) (49).

Arthur Kornberg already addressed the question whether yeast NMNAT would also accept the reduced form of NMN, NMNH, to directly synthesize NADH (3, 32). NMNH indeed serves as substrate for mammalian NMNATs, as it was suggested based on the observation that NMNH significantly inhibits the NAD^+ synthesis activity of purified human NMNAT (46). NADH synthesis by recombinant hNMNAT1 and 3 follows similar initial rate kinetics, although hNMNAT3 binds NMNH with about twofold higher affinity than hNMNAT1. Human NMNAT2 is least capable of synthesizing NADH. However, the actual existence and physiological role of NMNH is still unclear. Interestingly, some dehydrogenases might accept NMN as cofactor for electron transfer instead of NAD^+, for examples, L-glutamic dehydrogenase type III (72) and horse liver alcohol dehydrogenase (73).

3.4.2. Purine nucleotide substrates

Human NMNAT3 was also found be the isoform with the lowest selectivity towards purine nucleotide substrates. It is capable of transferring the nucleotidyl moiety of guanosine triphosphate (GTP) and inosine triphosphate (ITP) onto NMN to produce the guanine and hypoxanthine derivatives of pyridine dinucleotides, NGD^+ and NHD^+, respectively (71). The K_M values for both substrates are approximately one order of magnitude higher than for ATP (49). Human NMNAT1 and 2 exhibit only negligible activities with GTP and ITP (71). The specificity towards deoxy purine nucleotides has been investigated for hNMNAT1 using dATP. Like both yeast enzymes, hNMNAT1 is capable of converting dATP, however at a considerably lower rate than ATP (44). Yeast NMNATs do not utilize dGTP, dCTP, dUTP or dITP as substrates (35). These nucleotides have not been tested with the human enzymes.

3.5. The mechanism of adenylyltransfer by NMNATs

3.5.1. A ternary complex and a nucleophilic attack

It is generally accepted, that adenylyltransfer is initiated by the attack of the nucleophilic 5'-phosphate of NMN at the α-phosphate of ATP. The ester bond between the α- and β-phosphorous of ATP is thereby destabilized, NMN is displaced at the α-phosphate, and, finally, NAD^+ and inorganic pyrophosphate are released from the enzyme (74).

In 1951, Kornberg and Pricer already proposed that the catalysis of NAD synthesis by NMNAT does not require the formation of an adenylyl-enzyme intermediate after they had observed that only little phosphorous exchange occurs between ATP and inorganic pyrophosphate in the absence of NMN (3). This assumption was later supported by Lowe and Tansley (74). They revealed the stereospecificity of adenylyltransfer towards the R-configuration of the α-phosphate of ATP. The inversion to the S-configuration found in NAD^+ presumably occurs upon displacement of NMN within the ternary complex between the two substrates and the enzyme.

3.5.2. Substrate binding order

Studies by the group of Magni established that NAD synthesis proceeds in correspondence to an ordered sequential two-substrate mechanism based on the kinetic

properties of eukaryotic enzymes (33, 46, 49). This mechanism excludes the dissociation of one product before entry of the second substrate. As a consequence, only the first substrate and the last product bind to the same free form of the enzyme, thereby potentially competing with each other.

Archaeal NMNATs are thought to not follow an ordered sequential binding mechanism. The possibility to generate crystal structures of single substrate complexes with either Mg^{2+}-ATP (25) or NMN implied that they have the capacity to bind the two substrates independently of each other (26). Moreover, human NMNAT3 has been crystallized in complexes with either NMN or a non-cleavable ATP species (1), although kinetic evidence suggested ordered sequential substrate binding (49).

To determine the order of substrate binding, a detailed steady-state kinetic study of recombinant human NMNAT isoforms was recently conducted (49). For hNMNAT1 and 2, ATP is the substrate to be bound first, and PP_i is the first product to be released from the ternary complex, followed by NAD^+ or NaAD. Human NMNAT3 binds the pyridine mononucleotide first, whereas the order of product release is the same for all isoforms (49). NAD^+ and NaAD compete for ATP binding during adenylyltransfer by hNMNAT3, while hNMNAT1 and 2 driven ATP utilization is inhibited in a mixed fashion. Inorganic pyrophosphate affects NAD^+ synthesis either by non-competitive (hNMNAT1) or mixed inhibition (hNMNAT2 and 3) (49).
Synthesis of NAD^+ and NaAD catalyzed by human NMNAT2 is most sensitive to product inhibition. Interestingly, NaAD exhibits an even higher inhibitory effect than NAD^+ (49). Therefore, accumulation of NaAD as product might account for the lower NaAD synthesis capacity of hNMNAT2 (see above).

Substrate inhibition of the human NMNAT isoforms by NMN or PP_i could not be observed for NAD^+ synthesis or cleavage, respectively. NMNH, on the other hand, does inhibit NAD^+ synthesis efficiently (32, 46, 49, 71, 75). The K_M for ATP differs at varying NMN concentrations and so does the K_M for PP_i when using different NAD^+ concentrations (46).

3.6. Small molecule effectors of NMNATs

A number of intermediates of nucleotide metabolism, among them purine nucleotides or polyphosphates, are precursors, product or substrate analogs and, therefore, possibly interfere with NAD synthesis. Nicotinamide is the major product of NAD catabolism, i.e. mono- and poly(ADP-ribosylation), NAD-dependent protein deacetylation, or hydrolysis by NAD glycohydrolases (4), and inhibits several of these reactions directly. However, as precursor of the salvage pathway, nicotinamide does not inhibit the enzymatic activities of NAD biosynthetic enzymes such as NMNATs (71). NAD conversions lead to the formation of ADP-ribose, poly-ADP-ribose, AMP, ADP, and NADP, among others. None of these compounds was reported to stimulate NAD synthesis or pyrophosphorylysis by NMNATs.

For ADP-ribose, non-competitive inhibition of NAD binding was reported for NMNAT purified from human placenta. However, the respective K_i amounts to 950 μM and, thereby, by far exceeds the ADP-ribose concentrations *in vivo* (46). Sub-millimolar concentrations of both free ADP-ribose (250 μM) and poly(ADP-ribose) (20 μM) had no measurable influence on the NAD synthesizing activity of any of the human NMNAT isoforms *in vitro* (71). Nevertheless, hNMNAT1 is capable of binding poly(ADP-ribose), but presumably in a region rather distant from the catalytic site (53) (see *4.3.1.*).

ADP and AMP, but also NADP are slightly inhibitory for NMNATs, primarily NMNAT2 (71). The strongest effect was observed with ADP, which inhibited hNMNAT2 by about 67 % at a concentration of 1 mM.

The green tea polyphenol gallotannin reduces poly(ADP-ribose)glycohydrolase (PARG) activity to 50 % at a concentration of 25 μM (76, 77). It also potently inhibits the NAD synthesis activity of hNMNATs *in vitro*, at IC50s of 10 μM for hNMNAT1, 55 μM for hNMNAT2, and 2 μM for hNMNAT3 (71). Consequently, the interpretation of experiments using gallotannin to study NAD conversions needs to consider the inhibitory effect on both PARG and NMNATs.

Another polyphenol, the condensed tannin epigallocatechin gallate (EGCG) inhibits PARG less potently than gallotannin. Interestingly, 50 μM EGCG stimulates human NMNATs *in vitro*. It doubles the NAD synthesis activity of hNMNAT2, while the effect on hNMNAT1 and 3 are less strong (71). Thus, EGCG was the first compound shown to be capable of enhancing NMNAT activity. However, further studies are required to establish whether activation of NMNATs contributes to the positive effects exerted by EGCG (reviewed in 78 and 79).

Effective exogenous NMNAT inhibitors have not yet been discovered. Several substrate analogs carrying modifications in the pyridine or adenine ring were tested. For example, nicotinamide-2'-methyladenine dinucleotide, *N-2'-MeAD*, and its deamidated form *Na-2'-MeAD* selectively reduced the NAD synthesis activity of hNMNAT3 by about 80 %. However, the rather high IC50 values of 0.19 mM and 1.12 mM, respectively (80), classify them as non-potent inhibitors. The polyphosphate analogs *Np3AD*, *Np4AD* and *Nap4AD* inhibit *in vitro* NMNAT activity also at sub-millimolar concentrations. *Np3AD* and *Np4AD* reduce hNMNAT2-catalyzed NAD synthesis, while hNMNAT1 and 3 are preferentially affected by *Nap4AD* (49).

4. THE BIOLOGY OF NMNATs

4.1. Tissue and subcellular distribution of human NMNAT isoforms

4.1.1. Tissue specific expression

As shown in Table 1, human NMNATs exhibit a distinct tissue distribution. NMNAT1 appears to be the most abundant and widely expressed isoform. Its gene expression was shown in a wide variety of tissues with

highest levels in skeletal muscle and heart and strong expression in liver and kidney. Less pronounced transcription was detected in thymus and spleen as well as in a number of tumor cells (44).

In contrast to NMNAT1, NMNAT2 is strongly expressed in brain and, therefore, has originally been designated as brain-specific isoform. Its transcript is highly abundant in most brain sub-regions except for corpus callosum (47), medulla and spinal cord (62). NMNAT2 expression was also observed in heart, skeletal muscle and pancreas, but was hardly detectable in several other tissues (62). There are indications for the expression of hNMNAT2 protein in endocrine pancreatic tissue, possibly insulin-secreting β-cells (62). The distinct expression pattern clearly distinguishes hNMNAT2 from hNMNAT1, which is almost ubiquitously found.

The expression of NMNAT3 appears to negatively correlate with that of NMNAT2 (71). Indeed, tissues with relatively high expression of NMNAT3-encoding mRNA (e.g., lung and spleen) contained almost undetectable levels of transcripts encoding NMNAT2. Weak expression of NMNAT3, as detected by Northern Blots, was found in placenta and kidney. Nevertheless, the presence of one isoform does not exclude another, since at least in HeLa and HEK293 cells the presence of transcripts for all three NMNATs were detected (71).

Thus, according to mRNA analyses, human NMNATs 2 and 3 appear to be specifically expressed in some tissues, whereas NMNAT1 is virtually ubiquitously present, albeit at varying concentrations. However, so far there is little information regarding the actual presence of hNMNAT proteins in the tissues.

4.1.2. Subcellular distribution and compartment specific functions of hNMNATs

Research on NAD metabolism revealed intracellular nucleotide pools not only to be maintained by transport and shuttling systems, but also by a compartmented distribution of NAD synthesizing and degrading enzymes. In humans, NMNAT activity is present in the nucleus represented by hNMNAT1 (44, 45, 71), while hNMNAT2 and 3 were localized to the Golgi apparatus and mitochondria, respectively (71).

The enrichment of mammalian nuclear NMNAT was reported to result in the co-purification of nucleic acid (8), suggesting a binding to chromatin (81) or nuclear ribosomes (82). Furthermore, hNMNAT activity associates with the inner nuclear membrane (83). The ribose content of NMNAT purified from *Saccharomyces cerevisiae* implied a possible posttranslational modification of the enzyme by ADP-ribose (33). However, ADP-ribosylation has not been shown for any NMNAT.

Recently, hNMNAT1 was reported as target of protein kinase C-mediated phosphorylation at serine 136 (53). This residue is found in close proximity to the enzymes putative NLS ($_{123}$PGRKRKW$_{129}$). However, the phosphorylation state does not influence the nuclear targeting. Rather, it regulates the interaction between hNMNAT1 and poly(ADP-ribose), PAR. It was demonstrated that hNMNAT1 associates with automodified poly(ADP-ribose) polymerase 1, PARP1. This interaction resulted in the catalytic activation of PARP1. Moreover, overexpression of hNMNAT1 in HeLa S3 cells stimulated the relocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus upon genotoxic treatment (53). The AIF relocation has previously been shown to be a downstream event of PARP1 activity (84, 85). Interestingly, protein kinase C-mediated phosphorylation of hNMNAT1 at Ser136 diminished the interaction with automodified PARP-1, while the NAD synthesis activity remained unchanged (53). These observations indicate that at least hNMNAT1 has attained additional, regulatory functions. They also demonstrate a direct interaction between the critical step in NAD synthesis and the major NAD consumer, PARP1.

The nuclear localization signal and serine 136 are part of the structurally unresolved loop consisting of amino acids 111 to 147 of the hNMNAT1 apo-enzyme (1KKU; 41) (see Figure 3C and paragraph *3.3.1.*). This unique and highly flexible sequence is located at the outer surface of each subunit in the hexamer (42, 43) and, therefore, easy to access for interactions with components of the nuclear import machinery or other molecules like PAR. It would appear that the structural resolution of this stretch of amino acids might depend on the presence of an interacting partner such as PAR.

Within the hexameric structure of hNMNAT1, a patch of positively charged amino acids appears in spatial proximity to the unresolved loops of two neighboring dimers. It involves Arg228, Arg231, Arg232 of monomer A and Arg40, K205, R207 of monomer E and the same residues in the adjacent dimer (B-D). This patch might promote electrostatic interactions with poly(ADP-ribose) or nucleic acid, too (43). Phosphorylation at serine 136 could shield the positive charge and disable a putative interaction. According to the primary structure alignment in Figure 2, an even more extended amino acid insertion is present in hNMNAT2. However, the NMNAT2 primary structure is lacking a nuclear localization signal. Immunohistochemistry on human endocrine pancreas suggested hNMNAT2 to be cytosolic (62). Direct and indirect fluorescent cytochemistry on the overexpressed enzyme in eukaryotic cells confirmed its extranuclear localization (1, 47) and even indicated a more specific distribution to compartments of the secretory pathway, particularly the Golgi apparatus (71). This somehow puzzling localization of the human NMNAT isoform 2 is just another contribution to the list of its unique features among hNMNATs (see Table 1).

Predictions based on the primary structure would suggest a cytosolic localization of NMNAT3 which was also originally observed using an eGFP fusion protein (1). These authors also indicated a partial localization of the protein in mitochondria. Overexpression of the recombinant Flag-tagged hNMNAT3 protein showed a clear association with mitochondria, which was

immunocytochemically confirmed for the endogenous protein (71). The synthesis of mitochondrial NAD has been of particular interest, since most of the cellular NAD pool might be sequestered here (86). Since mitochondria of vertebrates do not appear to have a transporter for NAD, an autonomous biosynthetic pathway for pyridine nucleotides would in fact be required. In 1996, Barile *et al.* reported that NAD^+ synthesis from NMN and ATP within rat liver mitochondria was inhibited by PP_i, AMP and ADP-ribose (87). Accordingly, the characterization of the mitochondrial NMNAT in humans was the first molecular evidence for independent NAD synthesis within this compartment (1, 71). Still, the actual localization of hNMNAT3 within the matrix of the organelles has still to be demonstrated. Moreover, evidence that NMN directly enters the mitochondria has not been presented. Therefore, it may well be that NMNAT is not the only activity of a biosynthetic pathway of NAD within mitochondria.

Owing to compartmentation, tissue specificity and different kinetic properties of the three known NMNAT isoforms in humans, their simultaneous presence is unlikely to reflect functional redundancy. It might, in fact, be important for cell- or tissue-specific fine-tuning of particular NAD-mediated signaling processes, which are associated with the breakdown of the nucleotide (see Figure 1) (reviewed in 88).

4.2. Gene structure and expression

4.2.1. Identification of NMNATs from unicellular organisms

The identification of N-terminal sequences of NMNAT purified from the archaeon *Sulfolobus solfataricus* enabled the identification of an open reading frame (ORF; MJ0541orf) from *Methanocaldococcus jannaschii* by aligning the peptide sequence against the genome sequence of this related organism (89), which just had become available at that time. Indeed, expression of this ORF led to the generation of a protein exhibiting NMNAT activity. Analysis of internal peptide sequences from purified *S. solfataricus* further confirmed MJ0541orf to encode NMNAT. By a similar approach, the same group reported the first identification of a eukaryotic NMNAT gene (YLR328W), which was localized to chromosome XII of *Saccharomyces cerevisiae* (34). These studies can be regarded as crucial for the subsequent identification, characterization, and functional analyses of NMNATs from higher eukaryotes.

4.2.2. Human NMNAT1

Based on the purification of an NMNAT activity from placenta, peptide sequences were established. Comparison to ESTs in available databases then led to the cloning and characterization of the first human isoform, hNMNAT1 (44, 45). Fernando *et al.* extended the initial characterization, for example, by FISH analysis revealing that the NMNAT1 gene localizes to the short arm of human chromosome 1, where it was fine mapped to 1p36.2 and shown to be composed of 4 exons (90). FISH analysis using BAC clones also indicated the existence of sequence homologous to hNMNAT1 on chromosome 14. Moreover, additional BLAST searches indicated the existence of similar sequences on chromosome 3, 4 and 15. However, all those additional genomic sequences lack introns and contain several translational stops in each frame (90). In addition, Southern blot analysis indicated the existence of a single NMNAT1 gene in the human genome (44).

4.2.3. Human NMNAT2

Based on a BLAST search using the primary structure of NMNAT1 as query an ORF (KIAA0479) was identified, whose deduced amino acid sequence exhibited remarkable protein sequence similarity to hNMNAT1. The finding that the recombinant protein expressed from this ORF comprising 924 nucleotides in length exhibited NMNAT activity prompted Raffaelli *et al.* in 2002 to characterize this protein as a new NMNAT isoform (NMNAT2) (47). The gene encoding this ORF was localized to the long arm of chromosome 1 (1q25), thus the same chromosome which also harbors the NMNAT1 gene. It comprises eleven exons, of which the last one contains the major portion of the mature transcript. The gene spans about 171 kb and contains a large intron of about 125 kb between exon 1 and 2 (62). The existence of a corresponding transcript of 6.6 kb in length was initially shown in a study aimed at the identification of susceptibility genes involved in hereditary prostate cancer (91). Interestingly, this transcript was originally identified from a clone deriving from a cDNA library generated from brain (92). Its occurrence in tissue from different brain regions was confirmed by Raffaelli *et al.* and Yalowitz *et al.* (47, 62). However, the transcripts detected by Northern blot analyses in both reports showed different characteristics. While the hybridization probe used in the first report led to the detection of the described 6.6 kb mRNA and an additional mRNA of 4.8 kb in length, the latter study revealed a transcript of 5.6 kb in brain and heart tissue. In addition, RNA preparations from muscle tissue indicated the presence of a 3.2 kb transcript. Thus, the possibility of the existence of alternatively spliced transcripts cannot be ruled out. This is further supported by the description of two NMNAT2 transcript variants, which have been deposited in the NCBI database (Table 1). A comparison of these transcript variants reveals the possibility of an hNMNAT2 variant 2 containing an alternative N-terminus.

4.2.4. Human NMNAT3

Yet another human protein exerting NMNAT activity, isoform 3, was discovered and subjected to in-depth analyses both on structural and molecular levels by Zhang *et al.* (1). The sequence of 252 amino acids was identified as NMNAT based on sequence similarities using the hNMNAT1 amino acid sequence as query. Using the corresponding coding cDNA sequence as hybridization probe in Northern blot analysis, comparable low overall expression levels of this transcript could be observed. The NMNAT3 transcript detected by Northern blot analysis was estimated to consist of 1100 nucleotides. This result is at variance with NCBI database entries found for NMNAT3 cDNAs (Reference sequences: NM_178177, deposited as NMNAT3 mRNA; AF345564, deposited as FKSG76 mRNA), which give rise to cDNAs composed of 1886 bp and 1223 bp, respectively. Despite of these inconsistencies,

the successful amplification of NMNAT3 from cDNA generated from HEK293 cells and heart tissue in an independent study (71) supports the actual existence of the NMNAT3 protein annotated as FKSG76.

4.3. Pathophysiological implications of NMNAT activity

No diseases have been described so far which include an NMNAT isoform as a causative factor. However, since NAD is essential, it is conceivable that any severe defect in a key step of its biosynthesis would be lethal. Still, the potential importance of NMNATs in pathological states, in particular regarding the major human form, hNMNAT1, has already been demonstrated in several instances.

4.3.1. The switch between cell death and longevity

It is well known that niacin (collectively designating nicotinamide and nicotinic acid) deficiency causes pellagra (93). Since it is NAD synthesis for which this vitamin (B3) is required, any deficiency along the biosynthetic pathway would be expected to result in similar symptoms. In mammals, niacin and the essential amino acid tryptophan are precursors of NAD biosynthesis (5, 7, 94). Moreover, NMN may also be generated from nicotinamide riboside, which has recently been described as a potential nutrient (15). In contrast, there are no alternative routes for the formation of the dinucleotide by NMNATs suggesting these enzymes to be of critical importance (Figure 1). Recent research has established that NAD-dependent protein deacetylation represents a key mechanism in the regulation of life span. The first NAD-dependent deacetylase was discovered in yeast as silence information regulator 2 (Sir2). Increased activity of this enzyme has been shown to extend life span in yeast, and this has been confirmed for its homologs (Sirtuins) in a variety of other species (reviewed in 95). Interestingly, sirtuins are inhibited by nicotinamide, and release of this inhibition by increased nicotinamide reutilization for NAD synthesis has a positive effect on Sir2-mediated gene silencing. That is, increased NAD salvage (e.g., by overexpression of the enzymes involved) mimics the effect of Sir2 overexpression with regard to lifespan. On the other hand, hNMNAT1 accelerates poly(ADP-ribosylation) by PARP1 once the reaction is initiated (53). Strong activation of PARP1 results in cell death, either necrotic or apoptotic (reviewed in 96). It appears possible, therefore, that hNMNAT1 therefore, might have a critical role in the regulation of important cellular functions. This is also indicated by its PKC-dependent phosphorylation that abolishes its stimulating influence on PARP1 activity.

4.3.2. NMNAT and cancer

NAD synthesis activity is markedly decreased in tumor cell nuclei compared to normal cells (97). A homozygous 500 kb deletion of a tumor suppressor region at chromosome 1p36.2 – p36.3 has been described as one of the hallmarks of neuroblastoma (98, 99). Tumor development and progression are in part ascribed to a loss of or mutations in the polyubiquitin chain elongation factor UBE4B gene product (100), whose gene is located downstream of hNMNAT1 at least in man, rat and mouse. A gain of 70 N-terminal amino acids of UBE4B, C-terminally fused to full-length hNMNAT1, on the other hand, may have a beneficial effect, as revealed by slowed Wallerian degeneration in mice (see above).

Sorci *et al.* could observe an increase in transcription and relative activity of Golgi-localized hNMNAT2 with elevating degree of malignancy in cells derived from liver cancer. Within the neuroblastoma cell line SH-SY5Y, the transcripts of hNMNAT1 and hNMNAT3 were even virtually absent, in contrast to hNMNAT2 (49). The chromosomal region 1q25 – 31 was identified as metastasis-associated region, possibly due to a gain of the extracellular matrix component laminin 5 γ2 (LAMC2), which is involved in cell adhesion and motility (101). Interestingly, in human and mice, the NMNAT2 gene is head-to-head facing the LAMC2 gene, mapping to the same region.

The NMNATs also play a role in the treatment of leukemia by tiazofurin. The conversion of this nucleoside analog to its active form requires this enzyme activity. The resultant dinucleotide inhibits a critical step in (d)GTP generation and thereby cancer progression (102-104). It was recently established (49) that hNMNAT1 exhibits the highest activity towards tiazofurin mononucleotide, and analysis of the expression of this isoform could be helpful in predicting the responsiveness of a tumor to the drug.

4.4. Is NMNAT1 a key to neuronal survival? Chromosomal aberrancy in the Wallerian degeneration (slow) (Wld^S) mouse

Upon transection of nerve fibers of particularly the peripheral nerve system, the proximal and distal parts of the embedded neurons meet different fates. Attracted by growth factors produced by Schwann cells, the proximal part might still grow across the lesion into the dissected tube to eventually reinnervate the target tissue. The distal stump, however, is subject to degradation by e.g. macrophages and Schwann cells. This process was originally described by Augustus Volney Waller in 1850 (105) and is known as Wallerian degeneration.

Wallerian degeneration usually occurs within the first one to two days after transection. Interestingly, the process is delayed for up to three weeks in a mutant mouse strain ($C57BL/Wld^S$). Analysis of the genetic background of $C57BL/Wld^S$ mice revealed the *Wld^S* gene to localize to the distal chromosome 4. It was subsequently fine-mapped to a mutated gene locus with a tandem triplication repeat of 85 kbp in length, which is stably inherited in the $C57BL/Wld^S$ mice (106). Two transcripts were shown to be expressed from this mutated gene locus. One of them encoded a novel member of the retinoid binding protein family, Rbp7. Its expression pattern, however, revealed no obvious importance in a neuronal background. The second transcript could be identified as a chimeric mRNA of 3.2 kb in size, which contains exonic sequences of the *Ufd2* and *D4Cole1e* gene. In contrast to Rbp7, this mRNA is abundantly expressed from the *Wld^S* gene locus in the nerve system (107). Translation of this mRNA results in a chimeric protein consisting of the N-terminal 70 amino acids of ubiquitination factor E4B (Ube4b) fused by an

aspartic acid junction with the C-terminal 302 amino acids of the *D4Cole1e* gene. Importantly, the human homolog of the *D4Cole1e* gene mapped to human chromosome 1p36.2 and eventually turned out to comprise the open reading frame for all 279 amino acids of human NMNAT1. Due to the fusion with the *Ufd2* gene, the mouse *Wld*^{S} gene encodes the complete 285 amino acids of mouse NMNAT1 and some additional 17 amino acids of its 5'-untranslated region (90). Despite of this genetic rearrangement the *Wld*^{S} mice still express both the normal form of ubiquitination factor E4 (Ube4b) and NMNAT1. Nevertheless, the *Wld*^{S} gene was identified as the causative mutation leading to the phenotype of C57BL/Wld^S mice (108), which implicated the activity of NMNAT1 to be important for delayed Wallerian degeneration.

Expression of the *Wld*^{s} gene neither does lead to a different expression of components of the ubiquitin proteasome complex of the axons in *Wld*^{s} mice nor impairs the function of the normal form of Ube4b (109). Therefore, delayed Wallerian degeneration might rather depend on a novel function of the chimeric Ube4b/NMNAT1 protein (110) or a vital effect caused by overexpression of the complete NMNAT1 primary structure encoded by the *Ube4b/Nmnat*. Similar to NMNAT1, the *Wld*^{s} gene product is exclusively expressed in the nucleus (108). Thus, delayed Wallerian degeneration of the distal stump of axotomized neurons by a nuclear protein indicates that the phenotype of *Wld*^{s} mice is an indirect consequence of *Wld*^{s} gene expression. The indirect contribution of NMNAT1 activity to delayed Wallerian degeneration has been quite controversially discussed during the past years. In 2004, Araki *et al.* reported that both removal of cell body and exposure to neurotoxic stress prevented axonal degeneration only when cells overexpressed catalytically active NMNAT1, whereas no protective effect was observed following overexpression of the 70 amino acids of Ube4b (64). Moreover, axonal survival was promoted by pretreatment of explanted ganglia with NAD^+ and dependent on the activity of the NAD-dependent deacetylase SIRT1. Thus, the protective effect of the *Wld*^{S} gene product might be the result of an increased nuclear NAD biosynthesis owing to the overexpressed NMNAT1 portion particularly in neuronal cells. A study by Wang *et al.* confirmed that both NMNAT1 expression and exogenous NAD^+ can protect soma-free dorsal root ganglia (DRG) explants from Wld^s mice, but excluded this process to be mediated by SIRT1 using DRGs isolated from wild type and SIRT1$^{-/-}$ mice (111). Recent studies, however, challenged the view that the protective role of the Wld^s protein can be reduced to its NMNAT activity. For instance, transgenic mice overexpressing NMNAT1 at levels leading to comparable enzyme activity in brain tissue as in C57BL/Wld^S mice did not show delayed Wallerian degeneration after transecting neurons both *in vivo* and *in vitro* (112). Similar results were obtained for stably transfected Neuro2A cell lines overexpressing either NMNAT1 or the Wld^S protein (113). Experiments on Drosophila in which the fly homolog was expressed from a mutant *nmnat* locus suggested the possibility that delayed Wallerian degeneration might not require the catalytic activity of NMNAT itself (114). In Drosophila NMNAT expression is enriched in the nervous system. Complete lack of the *nmnat* gene severely affected the integrity of photoreceptors. However, catalytically inactive mutants retained the protective role on photoreceptors subjected to physical stress by prolonged exposure to light in a similar fashion as the wild type protein. These results would suggest that NMNAT might have an additional function beyond its activity to synthesize NAD^+ (114). However, these observations are in apparent conflict with a report by Araki *et al.*, who included a catalytically inactive NMNAT1 in their study (64). In addition, recent investigations, led to the identification of a critical phenylalanine at position 28 in NMNAT1 which is important for the catalytic activity of NMNAT1 itself but also for the fulfillment of its neuroprotective role in the Wld^S protein (60). Although further investigations are required to resolve these inconsistencies, the discovery and detailed study of the Wld^S mutation have already revealed important roles of NMNAT1, likely beyond its catalytic activity.

5. PERSPECTIVE

NMNATs have been established as key enzymes in the maintenance of the cellular NAD pool. The recognition of a permanent turnover of the pyridine nucleotides has prompted considerable efforts to understand their regeneration and, thereby, the important functional roles of NMNATs. Indeed, the progress since 2001, when the first primary structure of a human NMNAT was established, is impressive. Still, there are many open questions regarding these enzymes. Virtually nothing is known about the regulation of their activity in a cellular context. That relates to both physiological effectors and transcriptional regulation controlling the expression of NMNATs. The tissue mRNA expression levels of human NMNAT genes have indicated organ-specific needs. However, what these are and how differential expression of the isoforms is regulated has remained unknown.

There is also little information as to how NMNAT activities might change in response to external stimuli, in cellular stress situations or during development. The lack of specific inhibitors for any of the NMNATs represents a major obstacle to in-depth functional studies beyond the isolated enzymes. However, using molecular-genetic approaches (e.g., RNA interference) some of the major issues could be addressed. In particular, in mammalian cells the specific roles of the isoenzymes could be studied. For example, the presence of hNMNAT2 in the Golgi complex has still remained a mystery with regard to its functional meaning. Is it essential, at least for some cell types? Similarly, enhanced expression of NMNATs might be beneficial in some situations. Strong activation of PARP1 by DNA damage leads to a considerable NAD consumption, which might be compensated by elevated synthesis to prevent cell death.

Although clearly suggested, potential roles of NMNATs in diseases have not been thoroughly studied. For example, the remarkable decrease of NMNAT activity at least in some cancers has so far not been addressed with

regard to the mechanism of the down-regulation or its potential suitability for therapeutic applications. Since NMNATs generate NAD, the molecule at the crossroads of energy and signal transduction, their modulation is more than likely to influence important cellular processes. Understanding and exploiting the underlying mechanisms will be a major challenge in the future. The existence of three distinct human isoforms may be helpful rather than a problem in such studies.

6. ACKNOWLEDGEMENT

Research in our laboratory is supported by the Norwegian Research council and INTAS genomics (grant 05-1000004-7753).

7. REFERENCES

1. Zhang X, O. V. Kurnasov, S. Karthikeyan, N. V. Grishin, A. L. Osterman, H. Zhang: Structural characterization of a human cytosolic NMN/NaMN adenylyltransferase and implication in human NAD biosynthesis. *J Biol Chem* 278, 13503-13511 (2003)

2. Kornberg A, O. Lindberg: Diphosphopyridine nucleotide pyrophosphatase. *J Biol Chem* 176, 665-677 (1948)

3. Kornberg A, W. E. Pricer, Jr.: Enzymatic cleavage of diphosphopyridine nucleotide with radioactive pyrophosphate. *J Biol Chem* 191, 535-541 (1951)

4. Berger F, M. H. Ramirez-Hernandez, M. Ziegler: The new life of a centenarian: signalling functions of NAD(P). *Trends Biochem Sci* 29, 111-118 (2004)

5. Magni G, A. Amici, M. Emanuelli, N. Raffaelli, S. Ruggieri: Enzymology of NAD^+ synthesis. *Adv Enzymol Relat Areas Mol Biol* 73, 135-182, (1999)

6. Magni G, A. Amici, M. Emanuelli, G. Orsomando, N. Raffaelli, S. Ruggieri: Structure and function of nicotinamide mononucleotide adenylyltransferase. *Curr Med Chem* 11, 873-885 (2004)

7. Magni G, A. Amici, M. Emanuelli, G. Orsomando, N. Raffaelli, S. Ruggieri: Enzymology of NAD^+ homeostasis in man. *Cell Mol Life Sci* 61, 19-34 (2004)

8. Atkinson M R, J. F. Jackson, R. K. Morton: Adenosine triphosphate-nicotinamide mononucleotide adenylyltransferase of pig-liver nuclei. Extraction and purification of the enzyme. *Biochem J* 90, 433-438 (1964)

9. Imsande J: Pathway of diphosphopyridine nucleotide biosynthesis in Escherichia coli. *J Biol Chem* 236, 1494-1497 (1961)

10. Mehl R A, C. Kinsland, T. P. Begley: Identification of the Escherichia coli nicotinic acid mononucleotide adenylyltransferase gene. *J Bacteriol* 182, 4372-4374 (2000)

11. Olland A M, K. W. Underwood, R. M. Czerwinski, M. C. Lo, A. Aulabaugh, J. Bard, M. L. Stahl, W. S. Somers, F. X. Sullivan, R. Chopra: Identification, characterization, and crystal structure of Bacillus subtilis nicotinic acid mononucleotide adenylyltransferase. *J Biol Chem* 277, 3698-3707 (2002)

12. Zhang H, T. Zhou, O. Kurnasov, S. Cheek, N. V. Grishin, A. Osterman: Crystal structures of E. coli nicotinate mononucleotide adenylyltransferase and its complex with deamido-NAD. *Structure* 10, 69-79 (2002)

13. Raffaelli N, T. Lorenzi, P. L. Mariani, M. Emanuelli, A. Amici, S. Ruggieri, G. Magni: The Escherichia coli NadR regulator is endowed with nicotinamide mononucleotide adenylyltransferase activity. *J Bacteriol* 181, 5509-5511 (1999)

14. Kurnasov O V, B. M. Polanuyer, S. Ananta, R. Sloutsky, A. Tam, S. Y. Gerdes, A. L. Osterman: Ribosylnicotinamide kinase domain of NadR protein: identification and implications in NAD biosynthesis. *J Bacteriol* 184, 6906-6917 (2002)

15. Bieganowski P, C. Brenner: Discoveries of nicotinamide riboside as a nutrient and conserved NRK genes establish a Preiss-Handler independent route to NAD^+ in fungi and humans. *Cell* 117, 495-502 (2004)

16. Foster J W, Y. K. Park, T. Penfound, T. Fenger, M. P. Spector: Regulation of NAD metabolism in Salmonella typhimurium: molecular sequence analysis of the bifunctional nadR regulator and the nadA-pnuC operon. *J Bacteriol* 172, 4187-4196 (1990)

17. Zhu N, J. R. Roth: The nadI region of Salmonella typhimurium encodes a bifunctional regulatory protein. *J Bacteriol* 173, 1302-1310 (1991)

18. Zhu N, B. M. Olivera, J. R. Roth: Activity of the nicotinamide mononucleotide transport system is regulated in Salmonella typhimurium. *J Bacteriol* 173, 1311-1320 (1991)

19. Holley E A, M. P. Spector, J. W. Foster: Regulation of NAD biosynthesis in Salmonella typhimurium: expression of nad-lac gene fusions and identification of a nad regulatory locus. *J Gen Microbiol* 131, 2759-2770 (1985)

20. Zhu N, B. M. Olivera, J. R. Roth: Identification of a repressor gene involved in the regulation of NAD de novo biosynthesis in Salmonella typhimurium. *J Bacteriol* 170, 117-125 (1988)

21. Foster J W, T. Penfound: The bifunctional NadR regulator of Salmonella typhimurium: location of regions involved with DNA binding, nucleotide transport and intramolecular communication. *FEMS Microbiol Lett* 112, 179-183 (1993)

22. Penfound T, J. W. Foster: NAD-dependent DNA-binding activity of the bifunctional NadR regulator of Salmonella typhimurium. *J Bacteriol* 181, 648-655 (1999)

23. Singh S K, O. V. Kurnasov, B. Chen, H. Robinson, N. V. Grishin, A. L. Osterman, H. Zhang: Crystal structure of Haemophilus influenzae NadR protein. A bifunctional enzyme endowed with NMN adenyltransferase and ribosylnicotinimide kinase activities. *J Biol Chem* 277, 33291-33299 (2002)

24. Raffaelli N, F. M. Pisani, T. Lorenzi, M. Emanuelli, A. Amici, S. Ruggieri, G. Magni: Characterization of nicotinamide mononucleotide adenylyltransferase from thermophilic archaea. *J Bacteriol* 179, 7718-7723 (1997)

25. D'Angelo I, N. Raffaelli, V. Dabusti, T. Lorenzi, G. Magni, M. Rizzi: Structure of nicotinamide mononucleotide adenylyltransferase: a key enzyme in $NAD^{(+)}$ biosynthesis. *Structure* 8, 993-1004 (2000)

26. Saridakis V, D. Christendat, M. S. Kimber, A. Dharamsi, A. M. Edwards, E. F. Pai: Insights into ligand binding and catalysis of a central step in NAD^+ synthesis: structures of Methanobacterium thermoautotrophicum NMN adenylyltransferase complexes. *J Biol Chem* 276, 7225-7232 (2001)

27. Saridakis V, E. F. Pai: Mutational, structural, and kinetic studies of the ATP-binding site of Methanobacterium thermoautotrophicum nicotinamide mononucleotide adenylyltransferase. *J Biol Chem* 278, 34356-34363 (2003)

28. Raffaelli N, A. Amici, M. Emanuelli, S. Ruggieri, G. Magni: Pyridine dinucleotide biosynthesis in archaebacteria: presence of NMN adenylyltransferase in Sulfolobus solfataricus. *FEBS Lett* 355, 233-236 (1994)

29. Raffaelli N, F. M. Pisani, T. Lorenzi, M. Emanuelli, A. Amici, S. Ruggieri, G. Magni: Nicotinamide-mononucleotide adenylyltransferase from Methanococcus jannaschii. *Methods Enzymol* 331, 292-298 (2001)

30. Raffaelli N, T. Lorenzi, A. Amici, M. Emanuelli, S. Ruggieri, G. Magni: Synechocystis sp. slr0787 protein is a novel bifunctional enzyme endowed with both nicotinamide mononucleotide adenylyltransferase and 'Nudix' hydrolase activities. *FEBS Lett* 444, 222-226 (1999)

31. McLennan A G: The Nudix hydrolase superfamily. *Cell Mol Life Sci* 63, 123-143 (2006)

32. Kornberg A: Enzymatic synthesis of triphosphopyridine nucleotide. *J Biol Chem* 182, 805-813 (1950)

33. Natalini P, S. Ruggieri, N. Raffaelli, G. Magni: Nicotinamide mononucleotide adenylyltransferase. Molecular and enzymatic properties of the homogeneous enzyme from baker's yeast. *Biochemistry* 25, 3725-3729 (1986)

34. Emanuelli M, F. Carnevali, M. Lorenzi, N. Raffaelli, A. Amici, S. Ruggieri, G. Magni: Identification and characterization of YLR328W, the Saccharomyces cerevisiae structural gene encoding NMN adenylyltransferase. Expression and characterization of the recombinant enzyme. *FEBS Lett* 455, 13-17 (1999)

35. Emanuelli M, A. Amici, F. Carnevali, F. Pierella, N. Raffaelli, G. Magni: Identification and characterization of a second NMN adenylyltransferase gene in Saccharomyces cerevisiae. *Protein Expr Purif* 27, 357-364 (2003)

36. Hunt L, F. Lerner, M. Ziegler: NAD - new roles in signalling and gene regulation in plants. *New Phytologist* 136, 31-44 (2004)

37. Noctor G, G. Queval, B. Gakiere: NAD(P) synthesis and pyridine nucleotide cycling in plants and their potential importance in stress conditions. *J Exp Bot* 57, 1603-1620 (2006)

38. Noctor G: Metabolic signalling in defence and stress: the central roles of soluble redox couples. *Plant Cell Environ* 29, 409-425 (2006)

39. Wang G, E. Pichersky: Nicotinamidase participates in the salvage pathway of NAD biosynthesis in Arabidopsis. *Plant J* 49, 1020-1029 (2007)

40. Hashida S N, H. Takahashi, M. Kawai-Yamada, H. Uchimiya: Arabidopsis thaliana nicotinate/nicotinamide mononucleotide adenyltransferase (AtNMNAT) is required for pollen tube growth. *Plant J* 49, 694-703 (2007)

41. Garavaglia S, I. D'Angelo, M. Emanuelli, F. Carnevali, F. Pierella, G. Magni, M. Rizzi: Structure of human NMN adenylyltransferase. A key nuclear enzyme for NAD homeostasis. *J Biol Chem* 277, 8524-8530 (2002)

42. Werner E, M. Ziegler, F. Lerner, M. Schweiger, U. Heinemann: Crystal structure of human nicotinamide mononucleotide adenylyltransferase in complex with NMN. *FEBS Lett* 516, 239-244 (2002)

43. Zhou T, O. Kurnasov, D. R. Tomchick, D. D. Binns, N. V. Grishin, V. E. Marquez, A. L. Osterman, H. Zhang: Structure of human nicotinamide/nicotinic acid mononucleotide adenylyltransferase. Basis for the dual substrate specificity and activation of the oncolytic agent tiazofurin. *J Biol Chem* 277, 13148-13154 (2002)

44. Emanuelli M, F. Carnevali, F. Saccucci, F. Pierella, A. Amici, N. Raffaelli, G. Magni: Molecular cloning, chromosomal localization, tissue mRNA levels, bacterial expression, and enzymatic properties of human NMN adenylyltransferase. *J Biol Chem* 276, 406-412 (2001)

45. Schweiger M, K. Hennig, F. Lerner, M. Niere, M. Hirsch-Kauffmann, T. Specht, C. Weise, S. L. Oei, M. Ziegler: Characterization of recombinant human nicotinamide mononucleotide adenylyl transferase (NMNAT), a nuclear enzyme essential for NAD synthesis. *FEBS Lett* 492, 95-100 (2001)

46. Emanuelli M, P. Natalini, N. Raffaelli, S. Ruggieri, A. Vita, G. Magni: NAD biosynthesis in human placenta:

purification and characterization of homogeneous NMN adenylyltransferase. *Arch Biochem Biophys* 298, 29-34 (1992)

47. Raffaelli N, L. Sorci, A. Amici, M. Emanuelli, F. Mazzola, G. Magni: Identification of a novel human nicotinamide mononucleotide adenylyltransferase. *Biochem Biophys Res Commun* 297, 835-840 (2002)

48. The UniProt Consortium: The Universal Protein Resource (UniProt). *Nucleic Acids Res*, (2007)

49. Sorci L, F. Cimadamore, S. Scotti, R. Petrelli, L. Cappellacci, P. Franchetti, G. Orsomando, G. Magni: Initial-rate kinetics of human NMN-adenylyltransferases: substrate and metal ion specificity, inhibition by products and multisubstrate analogues, and isozyme contributions to NAD^+ biosynthesis. *Biochemistry* 46, 4912-4922 (2007)

50. Thisse B, C. Thisse: Fast Release Clones: A High Throughput Expression Analysis. ZFIN Direct Data Submission, World Wide Web URL: http://zfin.org (2004)

51. ZFIN; University of Oregon, Eugene, OR 97403-5274; World Wide Web URL: http://zfin.org

52. Murzin A G, S. E. Brenner, T. Hubbard, C. Chothia: SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J Mol Biol* 247, 536-540 (1995)

53. Berger F, C. Lau, M. Ziegler: Regulation of poly(ADP-ribose) polymerase 1 activity by the phosphorylation state of the nuclear NAD biosynthetic enzyme NMN adenylyl transferase 1. *Proc Natl Acad Sci U S A* 104, 3765-3770 (2007)

54. Christendat D, A. Yee, A. Dharamsi, Y. Kluger, A. Savchenko, J. R. Cort, V. Booth, C. D. Mackereth, V. Saridakis, I. Ekiel, G. Kozlov, K. L. Maxwell, N. Wu, L. P. McIntosh, K. Gehring, M. A. Kennedy, A. R. Davidson, E. F. Pai, M. Gerstein, A. M. Edwards, C. H. Arrowsmith: Structural proteomics of an archaeon. *Nat Struct Biol* 7, 903-909 (2000)

55. Izard T, A. Geerlof: The crystal structure of a novel bacterial adenylyltransferase reveals half of sites reactivity. *EMBO J* 18, 2021-2030 (1999)

56. Rossmann M G, A. Liljas, C. I. Brandén, L. J. Banaszak: Evolutionary and structural relationships among dehydrogenases. In: The Enzymes. Ed: P. D. Boyer, *Academic Press*, NY 3, 61-102 (1975)

57. Wierenga R K, M. C. H. De Maeyer, W. G. J. Hol: Interaction of pyrophosphate moieties with α-helixes in dinucleotide-binding proteins. *Biochemistry* 24, 1346-1357 (1985)

58. Bottoms C A, P. E. Smith, J. J. Tanner: A structurally conserved water molecule in Rossmann dinucleotide-binding domains. *Protein Sci* 11, 2125-2137 (2002)

59. Bork P, L. Holm, E. V. Koonin, C. Sander: The cytidylyltransferase superfamily: identification of the nucleotide-binding site and fold prediction. *Proteins* 22, 259-266 (1995)

60. Jia H, T. Yan, Y. Feng, C. Zeng, X. Shi, Q. Zhai: Identification of a critical site in Wld(s): essential for Nmnat enzyme activity and axon-protective function. *Neurosci Lett* 413, 46-51 (2007)

61. Veitch D P, D. Gilham, R. B. Cornell: The role of histidine residues in the HXGH site of CTP:phosphocholine cytidylyltransferase in CTP binding and catalysis. *Eur J Biochem* 255, 227-234 (1998)

62. Yalowitz J A, S. Xiao, M. P. Biju, A. C. Antony, O. W. Cummings, M. A. Deeg, H. N. Jayaram: Characterization of human brain nicotinamide 5'-mononucleotide adenylyltransferase-2 and expression in human pancreas. *Biochem J* 377, 317-326 (2004)

63. Izard T: The crystal structures of phosphopantetheine adenylyltransferase with bound substrates reveal the enzyme's catalytic mechanism. *J Mol Biol* 315, 487-495 (2002)

64. Araki T, Y. Sasaki, J. Milbrandt: Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science* 305, 1010-1013 (2004)

65. Yoon H J, H. L. Kim, B. Mikami, S. W. Suh: Crystal structure of nicotinic acid mononucleotide adenylyltransferase from Pseudomonas aeruginosa in its Apo and substrate-complexed forms reveals a fully open conformation. *J Mol Biol* 351, 258-265 (2005)

66. Han S, M. D. Forman, P. Loulakis, M. H. Rosner, Z. Xie, H. Wang, D. E. Danley, W. Yuan, J. Schafer, Z. Xu: Crystal structure of nicotinic acid mononucleotide adenylyltransferase from Staphyloccocus aureus: structural basis for NaAD interaction in functional dimer. *J Mol Biol* 360, 814-825 (2006)

67. Berman H M, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne: The Protein Data Bank. *Nucleic Acids Res* 28, 235-242 (2000)

68. Berman H, K. Henrick, H. Nakamura, J. L. Markley: The worldwide Protein Data Bank (wwPDB): ensuring a single, uniform archive of PDB data. *Nucleic Acids Res* 35, D301-D303 (2007)

69. Morris V K, T. Izard: Substrate-induced asymmetry and channel closure revealed by the apoenzyme structure of Mycobacterium tuberculosis phosphopantetheine adenylyltransferase. *Protein Sci* 13, 2547-2552 (2004)

70. Noreen I M A, J. M. Thornton: Diversity of protein-protein interactions. *EMBO J* 22, 3486-3492 (2003)

71. Berger F, C. Lau, M. Dahlmann, M. Ziegler: Subcellular compartmentation and differential catalytic

properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms. *J Biol Chem* 280, 36334-36341 (2005)

72. Fisher H F, L. L. McGregor: The ability of reduced nicotinamide mononucleotide to function as a hydrogen donor in the glutamic dehydrogenase reaction. *Biochem Biophys Res Commun* 34, 627-632 (1969)

73. Sicsic S, P. Durand, S. Langrene, F. Le Goffic: Activity of NMN, nicotinamide ribose and analogs in alcohol oxidation promoted by horse-liver alcohol dehydrogenase. Improvement of this activity and structural requirements of the pyridine nucleotide part of the NAD^+ coenzyme. *Eur J Biochem* 155, 403-407 (1986)

74. Lowe G, G. Tansley: The stereochemical course of nucleotidyl transfer catalysed by NAD pyrophosphorylase. *Eur J Biochem* 132, 117-120 (1983)

75. Balducci E, G. Orsomando, V. Polzonetti, A. Vita, M. Emanuelli, N. Raffaelli, S. Ruggieri, G. Magni, P. Natalini: NMN adenylyltransferase from bull testis: purification and properties. *Biochem J* 310 (Pt 2), 395-400 (1995)

76. Tsai Y J, T. Aoki, H. Maruta, H. Abe, H. Sakagami, T. Hatano, T. Okuda, S. Tanuma: Mouse mammary tumor virus gene expression is suppressed by oligomeric ellagitannins, novel inhibitors of poly(ADP-ribose) glycohydrolase. *J Biol Chem* 267, 14436-14442 (1992)

77. Aoki K, K. Nishimura, H. Abe, H. Maruta, H. Sakagami, T. Hatano, T. Okuda, T. Yoshida, Y. J. Tsai, F. Uchiumi: Novel inhibitors of poly(ADP-ribose) glycohydrolase. *Biochim Biophys Acta* 1158, 251-256 (1993)

78. Khan N, F. Afaq, M. Saleem, N. Ahmad, H. Mukhtar: Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. *Cancer Res* 66, 2500-2505 (2006)

79. Shankar S, S. Ganapathy, R. K. Srivastava: Green tea polyphenols: biology and therapeutic implications in cancer. *Front Biosci* 12, 4881-4899 (2007)

80. Franchetti P, R. Petrelli, L. Cappellacci, M. Pasqualini, P. Vita, L. Sorci, F. Mazzola, N. Raffaelli, G. Magni: Synthesis and biological evaluation of NAD analogs as human pyridine nucleotide adenylyltransferase inhibitors. *Nucleosides Nucleotides Nucleic Acids* 24, 477-479 (2005)

81. Siebert G: Enzymes of cancer nuclei. *Exp Cell Res* 24, SUPPL9, 389417 (1963)

82. Traub A, E. Kaufmann, Y. Ginzburg-Tietz: Studies on nuclear ribosomes. I. Association of DPN-pyrophosphorylase with nuclear ribosomes in normal and neoplastic tissues. *Exp Cell Res* 34, 371-383 (1964)

83. Humbert J P, N. Matter, J. C. Artault, P. Koppler, A. N. Malviya: Inositol 1,4,5-trisphosphate receptor is located to the inner nuclear membrane vindicating regulation of nuclear calcium signaling by inositol 1,4,5-trisphosphate. Discrete distribution of inositol phosphate receptors to inner and outer nuclear membranes. *J Biol Chem* 271, 478-485 (1996)

84. Yu S W, H. Wang, M. F. Poitras, C. Coombs, W. J. Bowers, H. J. Federoff, G. G. Poirier, T. M. Dawson, V. L. Dawson: Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* 297, 259-263 (2002)

85. Yu S W, S. A. Andrabi, H. Wang, N. S. Kim, G. G. Poirier, T. M. Dawson, V. L. Dawson: Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. *Proc Natl Acad Sci U S A* 103, 18314-18319 (2006)

86. Di Lisa F, M. Ziegler: Pathophysiological relevance of mitochondria in $NAD^{(+)}$ metabolism. *FEBS Lett* 492, 4-8 (2001)

87. Barile M, S. Passarella, G. Danese, E. Quagliariello: Rat liver mitochondria can synthesize nicotinamide adenine dinucleotide from nicotinamide mononucleotide and ATP via a putative matrix nicotinamide mononucleotide adenylyltransferase. *Biochem Mol Biol Int* 38, 297-306 (1996)

88. Ying W: NAD^+ and NADH in cellular functions and cell death. *Front Biosci* 11, 3129-3148 (2006)

89. Raffaelli N, M. Emanuelli, F. M. Pisani, A. Amici, T. Lorenzi, S. Ruggieri, G. Magni: Identification of the archaeal NMN adenylytransferase gene. *Mol Cell Biochem* 193, 99-102 (1999)

90. Fernando F S, L. Conforti, S. Tosi, A. D. Smith, M. P. Coleman: Human homologue of a gene mutated in the slow Wallerian degeneration (C57BL/Wld(s)) mouse. *Gene* 284, 23-29 (2002)

91. Sood R, T. I. Bonner, I. Makalowska, D. A. Stephan, C. M. Robbins, T. D. Connors, S. D. Morgenbesser, K. Su, M. U. Faruque, H. Pinkett, C. Graham, A. D. Baxevanis, K. W. Klinger, G. M. Landes, J. M. Trent, J. D. Carpten: Cloning and characterization of 13 novel transcripts and the human RGS8 gene from the 1q25 region encompassing the hereditary prostate cancer (HPC1) locus. *Genomics* 73, 211-222 (2001)

92. Seki N, M. Ohira, T. Nagase, K. Ishikawa, N. Miyajima, D. Nakajima, N. Nomura, O. Ohara: Characterization of cDNA clones in size-fractionated cDNA libraries from human brain. *DNA Res* 4, 345-349 (1997)

93. Elvehjem C A, R. J. Madden, F. M. Strong, D. W. Wolley: The isolation and identification of the anti-black tongue factor. *J Biol Chem* 123, 137-149 (1938)

94. Pollak N, C. Dolle, M. Ziegler: The power to reduce: pyridine nucleotides--small molecules with a multitude of functions. *Biochem J* 402, 205-218 (2007)

95. Haigis M C, L. P. Guarente: Mammalian sirtuins – emerging roles in physiology, aging, and calorie restriction. *Genes Dev* 20, 2913-2921 (2006)

96. Koh D W, T. M. Dawson, V. L. Dawson: Mediation of cell death by poly(ADP-ribose) polymerase-1. *Pharmacol Res* 52, 5-14 (2005)

97. Branster M V, R. K. Morton: Comparative rates of synthesis of diphosphopyridine nucleotide by normal and tumour tissue from mouse mammary gland; studies with isolated nuclei. *Biochem J* 63, 640-646 (1956)

98. Ohira M, H. Kageyama, M. Mihara, S. Furuta, T. Machida, T. Shishikura, H. Takayasu, A. Islam, Y. Nakamura, M. Takahashi, N. Tomioka, S. Sakiyama, Y. Kaneko, A. Toyoda, M. Hattori, Y. Sakaki, M. Ohki, A. Horii, E. Soeda, J. Inazawa, N. Seki, H. Kuma, I. Nozawa, A. Nakagawara: Identification and characterization of a 500-kb homozygously deleted region at 1p36.2-p36.3 in a neuroblastoma cell line. *Oncogene* 19, 4302-4307 (2000)

99. Brodeur G M: Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 3, 203-216 (2003)

100. Caren H, A. Holmstrand, R. M. Sjoberg, T. Martinsson: The two human homologues of yeast UFD2 ubiquitination factor, UBE4A and UBE4B, are located in common neuroblastoma deletion regions and are subject to mutations in tumours. *Eur J Cancer* 42, 381-387 (2006)

101. Ma J, M. Gao, Y. Lu, X. Feng, J. Zhang, D. Lin, T. Xiao, Z. Hu, J. Yuan, K. Su, J. Shipley, J. Xue, Y. Gao: Gain of 1q25-32, 12q23-24.3, and 17q12-22 facilitates tumorigenesis and progression of human squamous cell lung cancer. *J Pathol* 210, 205-213 (2006)

102. Boulton S, S. Kyle, B. W. Durkacz: Low nicotinamide mononucleotide adenylyltransferase activity in a tiazofurin-resistant cell line: effects on NAD metabolism and DNA repair. *Br J Cancer* 76, 845-851 (1997)

103. Jayaram H N, W. Zhen, K. Gharehbaghi: Biochemical consequences of resistance to tiazofurin in human myelogenous leukemic K562 cells. *Cancer Res* 53, 2344-2348 (1993)

104. Jayaram H N, A. O'Connor, M. R. Grant, H. Yang, P. A. Grieco, D. A. Cooney: Biochemical consequences of resistance to a recently discovered IMP dehydrogenase inhibitor, benzamide riboside, in human myelogenous leukemia K562 cells. *J Exp Ther Oncol* 1, 278-285 (1996)

105. Waller A: Experiments on the section of glossopharyngeal and hypoglossal nerves of the frog and observations of the alternatives produced thereby in the structure of their primitive fibres. *Philosophical Transactions of the Royal Society of London, Series B* 140, 423-429 (1850)

106. Mi W, J. D. Glass, M. P. Coleman: Stable inheritance of an 85-kb triplication in C57BL/WldS mice. *Mutat Res* 526, 33-37 (2003)

107. Conforti L, A. Tarlton, T. G. Mack, W. Mi, E. A. Buckmaster, D. Wagner, V. H. Perry, M. P. Coleman: A Ufd2/D4Cole1e chimeric protein and overexpression of Rbp7 in the slow Wallerian degeneration (WldS) mouse. *Proc Natl Acad Sci U S A* 97, 11377-11382 (2000)

108. Mack T G, M. Reiner, B. Beirowski, W. Mi, M. Emanuelli, D. Wagner, D. Thomson, T. Gillingwater, F. Court, L. Conforti, F. S. Fernando, A. Tarlton, C. Andressen, K. Addicks, G. Magni, R. R. Ribchester, V. H. Perry, M. P. Coleman: Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. *Nat Neurosci* 4, 1199-1206 (2001)

109. Fang C, M. Bernardes-Silva, M. P. Coleman, V. H. Perry: The cellular distribution of the Wld s chimeric protein and its constituent proteins in the CNS. *Neuroscience* 135, 1107-1118 (2005)

110. Gillingwater T H, T. M. Wishart, P. E. Chen, J. E. Haley, K. Robertson, S. H. Macdonald, S. Middleton, K. Wawrowski, M. J. Shipston, S. Melmed, D. J. Wyllie, P. A. Skehel, M. P. Coleman, R. R. Ribchester: The neuroprotective WldS gene regulates expression of PTTG1 and erythroid differentiation regulator 1-like gene in mice and human cells. *Hum Mol Genet* 15, 625-635 (2006)

111. Wang J, Q. Zhai, Y. Chen, E. Lin, W. Gu, M. W. McBurney, Z. He: A local mechanism mediates NAD-dependent protection of axon degeneration. *J Cell Biol* 170, 349-355 (2005)

112. Conforti L, G. Fang, B. Beirowski, M. S. Wang, L. Sorci, S. Asress, R. Adalbert, A. Silva, K. Bridge, X. P. Huang, G. Magni, J. D. Glass, M. P. Coleman: $NAD^{(+)}$ and axon degeneration revisited: Nmnat1 cannot substitute for Wld(S) to delay Wallerian degeneration. *Cell Death Differ* 14, 116-127 (2007)

113. Watanabe M, T. Tsukiyama, S. Hatakeyama: Protection of vincristine-induced neuropathy by WldS expression and the independence of the activity of Nmnat1. *Neurosci Lett* 411, 228-232 (2007)

114. Zhai R G, Y. Cao, P. R. Hiesinger, Y. Zhou, S. Q. Mehta, K. L. Schulze, P. Verstreken, H. J. Bellen: Drosophila NMNAT maintains neural integrity independent of its NAD synthesis activity. *PLoS Biol* 4, 2336-2348 (2006)

115. Chenna R, H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, J. D. Thompson: Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 31, 3497-3500 (2003)

116. International Human Genome Sequencing Consortium: Finishing the euchromatic sequence of the human genome. *Nature* 431, 931-945 (2004)

117. Yamada K, J. Lim, J. M. Dale, H. Chen, P. Shinn, C. J. Palm, A. M. Southwick, H. C. Wu, C. Kim, M. Nguyen, P. Pham, R. Cheuk, G. Karlin-Newmann, S. X. Liu, B.

Lam, H. Sakano, T. Wu, G. Yu, M. Miranda, H. L. Quach, M. Tripp, C. H. Chang, J. M. Lee, M. Toriumi, M. M. Chan, C. C. Tang, C. S. Onodera, J. M. Deng, K. Akiyama, Y. Ansari, T. Arakawa, J. Banh, F. Banno, L. Bowser, S. Brooks, P. Carninci, Q. Chao, N. Choy, A. Enju, A. D. Goldsmith, M. Gurjal, N. F. Hansen, Y. Hayashizaki, C. Johnson-Hopson, V. W. Hsuan, K. Iida, M. Karnes, S. Khan, E. Koesema, J. Ishida, P. X. Jiang, T. Jones, J. Kawai, A. Kamiya, C. Meyers, M. Nakajima, M. Narusaka, M. Seki, T. Sakurai, M. Satou, R. Tamse, M. Vaysberg, E. K. Wallender, C. Wong, Y. Yamamura, S. Yuan, K. Shinozaki, R. W. Davis, A. Theologis, J. R. Ecker: Empirical analysis of transcriptional activity in the Arabidopsis genome. *Science* 302, 842-846 (2003)

118. Edgar R C: MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32, 1792-1797 (2004)

119. Clamp M, J. Cuff, S. M. Searle, G. J. Barton: The Jalview Java alignment editor. *Bioinformatics* 20, 426-427 (2004)

120. DeLano W L: The PyMOL Molecular Graphics System. World Wide Web URL: http://www.pymol.org (2002)

Footnotes: [1]hNMNAT1, hNMNAT2, hNMNAT3 (UniProtKB entries: NMNA1_HUMAN, NMNA2_HUMAN, NMNA3_HUMAN), [2] mjNMNAT (NadM_METJA), [3] ecNMNAT (NadR_ECOLI), ecNaMNAT (NadD_ECOLI), [4] scNMA1 (NMA1_YEAST), scNMA2 (NMA2_YEAST), [5] atNMNAT, [6] NCBI Protein Reference Sequences NP_780669.1 (mouse), NP_001041507.1 (rat), [7] cDNA name MGC:73048, [8] Direct submission to the Zebrafish Information Network (ZFIN, 51); ZFIN ID: ZDB-GENE-030131-8201, [9] According to The Structural Classification of Proteins (SCOP, 52) database; http://scop.mrc-lmb.cam.ac.uk/scop

Key Words : Pyridine Nucleotides, Energy Metabolism, protein structure, Rossmann fold, Compartmentation, ADP-ribosylation, Review

Send correspondence to: Mathias Ziegler, Department of Molecular Biology, University of Bergen, Thormøhlensgate 55, N-5008 Bergen, Norway, Tel: 47-55584591, Fax: 47-55589683, E-mail: mathias.ziegler@mbi.uib.no

http://www.bioscience.org/current/vol14.htm