Structural elements determining NAMPT activity

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Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2023

UNIVERSITY OF BERGEN

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Date of defense: 20.06.2023

Year: 2023

Title: Structural elements determining NAMPT activity

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Print: Skipnes Kommunikasjon / University of Bergen

"*Fluctuat nec mergitur*" (beaten by the waves, but does not sink)

Motto of the city of Paris (France)

Scientific environment

The work presented in this dissertation was carried out between May 2015 and July 2019 at the Department of Biological Sciences at the Faculty of Mathematics and Natural Sciences, and at the Department of Biomedicine at the Faculty of Medicine. This work was supervised by Professor Mathias Ziegler and co-supervised by Professor Nathalie Reuter. The University of Bergen funded the project.

Acknowledgements

The PhD training requires perseverance, resilience, and pugnacity, but it is only once you are doing it that you truly understand it. The Parisian motto "*fluctuat nec mergitur*" (beaten by the waves, but does not sink) is a nice analogy to my PhD training, but I would not have managed to go through this journey on my own.

Firstly, I thank my main supervisor Professor Mathias Ziegler for giving me the opportunity to join his group. I express gratitude to you for your support and our scientific discussions during my training.

I want to thank my co-supervisor Professor Nathalie Reuter for her scientific input and her kind words that aided to keep me on track.

I also want to thank Professor Ines Heiland for allowing me to be part of this collaboration, for her involvement in the progress, and her patience.

I thank all the NAD members for their scientific and personal support. First of all, Øyvind, thanks for introducing me to the cool world of NMR. Then, I want to acknowledge Magali and Marc for their precious feedback in the writing process. I would also like to thank Lars, Barbara, Ingvill, Lena, Roberto and the members of the NAT group with whom it felt good to share the ups and downs of this journey (#crazyfarm, #coffeeiscoffeing, #fridaybeer, #Heidi's).

I also want to thank Professor Øyvind Halskau that helped me through the transition MBI – BIO – BBB. Thanks for your counseling even though I was far away from "home".

Many thanks to all my colleagues from the former MBI, and especially Grethe, Diana, and Espen. Teaching was a pleasant adventure, and I am glad that I shared it with you.

I thank the French connection, e.g., Daniel, Jean-Luc, Edmond-Paul, and our artist Pascal, as well as the ladies, Anne, Florence, and Katy! Your support was precious and helps me to get through that experience of life. I think this book proves that the "hyenas" were wrong…

Finally, I thank Charlotte and Aude for their friendship. Merci les filles !

Chers parents, je vous suis infiniment reconnaissante pour votre amour, soutien et patience. Toutes ces expériences m'ont faite mûrir et cet aboutissement est aussi grâce à vous deux.

Michael, dit "Le Flamboyant", oh toi, le vrai franco-belge normand du sud de la France, merci pour ces fou-rires et tout le reste ;) Tu as contribué à me rendre plus forte.

Dorothée Houry,

February 2020

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PAPER II Dorothée Houry*, Arne Raasakka*, Petri Kursula and Mathias Ziegler (2019): Identification of structural determinants of NAMPT activity and substrate selectivity.

(Manuscript in revision)

**Shared first authorship*

Selected abbreviations

ADP	adenosine diphosphate
ADPr	ADP-ribose
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BeF_3	beryllium fluoride
CD38/CD157	cluster of differentiation 38/cluster of differentiation 157
DNA	deoxyribonucleic acid
DNA-PKcs	DNA dependent protein kinase
ETC	electron transport chain
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK3	glycogen synthase kinase 3
HMG-CoA	3-hydroxy-3-methyl-glutaryl-Coenzyme A
HMGCS2	3-hydroxy-3-methyl-glutaryl- Coenzyme A synthase 2
HSP70	heat shock protein 70
LC-MS	liquid chromatography-mass spectrometry
MART	mono-ADP-ribosyltransferase
Mg^{2+}	magnesium
mRNA	messenger ribonucleic acid
NA	nicotinic acid
NAAD	nicotinic acid adenine dinucleotide
NAD	nicotinamide adenine dinucleotide
NADA	nicotinamidase
NADK	nicotinamide adenine dinucleotide kinase
NADP	nicotinamide adenine dinucleotide phosphate
NADS	nicotinamide adenine dinucleotide synthetase
Nam	nicotinamide
NAMN	nicotinic acid mononucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NAPRT	nicotinic acid phosphoribosyltransferase
NAR	nicotinic acid riboside
NFκB	nuclear factor-kappa B

NLS	nuclear localization signal
NMNAT	nicotinamide mononucleotide adenylyltransferase
NNMT	nicotinamide N-methyltransferase
NMR	nuclear magnetic resonance
NR	nicotinamide riboside
NRK	nicotinamide riboside kinase
OH	hydroxyl
PARP	poly-ADP-ribose polymerase
PBEF	pre-B cell colony-enhancing factor
PDB ID	Protein Data Bank identification
pHis	phosphorylated histidine
P_i	inorganic monophosphate
PP_i	inorganic pyrophosphate
PRPP	phosphoribosyl pyrophosphate
PRTase	phosphoribosyltransferase
PTM	post-translational modification
QA	quinolinic acid
QAPRT	quinolinic acid phosphoribosyltransferase
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
SAXS	small-angle X-ray scattering
SIRT	sirtuin
Tpt1	tRNA 2′-phosphotransferase
tRNA	transfer ribonucleic acid

Abstract

Nicotinamide adenine dinucleotide (NAD) is a hydride carrier involved in redox reactions in metabolism. Additionally, NAD^+ is a substrate of enzymes that mediate vital NAD^+-dependent processes, in which the dinucleotide is cleaved, releasing a nicotinamide moiety (Nam). The maintenance of these processes requires a continuous replenishment of NAD, which is mainly done by the Nam salvage pathway in mammals. The rate-limiting enzyme of this pathway, nicotinamide phosphoribosyltransferase (NAMPT), uses Nam and phosphoribosyl pyrophosphate (PRPP) as substrates for which the affinity increases following NAMPT's auto-phosphorylation on histidine 247 (pHis247), a key amino acid in the active site. Interestingly, the mammalian NAMPT has an exceptionally high affinity for Nam compared to bacterial enzymes. The goal of the study was to define the structural adaptations of NAMPT leading to such a high affinity for its substrate, as well as the structural basis of the regulation of Nam affinity.

Multiple protein sequence alignment identified in deuterostome NAMPT the occurrence of a stretch of ten amino acids which forms a flexible surface loop (β1-β2 loop). We investigated, therefore, the possible impact of this loop on the protein structure, activity and substrate affinity using a mutant lacking these ten amino acids. Deletion of the β1-β2 loop drastically reduced the affinity for Nam while the overall structure remained largely unaffected. Furthermore, a putative nuclear localization signal was identified in the β1-β2 loop. However, we could not confirm its involvement in NAMPT subcellular localization.

To understand how pHis247 regulates Nam affinity in NAMPT, we compared the binding of the native substrate and nicotinic acid, which has a far lower affinity, by X-ray crystallography and activity assays. The analyses led us to propose that the coordination of Mg^{2+} by the phosphate group of pHis247 optimizes the positioning of PRPP, thereby favoring the reaction with the pyridine base.

The evolutionary selection of Nam salvage by NAMPT in higher animals coincides with both a diversification of NAD-dependent signaling and the occurrence of the β1-β2 loop. This β1-β2 loop appears to be necessary to meet the need for highly efficient

Nam recycling, in addition to the phosphorylation of His247. This work provides new insights into the evolution and catalytic mechanism of an important NAD biosynthetic enzyme.

1. Introduction

Arising in the 18th century, in a context of extreme poverty, a fatal disease named Pellagra struck the less privileged populations in Europe and North America. The typical symptoms, also referred to as the "4Ds", were dermatitis, diarrhea, dementia, and eventually, death (1). Funk suggested in 1912 that Pellagra was caused by the lack of a vitamin, which was later confirmed by Goldberger who identified the vitamin B (2,3). However, it was only in 1937 that Conrad Arnold Elvehjem, the son of a Norwegian emigrate, identified the anti-pellagric properties of nicotinamide and nicotinic acid in the canine version of the disease (2,4). The supplementation with these compounds (called vitamin B3) to the target population eradicated the disease. But even nowadays, Pellagra-like symptoms sometimes re-emerge in situations of malnutrition (5,6). Vitamin B3 intake can originate from diverse food categories but the most enriched one is animal-based food, and notably red meat (7). The latter being of importance since it has even been suggested that vitamin B3 greatly contributed to the development of particular intellectual skills and robustness of the meat-addicted *Homo neanderthalensis* (8,9).

Vitamin B3 is essential to the organism because of its role as a precursor of nicotinamide adenine dinucleotide (NAD), which is a coenzyme and substrate required for the activity of a multitude of enzymes. According to the KEGG REACTION database (as of February 2020), NAD participates in ~2400 biochemical reactions, which is ~3 times more than ATP (10-15). Indeed, NAD is involved in fundamental cellular functions, making this dinucleotide an essential metabolite for the cell.

1.1. The functions of NAD in the cell

1.1.1. NAD is a hydride carrier

NAD is classically described as a hydride (a hydrogen atom with two electrons) carrier involved in major metabolic pathways (Table 1, in pink). The reduction of NAD^+ to NADH accompanies the activity of many dehydrogenases involved, for example, in

the β-oxidation of fatty acids, glycolysis, the citric acid cycle, and gluconeogenesis (16). The oxidation of NADH is required for the mitochondrial electron transport chain (ETC) which drives ATP production (17). NAD^+ can be further phosphorylated by NAD kinase at the OH-group of C2' of the adenosine moiety (Figure 1), forming $NADP^+$.

Table 1. Examples of enzymes using NAD^+/NADH and $NADP^+$/NADPH as redox agents.

Glycolysis	pyruvate dehydrogenase complex	reduction of NAD^+	Catabolism
	glyceraldehyde-3-phosphate dehydrogenase		
Fermentation	lactate dehydrogenase	oxidation of NADH	
	alcohol dehydrogenase		
β-oxidation (saturated fatty acids)	3-hydroxyacyl-CoA-dehydrogenase	reduction of NAD^+	
Citric acid cycle	isocitrate dehydrogenase		
	α-ketoglutarate dehydrogenase		
	malate dehydrogenase		
Glutamate deamination	glutamate dehydrogenase		
Mitochondrial ETC	NADH dehydrogenase (ubiquinone/complex I)	oxidation of NADH	
Degradation of (unsaturated) fatty acids	2,4-dienoyl-CoA-reductase	oxidation of NADPH	
Gluconeogenesis	lactate dehydrogenase	reduction of NAD^+	Anabolism
	malate dehydrogenase (formation of oxaloacetate		
	malate dehydrogenase (formation of malate)	oxidation of NADH	
	glycerol-3-phosphate dehydrogenase	reduction of NAD^+	
Pentose phosphate pathway	glucose-6-phosphate dehydrogenase	reduction of $NADP^+$	
	phosphogluconate dehydrogenase		
Lipid synthesis	3-keto-acyl-(ACP) reductase	oxidation of NADPH	
	enoyl-(ACP)-reductase		
Cholesterol synthesis	HMG-CoA reductase		
	squalene synthase		
	squalene mono-oxygenase		
Detoxification	cytochrome p450 reductase	oxidation of NADPH	Other
Anti-oxidant defense	glutathione reductase		

The reduced form of $NADP^+$, NADPH, is the predominant form in the cell. NADP is mainly involved in the reductive anabolism with, notably, the lipids and cholesterol synthesis, but also in the oxidative anabolism with the pentose phosphate pathway (Table 1, in grey), (18-20). Additionally, NADPH contributes to the catabolism of unsaturated fatty acids, the detoxification and the oxidative defense (21-24).

1.1.2. The signaling functions of NAD

A striking observation in evolution is the increased complexity of organisms which is accompanied with a rise in cellular processes and signaling pathways (25-29). Among them, the NAD^+-dependent signaling pathways are of particular importance since they are fundamental for organism survival.

In these pathways, NAD^+ is consumed by different classes of enzymes such as the ADP-ribosyltransferases, the sirtuins, and CD38/CD157. These NAD^+-dependent enzymes cleave NAD^+, releasing Nam and ADP-ribose (or cyclic ADP-ribose).

The first of these discovered NAD^+-consuming signaling pathways was ADP-ribosylation. This reaction consists in the addition of one (mono-) or several (poly-) ADP-ribose moieties mainly targeting proteins (30,31). This is catalyzed by ADP-ribosyltransferases (ARTs) which are either mono-ADP-ribosyltransferases (MARTs) or poly-ADP-ribosylpolymerases (PARPs). The PARP family contains the majority of known ARTs. It is composed of 17 members involved in various functions such as DNA repair, transcription regulation, cohesion of telomeres, and formation of the mitotic spindle, among others (32-36). ADP-ribosylation in these processes can consume a large amount of NAD. For instance, PARP1, whose activity is known to increase following DNA damage, can cause a decrease of 10-20% of total cellular NAD within 5-15 minutes in mouse leukemic cells following gamma-radiation (37-39). Recent studies have shown that nucleic acids are also the targets of ADP-ribosylation catalyzed by PARP10, -13, -15, and other enzymes. For instance, bacterial single-stranded DNA and fungal tRNAs are ADP-ribosylated by DarT and Tpt1, respectively (40-43).

NAD is also involved in the regulation of protein acetylation, a major post-translational modification (PTM) of protein. Discovered in the 1990s, the budding yeast sirtuin Sir2p was the first NAD^+-dependent histone deacetylase described for its role in the transcriptional silencing of the mating-type loci (44,45). Even though sirtuins are encountered in the three domains of life (i.e. Archaea, Bacteria, and Eukaryota), the number of orthologues varies from one kingdom to another. Indeed, only one orthologue is described in bacteria, while seven are identified in mammals. Sirtuins have many targets ranging from histones (e.g. histone H3 lysine 9) to transcription factors (e.g. p53), and metabolic enzymes (e.g. HMGCS2, (46-50)). They are therefore involved in numerous cellular processes, including gene silencing, circadian rhythm, metabolism regulation, and aging (51-53).

Calcium signaling is the third major NAD^+-dependent pathway involving two membrane proteins, CD38 and CD157. These enzymes, by cleaving NAD^+, generate cyclic and non-cyclic ADP-ribose, which bind to receptors or channels. The cyclic form stimulates calcium release from organelles into the cytosol, and the non-cyclic form supports the extracellular calcium intake (54-56). One of calcium's roles is to activate cytosolic signaling molecules (e.g. calcium-sensitive adenylate cyclase), initiating a cascade of downstream effectors, such as transcription factors (e.g. CREB, (57)). By regulating the cellular calcium pool, NAD indirectly regulates gene expression, as well as the release of neurotransmitters in synapses (58-60).

ADP-ribosylation, deacylation, and calcium signaling are the most studied NAD^+-consuming pathways. However, this dinucleotide is also involved in other functions. For instance, NAD^+ is an adenosine monophosphate donor for the bacterial ligase, forming DNA phosphodiester bonds in nicked DNA strands (61). Recently, it was shown that the entire molecule of NAD is used as an alternative system for the capping of RNAs. Around 13% of mRNAs in bacteria *Bacillus subtilis* and ~5% in *Arabidopsis thaliana* are capped with NAD^+ (62,63). The capping of RNAs by NAD seems to protect RNAs from degradation in bacteria while, on the contrary, it appears to promote RNA decay in mammals (64).

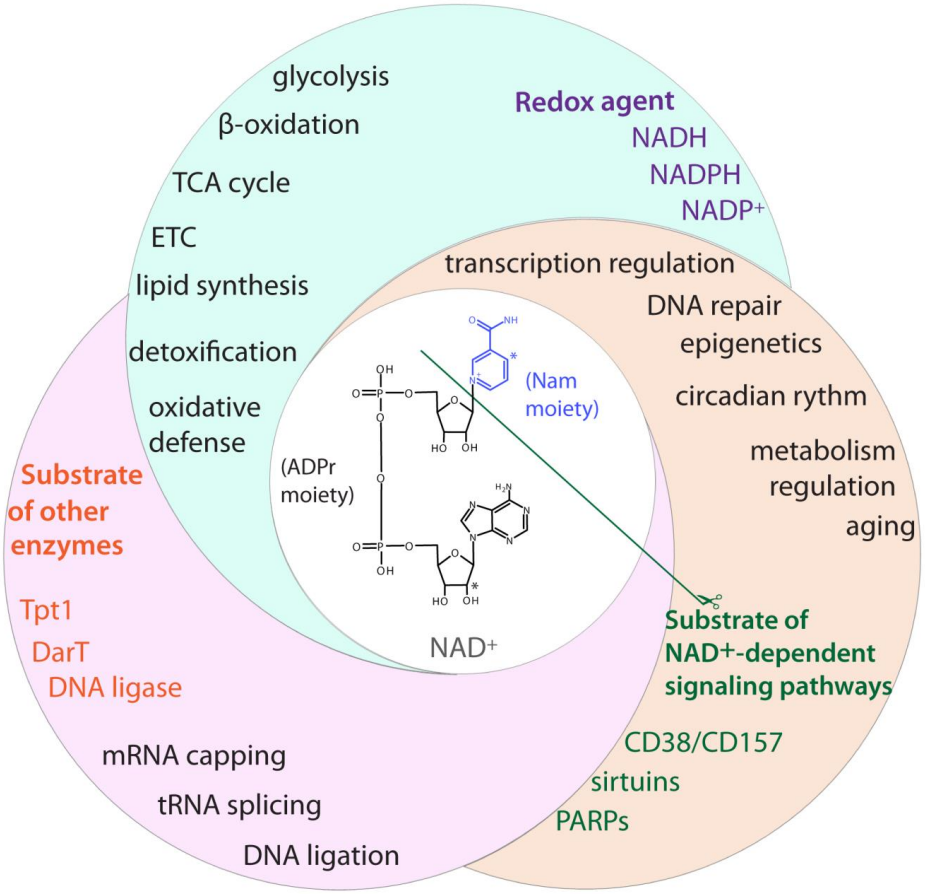

Figure 1: NAD is involved in various and important functions for the cell. (White circle): Nicotinamide adenine dinucleotide is a dinucleotide composed of a nicotinamide moiety (Nam, in blue) and an ADP-ribose moiety (ADPr, in black). The oxidized form (NAD^+) gains a hydride on C3 (blue asterisk) of the Nam moiety, forming the reduced form NADH. NAD can be phosphorylated by NAD kinase at the OH-group of C2' (black asterisk) of the adenosine moiety, forming $NADP^+$ and NADPH. (Green circle): NAD^+/NADH and $NADP^+$/NADPH are used as hydride carriers in many metabolic reactions, as well as in the detoxification and oxidative defense. (Orange circle) NAD^+ is a substrate for NAD^+-consuming enzymes (i.e. PARPs, sirtuins, and CD38/CD157). These enzymes cleave (green line) the glycosidic bond connecting C1' to the nitrogen atom of the Nam moiety, releasing Nam and ADP-ribose. The PARPs are notably involved in DNA repair and transcription regulation; the sirtuins regulate the circadian rhythm, epigenetics, regulation of the metabolism, and aging; CD38/CD157 generate ligands of receptors involved in calcium signaling. (Pink circle): NAD^+ supports the ADP-ribosylation of DNA (DarT) and tRNAs (Tpt1), capping of mRNAs as well as DNA ligation (bacterial DNA ligase). TCA cycle, citric acid cycle; ETC, electron transport chain.

1.2. NAD biosynthesis

NAD is at the crossroad of metabolism and signaling functions that are crucial for cell survival, proliferation, and differentiation. Whereas the interconversion of the oxidized and reduced forms of NAD does not alter its concentration, the signaling functions are accompanied by the cleavage of the molecule. The turnover of NAD^+ is tissue-dependent and can be shorter than an hour in murine spleen and gut (65). It is clear that, in order to fulfill all these functions, NAD consumption must be balanced accordingly to its anabolic rate.

1.2.1. The precursors of NAD

NAD can be synthesized by several pathways depending on the precursor used (Figure 2). These metabolites are nicotinamide (Nam) and nicotinic acid (NA), also referred to as niacin and vitamin B3. Additional precursors are quinolinic acid (QA) and the riboside form of Nam (NR) and NA (NAR).

The diet is the source of precursors either directly, or after the cleavage of NAD and NADP (7). L-tryptophan and L-aspartate are the precursors of QA in eukaryotes and prokaryotes, respectively. QA is the result of several reactions also known as the kynurenine pathway (66).

A common structural feature of these precursors is a pyridine ring (Figure 2, grey box). An amido group is present in Nam but not in NA, while a carboxylic group, conferring a negative charge at physiological pH, is present in NA.

These precursors are substrates of enzymes that initiate different pathways in the NAD synthesis, namely, the salvage pathway, the Preiss-Handler pathway, the *de novo* pathway, and the auxiliary pathways. Regardless of the precursor, the synthesis of the dinucleotide consists of two major steps that are the generation of a mononucleotide (Figure 2, pink box) followed by the formation of a dinucleotide (Figure 2, green box).

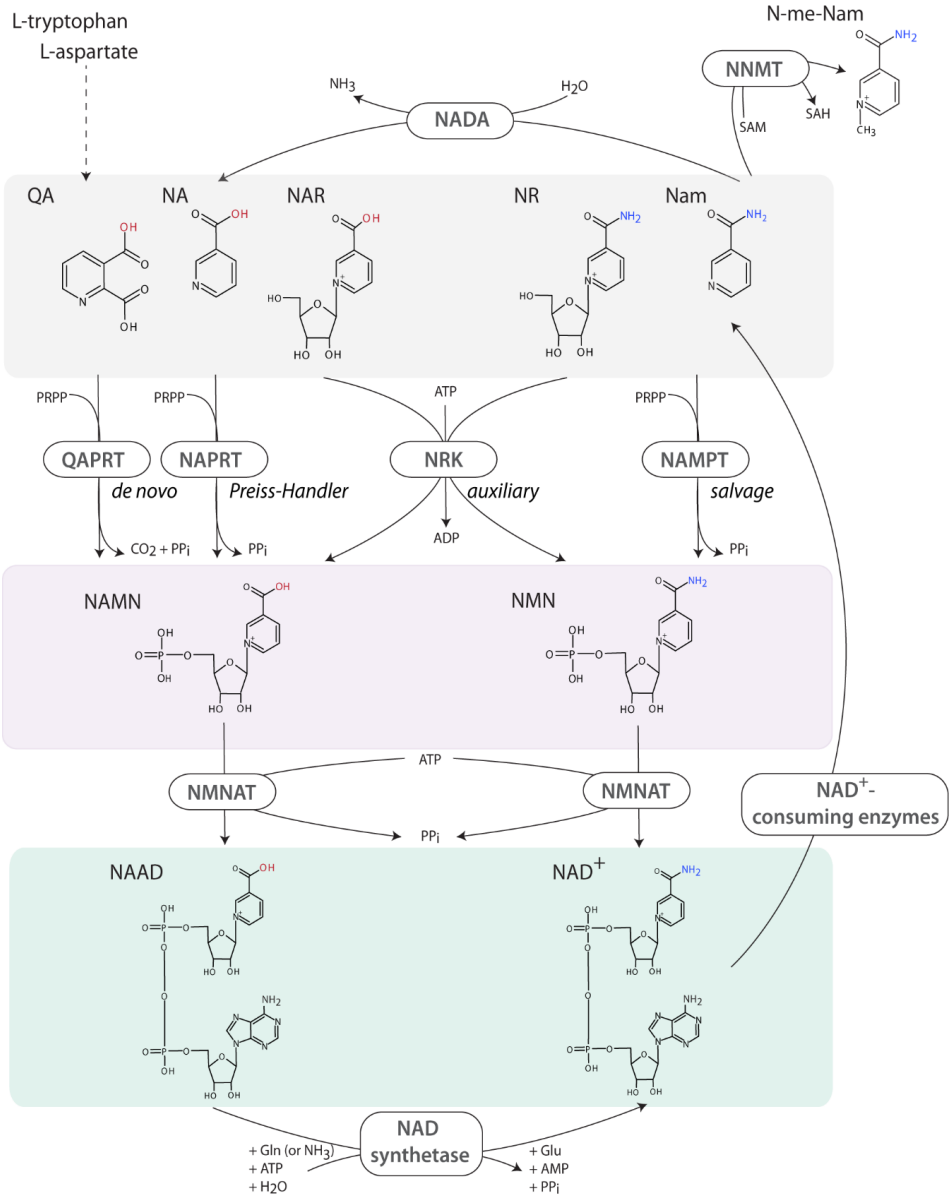

Figure 2: NAD biosynthesis in the cell. The amidated precursors, nicotinamide (Nam) and nicotinamide riboside (NR), are converted into nicotinamide mononucleotide (NMN) by Nam phosphoribosyltransferase (NAMPT) and NR kinase (NRK), respectively. NMN is further converted into NAD^+ by NMN adenylyltransferase (NMNAT). The acidic precursors, nicotinic acid (NA), nicotinic acid riboside (NAR), and quinolinic acid (QA), are converted into nicotinic acid mononucleotide (NAMN) by NAPRT, NRK, and QAPRT, respectively. NAMN is then converted into nicotinic acid adenine dinucleotide (NAAD) by NMNAT. Finally, NAAD is amidated into nicotinamide adenine dinucleotide (NAD^+) by NAD^+ synthetase (glutamine or ammonia can be the amide donor). NAD^+ is consumed by the sirtuins,

PARPs, and CD38/CD157 releasing the precursor Nam in the cell. The excess of Nam is methylated by the nicotinamide N-methyltransferase (NNMT), using the methyl donor S-adenosyl-L-methionine (SAM) that is then transformed into S-adenosyl-L-homocysteine (SAH) in the reaction. The precursor of QA in vertebrates is L-tryptophan, while in most of the plants, bacteria, and fungi the source of QA is L-aspartate. Most bacteria achieve the hydrolysis of Nam into NA by the nicotinamidase (NADA). The precursors, the mononucleotide intermediates, and the dinucleotides are represented in a grey, purple, and green box, respectively. The name of the pathway is italicized under the name of the enzyme. The abbreviations used are: ATP, adenosine triphosphate; ADP, adenosine diphosphate; PP_i, inorganic pyrophosphate; Gln, glutamine; Glu, glutamate; NH_3, ammonia.

1.2.2. The salvage pathway mediated by NAMPT

As mentioned previously, the degradation of NAD^+ by the different NAD^+-dependent signaling pathways is accompanied with the release of Nam, which in turn serves as a precursor for the salvage pathway. In the first step, Nam phosphoribosyltransferase (NAMPT) catalyzes the conversion of Nam and 5-phosphoribosyl-1-pyrophosphate (PRPP) to generate nicotinamide mononucleotide (NMN) and pyrophosphate (PP_i). NMN is subsequently converted to NAD^+ by nicotinamide mononucleotide adenylyltransferases (NMNATs, (67,68)).

1.2.3. The Preiss-Handler and *de novo* pathways

Both the Preiss-Handler and the *de novo* pathway comprise three steps for NAD biosynthesis, each starting with a different acidic precursor.

The Preiss-Handler pathway starts with the acidic form of vitamin B3. NA is converted with PRPP into nicotinic acid mononucleotide (NAMN) by the specific phosphoribosyltransferase NAPRT (69). In the *de novo* pathway, QA is converted with PRPP into NAMN by another phosphoribosyltransferase, QAPRT. The acidic mononucleotide is then converted by NMNATs in the presence of ATP into nicotinic acid adenine dinucleotide (NAAD). In the last step, NAAD is amidated to NAD^+ by ATP-dependent NAD^+ synthetase. This enzyme uses glutamine as donor of the amido group, however, some lower organisms (e.g. *Escherichia coli*) use ammonia instead (67,68,70-72).

1.2.4. The auxiliary pathways

Two additional precursors, the riboside form of NA (NAR) and Nam (NR), participate in the synthesis of NAD. Both metabolites can be phosphorylated by NA/Nam riboside kinase (NRK). ATP is used as a phosphate donor by NRK to convert NR to NMN and NAR to NAMN (73). Interestingly, the NR-based pathway was shown to be a reliable backup in the cell types that depend on the salvage pathway when NAMPT is inhibited (74).

1.2.5. Nam, a substrate of other enzymes

Nicotinamidase, NADA (encoded by the bacterial gene *PncA*), hydrolyzes Nam into its acidic counterpart, NA, generating ammonia in the process. Thereby, this reaction provides the precursor needed for the Preiss-Handler pathway and represents another pathway for Nam salvage in bacteria, plants, and fungi (75,76). Being a NAD biosynthetic enzyme, *Mycobacterium tuberculosis* NADA was proposed to be a promising target for the treatment of tuberculosis. The enzyme converts the prodrug pyrazinamide into its acidic counterpart pyrazinoic acid that possesses bactericidal activity (77).

Furthermore, Nam can also be used by another enzyme called nicotinamide N-methyltransferase (NNMT). NNMT catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to the pyridinic-nitrogen of Nam, converting SAM into S-adenosyl-L-homocysteine (SAH, (78,79)). Methylated Nam (N-me-Nam) can be oxidized into pyridones or directly secreted from the cell and excreted with the urine (80,81). The elimination of Nam by NNMT may be beneficial for maintaining the efficiency of the NAD^+-dependent signaling pathways. Indeed, the Nam newly released from NAD^+ cleavage exerts negative feedback on the activity of NAD^+-dependent enzymes, notably, for the PARPs and sirtuins (82-84).

1.3. NAD biosynthesis in vertebrates compared to bacteria, plants, and fungi

The precursors, intermediates, and dinucleotides involved in NAD biosynthesis can be divided into two groups: the amidated (including Nam, NR, NMN, and NAD) and the acidic metabolites (including NA, NAR, QA, NAMN, and NAAD). The selection of pathways leading to amidated or acidic intermediates interestingly diverges between species.

Vertebrates favor the NAD synthesis through the Nam salvage pathway mediated by NAMPT. This can be explained by the availability of Nam due to a supply from the diet, a constant synthesis by the liver, and the degradation of NAD^+ resulting from the NAD^+-dependent signaling (65,85,86). Then, NAMPT binds Nam with a particularly high affinity within the nanomolar range, contributing to make that pathway predominant in the cells (87). However, the tissue-specificity of the gene expression of NAD biosynthetic enzymes can dictate the pathways used in the cell. For example, the low expression of *NAPRT* and *NAMPT* in neurons is associated with the preference for the *de novo* pathway from tryptophan and salvage from NR (37,88,89).

On the contrary, most of the bacteria, plants, and fungi favor the synthesis of the acidic intermediate NAMN to achieve NAD synthesis. The NAMN formation is either the result of the *de novo* pathway from aspartate, or Nam salvage mediated by NADA (90). The preference for the synthesis of acidic intermediates is surprising since it requires the consumption of ATP during the final conversion of NAAD into NAD^+. This makes the acidic precursor-based pathway more energy-demanding than the salvage from Nam via NAMPT.

The transition in the Nam salvage from the four-step pathway with NADA in most bacteria, plants, and fungi to the two-step pathway with NAMPT in vertebrates raises the question about the event causing this evolution towards this “simpler” pathway.

1.4. The occurrence of *NAMPT, NADA, NNMT*, and the NAD^+-dependent enzymes in evolution

NAMPT and *NADA* are both spread across the kingdoms (Figure 3) but their occurrence differs from a kingdom to another. *NAMPT* has the lowest abundance in bacteria, plants and fungi, and the highest in metazoans. On the contrary, *NADA* has the highest abundance in bacteria, plants and fungi, and the lowest in metazoans. Within the metazoans, the occurrence of *NADA* drops drastically in deuterostomes, while it is maintained in cnidarians and prevails in protostomes (Figure 3). On the other hand, the occurrence of *NAMPT* is favored in deuterostomes, maintained in cnidarians, and drops in protostomes (90-92). Even though *NAMPT* and *NADA* are rarely seen within the same organism, they are not mutually exclusive. Indeed, both genes are present in a few marine organisms (cnidarians), indicating that the two pathways for Nam salvage via NADA and NAMPT can function simultaneously (93,94).

In parallel to the switch to the NAMPT-based salvage pathway in deuterostomes, new NAD^+-dependent enzymes have arisen such as the ADP-ribosyl cyclase and the Protein-arginine ADP-ribosyltransferase. In addition to the new NAD^+-degrading activities, deuterostomes display an increase in the number of PARP and sirtuin members (91,95). The diversification and increase in NAD^+-dependent signaling pathways lead to a rise in NAD consumption, which requires an efficient NAD synthesis. This suggests that the transition from a four-step to a two step-pathway may have been a more advantageous evolutionary adaptation to maintain the cellular NAD pool under increasing demands for NAD.

Besides the increasing number of NAD^+-consuming activities, the Nam-consuming enzyme NNMT emerged along with NAMPT (91). Selecting NNMT which targets Nam for secretion seems paradoxical. One can question the efficiency of a system that reduces the availability of the main precursor in NAD synthesis when there is an increase in NAD consumption.

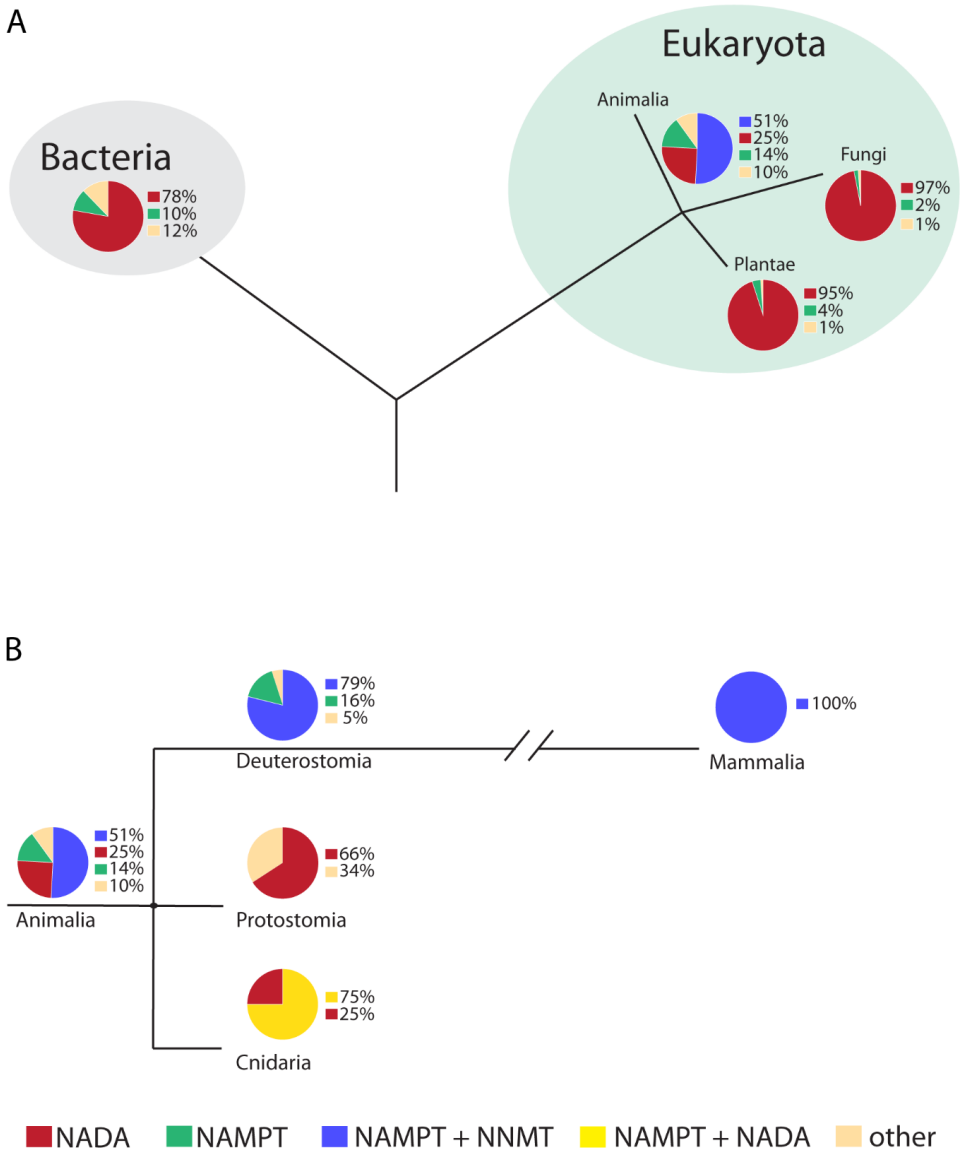

Figure 3: The occurrence of *NAMPT*, *NNMT*, and *NADA* in Bacteria and Eukaryota. The genes encoding NAMPT, NNMT, and NADA are found in all the domains of life as shown in the general (A) or focused on the Animalia kingdom (B). In (A) Bacteria and Eukaryota are represented in grey and light green, respectively. The pie charts represent the distribution of species in which *NADA* (red), *NAMPT* (green), *NAMPT* and *NNMT* (yellow), *NAMPT* and *NADA* (blue), and other combinations of NAD biosynthetic enzyme-encoding genes (black) occur. Among the three kingdoms of Eukaryota, Animalia shows a significant loss in *NADA* and, on the contrary, a gain in *NAMPT* as well as in the combination of *NAMPT* and *NNMT*, the latter being conserved in Mammalia. However, other combinations of genes exist such as *NADA-NAMPT-NNMT* in hemichordates and *NADA-NNMT* in some nematodes (not shown). Figure adapted from Figure 2 of (96).

The co-occurrence of NAMPT and NNMT is frequent but not mandatory. Some nematodes exhibit the co-occurrence of NADA-NNMT while other organisms like cyanobacteria only possess NAMPT (92). This is the case for two specific strains of cyanobacteria, namely, *Synechocystis* sp. strain PCC 6803 and *Synechococcus elongatus*, that both possess *NAMPT* without *NNMT*. Unlike most bacteria, these two strains can directly convert Nam into NMN instead of using the hydrolysis of Nam into NA. The characterization of the NAMPT from *Synechocystis* sp. strain PCC 6803 by Gerdes*, et al.* (2006) revealed this enzyme to display a far lower affinity for Nam than the human orthologue. In this organism, the absence of *NADA* and the selection for *NAMPT* break the pattern of the traditional four-step route for the NAD synthesis in bacteria (97).

The increasing number of NAD^+-dependent signaling activities, together with the co-occurrence of *NAMPT* and *NNMT*, emerged along with the organismal complexity. In this system, NAMPT is the rate-limiting step of the favored salvage pathway. However, this central role of NAMPT does not fit with the low affinity for Nam observed in the bacterial enzyme. The evolutionary events that made of NAMPT such an important enzyme in vertebrates remain unknown. Since *NNMT* does not exist in cyanobacteria, one may hypothesize that the appearance of *NNMT*, competing for the same substrate, supported the evolution of a NAMPT with a higher affinity for Nam in vertebrates.

1.5. Protein structure and enzymatic activity of NAMPT

The activity of NAMPT as part of mammalian NAD biosynthesis was discovered in the 1950s (98,99). However, the molecular identity of the enzyme was identified much later. Also, the enzyme has been characterized twice in another context than NAD biosynthesis. In 1994, the protein was identified as pre-B cell colony-enhancing factor (PBEF) which, after secretion from immune cells, is involved in the activation of lymphocytes (100). Eleven years later, a hormone-like function of the protein was suggested, referred to as visfatin. A study from a Japanese group reported the protein to exhibit insulin-like functions upon its secretion from visceral fat. However, this publication was retracted due to a lack of reproducibility (101). It was only in 2001,

that these different functions were attributed to the same protein, NAMPT, thanks to the identification of the gene (102,103). The central role of NAMPT in NAD synthesis led to numerous studies, providing an extensive source of information, including its protein structure.

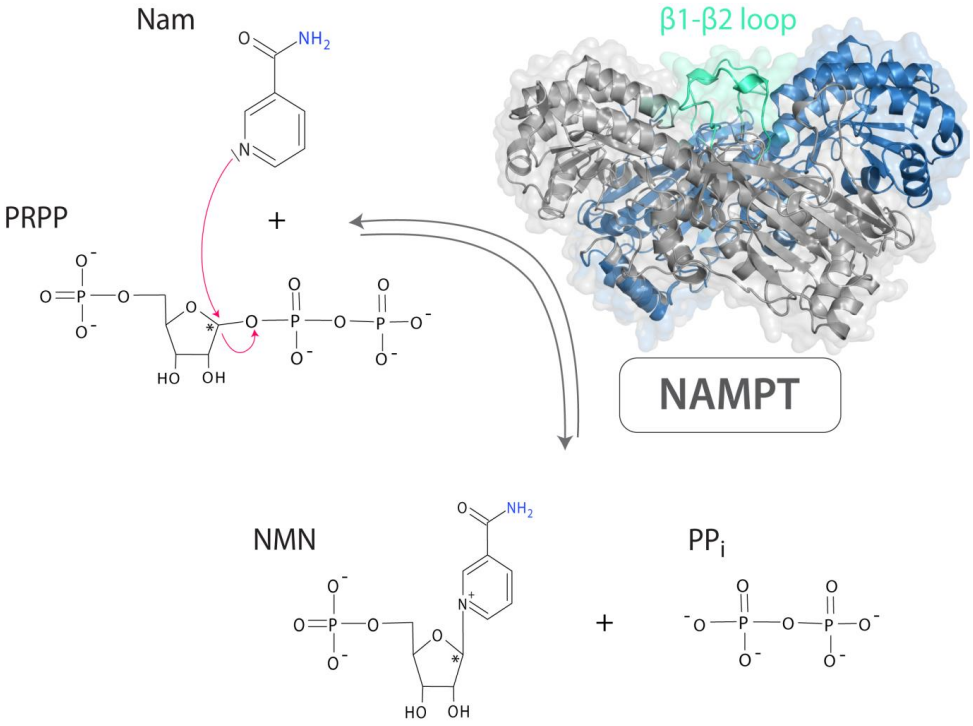

Figure 4: NAMPT-catalyzed reaction. NAMPT catalyzes the reversible transfer of the phosphoribose moiety from 5-phosphoribosyl-1-pyrophosphate (PRPP) to nicotinamide (Nam), which produces nicotinamide mononucleotide (NMN) and releases inorganic pyrophosphate (PP_i). The nucleophilic substitution occurs on the anomeric carbon C1' (asterisk) of the ribose moiety. The red arrows indicate the migration of pairs of electrons for the forward reaction. NAMPT is a homodimer (the monomers are represented in blue and grey). The β1-β2 loop (green) of each monomer is localized on the same side of human NAMPT, in the vicinity of the entrance of the active site. The β1-β2 surface loops were modeled with *SWISS-MODEL*.

1.5.1. Catalytic mechanism and substrate affinity

NAMPT catalyzes the reversible Mg^{2+}-dependent formation of NMN and its catalytic mechanism is a nucleophilic substitution by electrophile migration (Figure 4). The C1' of the ribose moiety of PRPP and NMN is the anomeric carbon that binds to the nucleophiles, Nam and PP_i. This mechanism appears associated with the formation of a ribo-oxocarbenium ion in the transition state (104).

Studies performed on murine and human NAMPT revealed a high affinity of the enzyme for Nam with a K_M ranging from 855 nM to 1.24 μM (93,103,105). The affinity of the human enzyme appears even higher for the substrate PRPP with K_M of 7 nM (105). This suggests that PRPP is the first substrate binding to the active site.

With this high affinity, NAMPT appears to be Nam specific (93). However, it was reported that the human enzyme can also catalyze the formation of NAMN from NA *in vitro* but with lower efficiency compared to the NMN synthesis (106). Finally, human NAMPT was also shown to convert the rodenticide agent Vacor into Vacor mononucleotide, but this molecule has been withdrawn from the market due to its acute toxicity on the population (107).

1.5.2. Protein structure and substrate binding

NAMPT is a type II phosphoribosyltransferase (PRTase) containing an irregular five-stranded α/β-barrel and a phosphate-binding motif (referred to as "PRPP-loop") which does not contain the established 13 conserved residues present in type I PRTases (108,109).

NAMPT is a homodimer with two active sites, each of them formed at the interface of two protomers, implying that the monomeric form is inactive (Figure 5A). The conserved α/β-barrel forms a tunnel corresponding to the active site. One entrance of the tunnel is rather small and due to its geometry, is considered to confer specificity for Nam. The second entrance, larger, allows access for PRPP and the products, namely, NMN and PP_i (110).

The binding of Nam participates in the maintenance of the oligomeric state. Indeed, the aromatic ring of Nam is involved in a π-stacking interaction between Tyr18 and Phe193, each residue belonging to a different protomer. In addition, the carbonyl group of Nam interacts with Arg311 whereas the nitrogen atom from the amide group forms a hydrogen bond with Asp219 (Figure 5B).

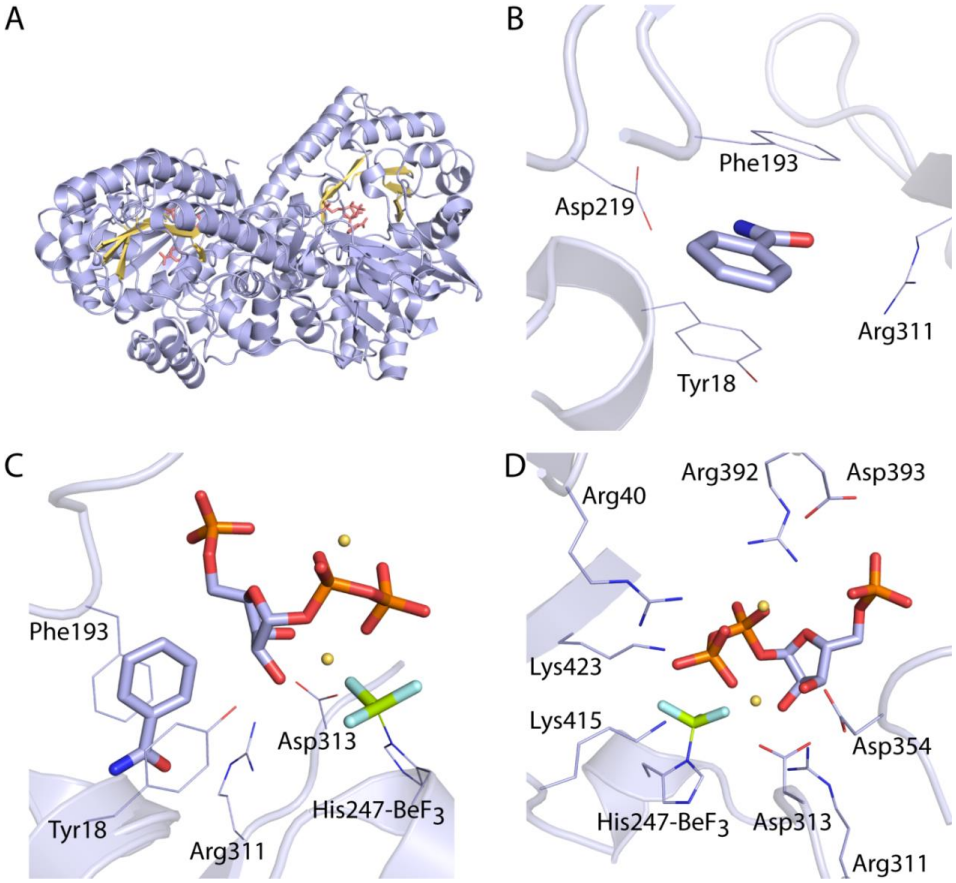

Figure 5: Substrates binding in human NAMPT. Crystal structure of human NAMPT in complex with benzamide, PRPP, and Mg^{2+} (PDB ID 3DKL, (87)). Cartoon representation of the dimer (A) where the strands of the α/β-barrel tunnel are highlighted in yellow and the substrates in red. Zoom into the active site and view focused on the binding of benzamide (B), benzamide and PRPP (C), and PRPP (D). The conjugate His247-BeF_3 mimics the phosphorylation of His247. Mg^{2+} is shown as yellow spheres.

As illustrated in figure 5C-D, the ribose moiety interacts with Arg311, Asp313, and Asp354 but remains flexible, allowing its rotation towards Nam or PP_i during the nucleophilic substitution. The pyrophosphate moiety is mainly stabilized by positively charged residues, i.e. Arg40, Arg392, Lys415 and Lys423 (106,111). Moreover, the OH-groups from the pyrophosphoribose moiety coordinate a molecule of Mg^{2+}, which is in the vicinity of His247.

1.5.3. Factors regulating the enzymatic activity

1.5.3.1. Increased substrate affinity by auto-phosphorylation

In 1982, Elliott, *et al.* discovered that human fibroblasts in culture with ATP increased the activity of endogenous NAMPT (112). The positive effect of ATP on NAMPT activity was explained by the auto-phosphorylation of the enzyme. This property was based on sequence similarities shared with another NAD biosynthetic enzyme, NAPRT, whose activity is upregulated by ATP.

A sequence alignment between *Salmonella typhimurium* NAPRT and human NAMPT indicated a histidine residue at position 247 (His247) in human NAMPT to be similar to histidine 219 (His219) in the NAPRT from *Salmonella typhimurium*. Based on the described auto-phosphorylation of His219 in the *Salmonella typhimurium* NAPRT, His247 of human NAMPT was investigated (112). Site-directed mutagenesis of His247 to glutamate or alanine reduced the enzymatic activity of human NAMPT in the presence of ATP, substantiating the importance of that residue in the catalysis (106,111).

The apparent phosphorylation of His247 increases the affinity of NAMPT by a ~160-fold factor for Nam and a ~10-fold factor for PRPP. In the presence of ATP, the K_M for Nam and PRPP is lowered to 5 nM and 0.6 nM, respectively (105,113). The precise mechanism by which the auto-phosphorylation increases the substrate affinity is not fully understood. However, it is assumed that the binding of the substrates and ATP cannot happen simultaneously in the active site. A crystal structure of the enzyme with

a non-hydrolyzable ADP analog (AMPcP) revealed that this ligand would compete with the binding of both substrates Nam and PRPP (87).

1.5.3.2. Regulation of the activity by pharmacological compounds

Several NAMPT inhibitors have been generated starting with APO866, also known as FK866 (114). This inhibitor remains widely used in research. FK866 is a competitive inhibitor that shares a common pyridine ring with Nam that binds between Tyr18 and Phe193 where Nam is stacked. The rest of the molecule is protruding outside the active site via the small entry of the tunnel, making FK866 a NAMPT-specific inhibitor. With a high affinity of NAMPT for the inhibitor (K_i = 0.2 nM, (69,113)), only 24h of FK866 treatment (as low as 10 nM) depletes the NAD pool of cells in culture, impairing their ATP production and eventually leading to cell death by autophagy (115,116). Due to its drastic effect on cell viability, FK866 was used in clinical trials to treat NAMPT-overexpressing cancer types. Unfortunately, FK866 as monotherapy was not efficient to kill cancerous cells in solid tumors (74,117,118).

A recent study from Gardell, *et al*. (2019) identified a new NAMPT effector, namely, SBI-797812. Originally designed as an inhibitor and structurally similar to the inhibitor GNI-50, SBI-797812 is surprisingly an activator (119). This compound enhances the formation of NMN in a dose-dependent manner in the presence of ATP *in vitro* and increases the level of NAD in the liver of mice (120).

1.5.3.3. Modulation by post-translational modification

The activity of NAMPT can be modulated by post-translational modification (PTM) occurring on the protein. A study by Yoon, *et al.* (2015) revealed the existence of several acetylated lysine residues (Lys), and identified Lys53 with a critical impact on NAMPT. Using mutagenesis, the authors showed that the acetylation of Lys53 decreases the enzymatic activity as well as the secretion of the enzyme in the extracellular environment (121). Acetylation was shown to be involved in the sorting of several exosome-secreted proteins (122-125), but how the PTM of Lys53 regulates the enzymatic activity of NAMPT remains unknown.

Even though NAMPT has gathered a lot of attention, several questions remain unanswered. Why does the vertebrate NAMPT have a higher affinity for Nam than the bacterial enzyme? Are there any genetic and structural events causing this increase in affinity? Moreover, the exact mechanism behind the auto-phosphorylation of NAMPT remains obscure. What is the structural basis for the higher affinity of NAMPT for Nam provided by ATP? Does the ATP have the same effect on a less reactive substrate such as NA?

2. Aims of the study

The work of this thesis focused on the structural properties and enzymatic activity of NAMPT, the rate-limiting enzyme in the salvage pathway. First, the study aimed at defining structural adaptations of NAMPT throughout evolution, which resulted in the development of an enzyme with a very high affinity for its substrate Nam in vertebrates. Secondly, the study aimed at determining the structural basis of the regulation of Nam affinity, in particular, with regard to the effect of auto-phosphorylation of His247.

An evolutionary gain in Nam affinity

Synechocystis sp. strain PCC 6803 is one of the few bacterial species, whose genome encodes a functional NAMPT. The characterization of the enzymatic properties revealed a lower affinity for Nam compared to the human enzyme. Remarkably, the selection for NAMPT coincides with a time of evolution when there is a rise in the number of NAD^+-consuming enzymes and the occurrence of another Nam-consuming enzyme, NNMT. The **first goal** of the thesis was to define the structural changes that resulted in the increased affinity of NAMPT for Nam during evolution. In this regard, we wanted to understand whether the augmentation in the affinity of NAMPT to Nam was associated with the emergence of NNMT.

Structural basis of the regulation of the affinity of NAMPT for Nam

The auto-phosphorylation of NAMPT is well known to increase the affinity for Nam. However, the precise mechanism of action and its combination with the PRTase activity remain obscure. The **second goal** of the study was, therefore, to reveal the structural basis underlying the increase of affinity for Nam in the phospho-enzyme, as well as to characterize the critical structural features distinguishing mammalian NAMPTs from those of lower organisms.

3. Summary of the results

3.1. Selection for higher Nam affinity during evolution

The goal of paper I was to determine the evolutionary events causing the increased affinity of NAMPT for Nam which is observed in evolution.

3.1.1 Identification of a structural element affecting the enzymatic activity of NAMPT

The first objective was to define structural changes causing a change in the affinity of NAMPT for Nam. In paper I, an initial multiple sequence alignment of NAMPTs from deuterostomes performed by our collaborators at the University of Sheffield and the Arctic University of Norway (UiT) identified a ten-amino acid insertion in deuterostome NAMPT that forms a surface loop (the β1-β2 loop, see Figure 4). As a potentially interesting feature, this insertion was predicted to contain a weak nuclear localization signal (NLS). In order to explore the impact of the β1-β2 loop on the subcellular localization, the protein structure, and activity of human NAMPT, we generated a mutated version of the enzyme lacking this peptide sequence.

Immunocytochemical investigations did not reveal any participation of the β1-β2 loop in the subcellular localization in HeLaS3 cells. Both wild-type and mutant (Δ42-51) NAMPT exhibited a nuclear-cytosolic localization with predominance in the cytosol. Biochemical analyses of the wild-type and mutant recombinant proteins, expressed in *Escherichia coli*, showed that the deletion of the fragment did not alter the dimerization of the protein but affected the enzymatic activity. Indeed, after incubation with saturating concentrations of substrates, a lower enzymatic activity was observed for the mutant enzyme using one-dimensional ^{1}H NMR for product detection. Further analyses by liquid chromatography-mass spectrometry (LC-MS) revealed a reduced affinity for the substrate Nam.

3.1.2 The occurrence of NNMT has driven the increase in substrate affinity of NAMPT

The occurrence of NNMT in deuterostomes is enigmatic as it competes with NAMPT for the same substrate, Nam. In collaboration with our partners from UiT, who generated a mathematical model to simulate NAD metabolism, it was found that the presence of NNMT enables a higher NAD consumption flux provided that the affinity of NAMPT to Nam is very high. This conclusion matches very well with our experimental findings. Indeed, the sequence insertion forming the β1-β2 loop increased Nam affinity of NAMPT, which emerged concomitantly with the occurrence of NNMT, as well as a profound diversification of NAD^+-consuming signaling pathways.

3.2. The structural basis for the regulation of the affinity of NAMPT for Nam

The goal of paper II was to determine the structural basis for the increased affinity of NAMPT towards Nam, mediated on one hand by the occurrence of the β1-β2 loop that appeared in vertebrates, and, on the other hand, by the auto-phosphorylation of His247 (pHis247).

3.2.1. The β1-β2 loop increases NAMPT overall flexibility

The first objective of paper II was to establish the structural outcome of the deletion of the β1-β2 loop. Structural analyses confirmed the dimerization of the protein lacking this stretch of amino acids. It also showed that the binding mode of NMN and PP_i, as well as the interacting amino acids in the active site, were similar between the wild-type and mutant enzyme. Small-angle X-ray scattering (SAXS) revealed that the mutant enzyme was more compact in solution compared to the wild-type. Combined with normal mode analysis, these results suggest that the deletion of the β1-β2 loop reduced the overall flexibility of the protein in the mutant enzyme.

3.2.2. Proposed stabilization of PRPP by phosphorylation of His247

The second objective of the study was to establish the mechanism by which pHis247 participates in the increase of affinity for Nam. In paper II, we showed by X-ray crystallography that NA binding to NAMPT is comparable to that of Nam. However, the formation of NAMN is strictly ATP-dependent (K_M = 158 (± 27) μM). We found that Mg^{2+} is essential for reducing the conformational heterogeneity of PRPP, in particular with regard to its pyrophosphate moiety (Figure 6A). By replacing a water molecule in the coordination of Mg^{2+}, we suggest that pHis247 aligns the phosphoribose moiety of PRPP with NA or Nam, conferring to PRPP a conformation that is favorable for catalysis.

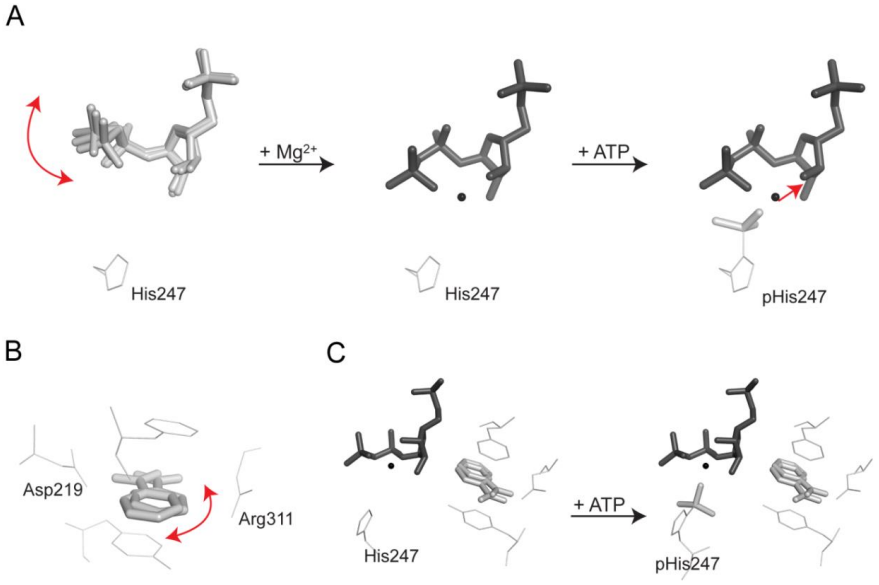

Figure 6: Proposed role of pHis247 in optimizing substrate conformation in NAMPT active site. (A) Different conformations of PRPP from observed crystal structures in the absence of Mg^{2+} show the flexibility (arrow) of the distal phosphate of PRPP (left). In the presence of Mg^{2+}, the ion is coordinated by the hydroxyl groups of PRPP, increasing conformational homogeneity (center). In the presence of ATP, His247 is phosphorylated to pHis247, which coordinates with Mg^{2+} and stabilizes PRPP (right). (B) NA may fluctuate (arrow) in the active site due to the presence of Asp219, making it a less reactive substrate. (C) pHis247 limits the fluctuations of the PRPP-Mg^{2+} complex, which increases the probability of successful catalysis with NA. Mg^{2+} ion is shown as a black sphere.

4. General discussion

The evolution of organisms is associated with an increased complexity that can be correlated with different factors: increased number of genes, diversification of transcriptome, cellular organization, metabolic network, and PTMs of protein (25-28). If evolutionists still debate about which criteria weigh the most in the definition of organismal complexity, an accepted consensual observation is the increased number of signaling pathways (29), and among those, the NAD^+-dependent signaling pathways (91). Consequently, the NAD consumption is more developed in higher animals than in "simpler" organisms. With this increased number of NAD^+-consuming enzymes, one may expect the same trend for the NAD^+-synthesizing enzymes, and yet, it remains relatively constant. This suggests that some adaptations at the genetic and/or enzymatic level occurred to achieve a continuous supply of NAD.

4.1. The β1-β2 loop is a critical element for the enzymatic activity of human NAMPT

The identified sequence insertion in deuterostome NAMPT is the β1-β2 loop, a surface loop localized in the vicinity of the active site's entrance. No resolved structure of the β1-β2 loop of NAMPT is observed in our crystal structures as well as those from the PDB database (as an apoenzyme or in complex with ligand(s)). The β1-β2 loop is a disordered region of NAMPT that apparently does not bind to the substrates, products, or inhibitors. However, the absence of structure does not mean that this region is not functional. Despite the dogma of the "structure-function", it is now accepted that intrinsically disordered regions of protein are also functional elements (126). They can be involved in protein-protein interaction, nucleic acid binding, allosteric regulation, and subcellular localization (127-129).

A potential role of the β1-β2 loop in NAMPT's subcellular localization was considered based on the prediction of a weak NLS that comes in addition to another C-terminal NLS. In the presence and absence of the β1-β2 loop, and hence the putative NLS, the protein had a nuclear-cytosolic localization, however, the protein was mainly in the

cytosol. This indicates the putative NLS from the β1-β2 loop did not participate in the nuclear translocation of NAMPT and the C-terminal NLS was used. The results are confirmed by Svoboda, *et al.* (2019) who also investigated the role of this putative NLS by the means of a mutagenesis strategy (130). Interestingly, a stronger cytosolic localization of NAMPT was suggested in the case of proliferative cells, which is consistent with our observations (130,131). However, several studies reported that the affinity of NLS binding to the import receptor can be regulated by post-translational modifications (PTMs), and notably, by lysine acetylation (132-136). The β1-β2 loop is composed of several lysines in positions -42, -43, -48, and -51, and among those, acetylation of Lys48 has been reported in the database PhosphosSitePlus (137-141). PTMs on the β1-β2 loop may regulate the putative NLS binding in NAMPT, which may deserve more scrutiny. However, a recent study reported that NAMPT forms a complex with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The formation of a NAMPT-GAPDH complex was needed for NAMPT's nuclear translocation. They also evidenced the contribution of the lysines from the β1-β2 loop, and notably Lys48, in the interaction of NAMPT with GAPDH (142). PTMs on these lysines may affect the interaction of NAMPT with its protein partner and consequently, the nuclear translocation.

In addition to the regulation of the subcellular localization, PTMs on the β1-β2 loop may also regulate the protein secretion. Acetylation of Lys53 reduces the secretion of NAMPT outside the cell and, interestingly, Lys48 has been reported to be the target of acetylation and also ubiquitination (121,137-141,143). The interplay between these two marks is known to be involved in the sorting of several exosome-secreted proteins (122-124). Consequently, the study of Lys48 in the regulation of the secretion of NAMPT may need further investigation.

The role of the β1-β2 loop on the protein activity was also investigated. A mutant of human NAMPT lacking the β1-β2 loop was designed, resembling the ancestral form of NAMPT. The deletion of the β1-β2 loop neither altered the dimerization of the enzyme, nor the overall structure of the protein. Moreover, the deletion mutant exhibited enzymatic activity. Altogether, this suggests that the mutant enzyme is a functional protein. However, our study showed a significantly reduced affinity for Nam

in the mutant enzyme. This is in agreement with the observed lower affinity for Nam of the cyanobacteria *Synechocystis* sp. strain PCC 6803 NAMPT, which also lacks the β1-β2 loop (97,105). This result is consistent with the NAD biosynthesis model of our collaborators: deuterostome NAMPT that contains the β1-β2 loop is in competition with NNMT for Nam binding. In this context, NAMPT needed to increase its substrate affinity to ensure the production of NMN for the NMNATs activity, and hence, NAD synthesis.

However, the residues in the active site are conserved between bacterial and human enzymes and therefore could not account for the difference in Nam affinity. This raises the questions of how the β1-β2 loop can increase the affinity of NAMPT for Nam. The mutant enzyme revealed a decreased overall protein flexibility that may explain the reduced affinity. Indeed, substrate binding, catalysis, or even product release in enzymes can be affected by protein flexibility (144-146). In addition, loops and linkers can affect the conformational dynamics of protein (147,148). The occurrence of the β1-β2 loop in deuterostome NAMPT appears to be an evolutionary event that enhanced overall protein flexibility thereby increasing the affinity for Nam. It would be interesting to insert the vertebrate β1-β2 loop into the bacterial enzyme to test whether the protein flexibility and the substrate affinity increases.

The localization of the β1-β2 loop is also intriguing since it is in the vicinity of the active site's entrance. This raises the question of whether this loop may be involved in the recruitment of substrates and/ or holding them in the active site. The β1-β2 loop could be used as a "lid" but there is no evidence of a closed state in the presence of any ligand in the crystal structures. Therefore, the occurrence of lid-gated active sites cannot be confirmed on the basis of the present data. The β1-β2 loop may be involved in the electrostatic attraction of the ligands. As mentioned previously, the loop is composed of several positively charged residues which may attract the negatively charged phosphate groups of PRPP, ATP, PP_i, and NMN. Such a mechanism has been described for the prokaryotic Cu, Zn superoxide dismutase and human Chymotrypsin C (149,150). In addition, the acetylation of Lys53, which is close to the entrance of the active site, is associated with a reduced enzymatic activity of NAMPT, which may suggest the participation of this residue in such a mechanism (121).

More PTMs are predicted on this surface loop and surrounding areas (e.g. phosphorylation Ser47, SUMOylation Lys53, (151-154)). Further examination of these marks should be done as they may affect catalysis, through substrate attraction/product release, or interference with the protein flexibility. They may also influence interactions with protein partners and (sub)cellular localization.

NAMPT is not the only protein in which a surface loop is critical for the enzymatic activity. For instance, a 24-amino acid insertion was identified in the imidazole glycerol phosphate dehydratase (IGDP) in *Saccharomyces cerevisiae* (155). This insertion forms a surface loop between two β-strands, close to the active site, which becomes an additional strand that participates in the stabilization of substrate binding. Another striking similarity with NAMPT is the conservation of the *Saccharomyces cerevisiae* IGDP's loop across species. The conservation of sequences or chemical properties of the amino acids in loops is usually related to the importance of their function (156-158). Consequently, the sequence conservation of the β1-β2 loop in vertebrate NAMPTs stresses how critical this structural element is for NAMPT in higher animals. Further examination of its implication on the regulation of NAMPT activity and potential interaction with protein partners should be addressed.

4.2. The auto-phosphorylation of NAMPT may stabilize PRPP in a favorable conformation

As discussed previously, our study showed that the occurrence of the β1-β2 loop contributed to increasing NAMPT affinity to Nam. However, this structural element is not the only regulatory factor of NAMPT activity. Indeed, ATP is well known to increase the affinity of NAMPT for its substrates, but the precise mechanism remains unclear (87,105). To address this question, a lesser reactive substrate of NAMPT, NA, was used in the absence and the presence of ATP (106). Both NA and PRPP bound to the active site of NAMPT. The carboxylic group on NA in the vicinity of Asp219 is thought to reduce the binding of the substrate. That is why the similar stacking of NA between Phe193 and Tyr18 as Nam is surprising. However, one should bear in mind that protein structures in crystals tend to display the most favorable low free energy

conformation, meaning that NA binding to NAMPT may not be stable in solution. Besides the binding of NA and PRPP, no reaction occurs. It is only in the presence of ATP that the formation of NAMN is observed. The major finding of the thesis is the ATP-dependent conversion of NA by NAMPT, which highlights the importance of the phosphorylated His247 (pHis247) for the reaction.

Another important observation of this study is that ATP, and supposedly through the phosphorylation of His247, supports the catalysis by providing a favorable conformation for PRPP in the active site. This is achieved by the phosphate group of pHis247 that replaces the water molecule as the sixth ligand of Mg^{2+}. The coordination of Mg^{2+} by pHis247 orients subsequently the ribose moiety of PRPP in a favorable conformation for the catalysis (Figure 6). Indeed, in our Mg^{2+}-free crystal structure of NAMPT in complex with NAMN, the ribose moiety does not align with the one seen for NMN in the presence of Mg^{2+} (Paper II, Figure 2D). In addition, following the cleavage of the PP_i moiety from PRPP, the coordination of Mg^{2+} by pHis247 probably anchors the ribo-oxocarbenium ion formed in the transition state, favoring the nucleophilic attack. Altogether, the NMN synthesis would combine a stable position of Nam and a favorable conformation of the ribose moiety for the nucleophilic attack. This would explain the increased affinity for Nam in the phosphorylated enzyme. Regarding the acidic precursor, the reduced binding of NA in solution would be compensated by the favorable position of the ribose moiety, enabling NAMN formation. However, one should bear in mind that the presence of pHis247 was not directly shown but extrapolated from previous studies (111,120). In addition, the structural analysis of the pHis247 is based on structures of NAMPT in complex with BeF_3 that mimics the phosphate group of pHis247 (87).

4.3. Allosteric regulation of NAMPT

The increased substrate affinity mediated by ATP indicates that it is an allosteric effector of NAMPT. Allostery is a mechanism described in other PRTases (e.g. glutamine PRPP amidotransferase, ATP phosphoribosyltransferase, and NAPRT, (159-162)). Besides their structural similarity and the almost identical chemical

reaction they catalyze, NAMPT and NAPRT share several other properties. First, NAPRT is a dimer, then, the affinity of the human and bovine enzymes increases for its substrates in the presence of ATP, and finally, this effect is mediated by the phosphorylation of a His residue in the active site (161,162).

In our study, the mutant NAMPT lacking the β1-β2 loop displayed no formation of NAMN and no increased NMN formation in the presence of ATP. The positive regulation mediated by ATP was lost in the absence of the β1-β2 loop. However, our crystal structure of the mutant enzyme showed the same geometry in the active site compared to the wild-type enzyme. This may indicate that the flexibility brought by the β1-β2 loop enhances the pHis247-mediated effect on the catalysis. In this perspective, the β1-β2 loop could be considered an allosteric loop. Several enzymes have flexible connectors that mediate their allosteric regulation (e.g. HIV-1 reverse transcriptase, cytochrome c, Cdc34-like E2 ubiquitin-conjugating enzymes. phosphocholine cytidylyltransferase isoform α, PKA, and HSP70, (147,163-169)). In deuterostomes, the β1-β2 loop may exert a synergic effect with ATP on NAMPT's activity. This raises the question of whether the magnitude of ATP activation is the same in the bacterial and human enzymes.

Gardell, *et al.* (2019) recently reported another important allosteric property of NAMPT. They found that SBI-797812 amplifies product formation in the presence of ATP, suggesting a mechanism of cooperativity between SBI-797812 and ATP (120). In their study, they also suggest a simultaneous binding of NMN, PP_i, SBI-797812, and the presence of pHis247 in NAMPT. The binding of ATP into one catalytic site at a time was already suggested by Smith and Gholson in the bovine NAPRT (161). It is characteristic of the "half-site" reactivity of enzymes with paired active sites. In other words, the ligand binding in one active site triggers an allosteric conformational change, which prevents the binding in the second site (170-172). Even though no half occupancy was observed in our crystal structures, more studies like molecular and quantum mechanics should be done to confirm whether NAMPT is a "half-site" enzyme. Moreover, investigation of additional effectors of NAMPT activity should be done, especially with AMP. Indeed, this metabolite was reported to be an allosteric

inhibitor that binds to the PRPP loop of glutamine PRPP amidotransferase and ATP phosphoribosyltransferase, as well as an inhibitor of NAPRT (159-161).

In our study, ATP supported the conversion of NA into NAMN by NAMPT. It should be noted that the physiological relevance of this reaction seems limited. The affinity of NAMPT for NA is 3-fold lower compared to the human NAPRT, making the NAMN formation unlikely *in vivo* (173). However, ATP may support the conversion of other substrates. For instance, Vacor is known to be converted by NAMPT into Vacor mononucleotide that leads to the formation of a NAD analog, causing dramatic outcomes (107). It would be interesting to study whether pHis247 also increases the affinity of Vacor.

4.4. Selection of a shorter and more efficient Nam salvage pathway in higher animals

Our collaborators revealed a major evolutionary adaptation in NAD biosynthesis, which is the co-occurrence of *NAMPT* and *NNMT* accompanying the increase in NAD^+-dependent signaling pathways (paper I). This observation seems to be contradictory, but the occurrence of this new enzyme NNMT eventually benefits the synthesis of NAD. Methylation of Nam contributes to the removal of excess intracellular Nam, which maintains the efficiency of the NAD^+-dependent signaling pathways. In addition, NAMPT was forced to adapt by increasing its affinity to Nam given the competition with NNMT for the same substrate. Altogether, the combination of NAMPT and NNMT makes the NAD synthesis more efficient, conferring an evolutionary advantage for the organisms with an increased NAD consumption. Even though this *in silico* modeling of NAD biosynthesis does not take into account the protein or at least transcript levels of the NAD biosynthetic enzymes, nor all the NAD^+-consuming enzymes, the results are consistent with the observations obtained from the phylogenetic analysis. NAD synthesis from Nam switches from a four-step to two-step pathway, making the Nam salvage shorter and less energy-demanding (90,91,174).

5. Concluding remarks and future perspectives

George Gaylord Simpson, a prominent American paleontologist, said that "there is in evolution a continual balancing of the two advantages: the advantage of increasing specialization in sufficiently stable conditions, the advantage of versatility in changing conditions" (175). This statement fits with the evolution of the Nam salvage. Indeed, some NAD^+-dependent signaling pathways were encountered in less complex forms of life. They were maintained, increased, and even diversified in higher organisms. To ensure the stable functioning of these pathways, different evolutionary events happened regarding the NAD synthesis. In addition to the occurrence of NNMT, a new gene encoding a Nam-converting enzyme, the insertion of the β1-β2 loop into NAMPT took place. These evolutionary events eventually increased the substrate affinity of NAMPT, shortened the global pathways of the Nam salvage and amplified the NAD fluxes in higher organisms.

Our study showed that the β1-β2 loop is a critical element for the enzymatic activity of NAMPT by providing flexibility to the protein. The loop could be a new angle for the research on NAMPT inhibitors which has mainly focused on molecules binding to the active site so far. It would be interesting to investigate the consequence of the observed PTMs on the β1-β2 loop, which might be a novel way to regulate the enzymatic activity or the (sub)cellular localization of the enzyme.

Our study also suggests that the β1-β2 loop in human NAMPT enhances the stimulating effect of ATP on NMN and NAMN formation. It would be interesting to investigate whether the magnitude of stimulation by ATP is the same for the NAMPTs in lower organisms such as cyanobacteria and marine organisms. Furthermore, the existence of other allosteric regulators of NAMPT should be addressed as well as the "half-site" reactivity of the enzyme.

Finally, NAMPT was shown in our study to catalyze the conversion of NA into NAMN in the presence of ATP. This suggests that the phosphorylation of NAMPT increases the substrate affinity at the expense of the substrate selectivity. A screening with

different pyridine-based substrates should also be instigated to identify new potential substrates of the phosphorylated enzyme, as well as their conversion *in vivo*.

6. References

1. Barrett-Connor, E. (1967) The etiology of pellagra and its significance for modern medicine. *Am J Med* **42**, 859-867
2. Funk, C. (1975) The Journal of State Medicine. Volume XX: 341-368, 1912. The etiology of the deficiency diseases. Beri-beri, polyneuritis in birds, epidemic deopsy, scurvy, experimental scurvy in animals, infantile scurvy, ship beri-beri, pellagra. *Anal Chim Acta* **76**, 176-177
3. Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M. (1926) A Further Study of Butter, Fresh Beef, and Yeast as Pellagra Preventives, withConsideration of the Relation of Factor P-P of Pellagra (And Black Tongue of Dogs) toVitamin B. *Pub. Health Rep.* **41**, 297-318
4. Elvehjem, C. A., Madden, R. J., Strong, F. M., and Woolley, D. W. (1974) The isolation and identification of the anti-black tongue factor. *Nutr Rev* **32**, 48-50
5. Ng, E., and Neff, M. (2018) Recognising the return of nutritional deficiencies: a modern pellagra puzzle. *BMJ Case Rep* **11**
6. Pique-Duran, E., Perez-Cejudo, J. A., Cameselle, D., Palacios-Llopis, S., and Garcia-Vazquez, O. (2012) Pellagra: a clinical, histopathological, and epidemiological study of 7 cases. *Actas Dermosifiliogr* **103**, 51-58
7. Kirkland, J. B. (2014) Niacin. *Modern Nutrition in Health and Disease* **11th ed. Baltimore**, 331-340
8. Hill, L. J., and Williams, A. C. (2017) Meat Intake and the Dose of Vitamin B3 - Nicotinamide: Cause of the Causes of Disease Transitions, Health Divides, and Health Futures? *Int J Tryptophan Res* **10**, 1178646917704662
9. Williams, A. C., and Hill, L. J. (2017) Meat and Nicotinamide: A Causal Role in Human Evolution, History, and Demographics. *Int J Tryptophan Res* **10**, 1178646917704661
10. Hattori, M., Okuno, Y., Goto, S., and Kanehisa, M. (2003) Development of a chemical structure comparison method for integrated analysis of chemical and genomic information in the metabolic pathways. *Journal of the American Chemical Society* **125**, 11853-11865
11. Kotera, M., Okuno, Y., Hattori, M., Goto, S., and Kanehisa, M. (2004) Computational assignment of the EC numbers for genomic-scale analysis of enzymatic reactions. *Journal of the American Chemical Society* **126**, 16487-16498
12. Moriya, Y., Shigemizu, D., Hattori, M., Tokimatsu, T., Kotera, M., Goto, S., and Kanehisa, M. (2010) PathPred: an enzyme-catalyzed metabolic pathway prediction server. *Nucleic Acids Res* **38**, W138-143
13. Muto, A., Kotera, M., Tokimatsu, T., Nakagawa, Z., Goto, S., and Kanehisa, M. (2013) Modular architecture of metabolic pathways revealed by conserved sequences of reactions. *J Chem Inf Model* **53**, 613-622
14. Oh, M., Yamada, T., Hattori, M., Goto, S., and Kanehisa, M. (2007) Systematic analysis of enzyme-catalyzed reaction patterns and prediction of microbial biodegradation pathways. *J Chem Inf Model* **47**, 1702-1712
15. Yamanishi, Y., Hattori, M., Kotera, M., Goto, S., and Kanehisa, M. (2009) E-zyme: predicting potential EC numbers from the chemical transformation pattern of substrate-product pairs. *Bioinformatics* **25**, i179-186
16. Burns, J. S., and Manda, G. (2017) Metabolic Pathways of the Warburg Effect in Health and Disease: Perspectives of Choice, Chain or Chance. *Int J Mol Sci* **18**
17. van der Bliek, A. M., Sedensky, M. M., and Morgan, P. G. (2017) Cell Biology of the Mitochondrion. *Genetics* **207**, 843-871
18. Singh, P., Saxena, R., Srinivas, G., Pande, G., and Chattopadhyay, A. (2013) Cholesterol biosynthesis and homeostasis in regulation of the cell cycle. *PLoS One* **8**, e58833
19. Beld, J., Lee, D. J., and Burkart, M. D. (2015) Fatty acid biosynthesis revisited: structure elucidation and metabolic engineering. *Mol Biosyst* **11**, 38-59

20. Moritz, B., Striegel, K., De Graaf, A. A., and Sahm, H. (2000) Kinetic properties of the glucose-6-phosphate and 6-phosphogluconate dehydrogenases from Corynebacterium glutamicum and their application for predicting pentose phosphate pathway flux in vivo. *Eur J Biochem* **267**, 3442-3452
21. Berger, F., Ramirez-Hernandez, M. H., and Ziegler, M. (2004) The new life of a centenarian: signalling functions of NAD(P). *Trends Biochem Sci* **29**, 111-118
22. Kim, S. J., Jung, H. J., Hyun, D. H., Park, E. H., Kim, Y. M., and Lim, C. J. (2010) Glutathione reductase plays an anti-apoptotic role against oxidative stress in human hepatoma cells. *Biochimie* **92**, 927-932
23. Manikandan, P., and Nagini, S. (2018) Cytochrome P450 Structure, Function and Clinical Significance: A Review. *Curr Drug Targets* **19**, 38-54
24. Wanders, R. J., and Waterham, H. R. (2006) Biochemistry of mammalian peroxisomes revisited. *Annu Rev Biochem* **75**, 295-332
25. Weng, G., Bhalla, U. S., and Iyengar, R. (1999) Complexity in biological signaling systems. *Science* **284**, 92-96
26. Szathmary, E., Jordan, F., and Pal, C. (2001) Molecular biology and evolution. Can genes explain biological complexity? *Science* **292**, 1315-1316
27. Tenaillon, O., Silander, O. K., Uzan, J. P., and Chao, L. (2007) Quantifying organismal complexity using a population genetic approach. *PLoS One* **2**, e217
28. Ekstig, B. (2015) Complexity, Natural Selection and the Evolution of Life and Humans. *Found Sci* **20**, 175-187
29. Soyer, O. S., and Bonhoeffer, S. (2006) Evolution of complexity in signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 16337-16342
30. Chambon, P., Weill, J. D., and Mandel, P. (1963) Nicotinamide mononucleotide activation of new DNA-dependent polyadenylic acid synthesizing nuclear enzyme. *Biochem Biophys Res Commun* **11**, 39-43
31. Crawford, K., Bonfiglio, J. J., Mikoc, A., Matic, I., and Ahel, I. (2018) Specificity of reversible ADP-ribosylation and regulation of cellular processes. *Crit Rev Biochem Mol Biol* **53**, 64-82
32. Ame, J. C., Spenlehauer, C., and de Murcia, G. (2004) The PARP superfamily. *Bioessays* **26**, 882-893
33. Schreiber, V., Dantzer, F., Ame, J. C., and de Murcia, G. (2006) Poly(ADP-ribose): novel functions for an old molecule. *Nat Rev Mol Cell Biol* **7**, 517-528
34. Morales, J., Li, L., Fattah, F. J., Dong, Y., Bey, E. A., Patel, M., Gao, J., and Boothman, D. A. (2014) Review of poly (ADP-ribose) polymerase (PARP) mechanisms of action and rationale for targeting in cancer and other diseases. *Crit Rev Eukaryot Gene Expr* **24**, 15-28
35. Ray Chaudhuri, A., and Nussenzweig, A. (2017) The multifaceted roles of PARP1 in DNA repair and chromatin remodelling. *Nat Rev Mol Cell Biol* **18**, 610-621
36. Rouleau, M., Patel, A., Hendzel, M. J., Kaufmann, S. H., and Poirier, G. G. (2010) PARP inhibition: PARP1 and beyond. *Nat Rev Cancer* **10**, 293-301
37. Houtkooper, R. H., Canto, C., Wanders, R. J., and Auwerx, J. (2010) The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* **31**, 194-223
38. Goodwin, P. M., Lewis, P. J., Davies, M. I., Skidmore, C. J., and Shall, S. (1978) The effect of gamma radiation and neocarzinostatin on NAD and ATP levels in mouse leukaemia cells. *Biochim Biophys Acta* **543**, 576-582
39. Skidmore, C. J., Davies, M. I., Goodwin, P. M., Halldorsson, H., Lewis, P. J., Shall, S., and Zia'ee, A. A. (1979) The involvement of poly(ADP-ribose) polymerase in the degradation of NAD caused by gamma-radiation and N-methyl-N-nitrosourea. *Eur J Biochem* **101**, 135-142
40. Jankevicius, G., Ariza, A., Ahel, M., and Ahel, I. (2016) The Toxin-Antitoxin System DarTG Catalyzes Reversible ADP-Ribosylation of DNA. *Mol Cell* **64**, 1109-1116

41. Banerjee, A., Munir, A., Abdullahu, L., Damha, M. J., Goldgur, Y., and Shuman, S. (2019) Structure of tRNA splicing enzyme Tpt1 illuminates the mechanism of RNA 2'-PO4 recognition and ADP-ribosylation. *Nat Commun* **10**, 218
42. Munir, A., Banerjee, A., and Shuman, S. (2018) NAD+-dependent synthesis of a 5'-phospho-ADP-ribosylated RNA/DNA cap by RNA 2'-phosphotransferase Tpt1. *Nucleic Acids Res* **46**, 9617-9624
43. Munnur, D., Bartlett, E., Mikolcevic, P., Kirby, I. T., Matthias Rack, J. G., Mikoc, A., Cohen, M. S., and Ahel, I. (2019) Reversible ADP-ribosylation of RNA. *Nucleic Acids Res* **47**, 5658-5669
44. Bi, X., Braunstein, M., Shei, G. J., and Broach, J. R. (1999) The yeast HML I silencer defines a heterochromatin domain boundary by directional establishment of silencing. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 11934-11939
45. Braunstein, M., Rose, A. B., Holmes, S. G., Allis, C. D., and Broach, J. R. (1993) Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev* **7**, 592-604
46. Vaquero, A., Scher, M., Lee, D., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2004) Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. *Mol Cell* **16**, 93-105
47. Adamkova, K., Yi, Y. J., Petr, J., Zalmanova, T., Hoskova, K., Jelinkova, P., Moravec, J., Kralickova, M., Sutovsky, M., Sutovsky, P., and Nevoral, J. (2017) SIRT1-dependent modulation of methylation and acetylation of histone H3 on lysine 9 (H3K9) in the zygotic pronuclei improves porcine embryo development. *J Anim Sci Biotechnol* **8**, 83
48. Luo, J., Nikolaev, A. Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001) Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* **107**, 137-148
49. Vaziri, H., Dessain, S. K., Ng Eaton, E., Imai, S. I., Frye, R. A., Pandita, T. K., Guarente, L., and Weinberg, R. A. (2001) hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* **107**, 149-159
50. Shimazu, T., Hirschey, M. D., Hua, L., Dittenhafer-Reed, K. E., Schwer, B., Lombard, D. B., Li, Y., Bunkenborg, J., Alt, F. W., Denu, J. M., Jacobson, M. P., and Verdin, E. (2010) SIRT3 deacetylates mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2 and regulates ketone body production. *Cell Metab* **12**, 654-661
51. Kitada, M., Kume, S., Takeda-Watanabe, A., Kanasaki, K., and Koya, D. (2013) Sirtuins and renal diseases: relationship with aging and diabetic nephropathy. *Clin Sci (Lond)* **124**, 153-164
52. Kelly, G. (2010) A review of the sirtuin system, its clinical implications, and the potential role of dietary activators like resveratrol: part 1. *Altern Med Rev* **15**, 245-263
53. Haigis, M. C., and Sinclair, D. A. (2010) Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol* **5**, 253-295
54. Lee, H. C. (1997) Mechanisms of calcium signaling by cyclic ADP-ribose and NAADP. *Physiol Rev* **77**, 1133-1164
55. Quarona, V., Zaccarello, G., Chillemi, A., Brunetti, E., Singh, V. K., Ferrero, E., Funaro, A., Horenstein, A. L., and Malavasi, F. (2013) CD38 and CD157: a long journey from activation markers to multifunctional molecules. *Cytometry B Clin Cytom* **84**, 207-217
56. Malavasi, F., Deaglio, S., Funaro, A., Ferrero, E., Horenstein, A. L., Ortolan, E., Vaisitti, T., and Aydin, S. (2008) Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol Rev* **88**, 841-886
57. West, A. E., Chen, W. G., Dalva, M. B., Dolmetsch, R. E., Kornhauser, J. M., Shaywitz, A. J., Takasu, M. A., Tao, X., and Greenberg, M. E. (2001) Calcium regulation of neuronal gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11024-11031
58. Fatt, P., and Katz, B. (1952) Spontaneous subthreshold activity at motor nerve endings. *J Physiol* **117**, 109-128
59. Katz, B., and Miledi, R. (1967) Ionic requirements of synaptic transmitter release. *Nature* **215**, 651

60. Sudhof, T. C. (2012) Calcium control of neurotransmitter release. *Cold Spring Harb Perspect Biol* **4**, a011353
61. Wilkinson, A., Day, J., and Bowater, R. (2001) Bacterial DNA ligases. *Mol Microbiol* **40**, 1241-1248
62. Frindert, J., Zhang, Y., Nubel, G., Kahloon, M., Kolmar, L., Hotz-Wagenblatt, A., Burhenne, J., Haefeli, W. E., and Jaschke, A. (2018) Identification, Biosynthesis, and Decapping of NAD-Capped RNAs in B. subtilis. *Cell Rep* **24**, 1890-1901 e1898
63. Zhang, H., Zhong, H., Zhang, S., Shao, X., Ni, M., Cai, Z., Chen, X., and Xia, Y. (2019) NAD tagSeq reveals that NAD(+)-capped RNAs are mostly produced from a large number of protein-coding genes in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **116**, 12072-12077
64. Jiao, X., Doamekpor, S. K., Bird, J. G., Nickels, B. E., Tong, L., Hart, R. P., and Kiledjian, M. (2017) 5' End Nicotinamide Adenine Dinucleotide Cap in Human Cells Promotes RNA Decay through DXO-Mediated deNADding. *Cell* **168**, 1015-1027 e1010
65. Liu, L., Su, X., Quinn, W. J., 3rd, Hui, S., Krukenberg, K., Frederick, D. W., Redpath, P., Zhan, L., Chellappa, K., White, E., Migaud, M., Mitchison, T. J., Baur, J. A., and Rabinowitz, J. D. (2018) Quantitative Analysis of NAD Synthesis-Breakdown Fluxes. *Cell Metab* **27**, 1067-1080 e1065
66. Lima, W. C., Varani, A. M., and Menck, C. F. (2009) NAD biosynthesis evolution in bacteria: lateral gene transfer of kynurenine pathway in Xanthomonadales and Flavobacteriales. *Mol Biol Evol* **26**, 399-406
67. Magni, G., Amici, A., Emanuelli, M., Orsomando, G., Raffaelli, N., and Ruggieri, S. (2004) Enzymology of NAD+ homeostasis in man. *Cell Mol Life Sci* **61**, 19-34
68. Magni, G., Amici, A., Emanuelli, M., Raffaelli, N., and Ruggieri, S. (1999) Enzymology of NAD+ synthesis. *Adv Enzymol Relat Areas Mol Biol* **73**, 135-182, xi
69. Marletta, A. S., Massarotti, A., Orsomando, G., Magni, G., Rizzi, M., and Garavaglia, S. (2015) Crystal structure of human nicotinic acid phosphoribosyltransferase. *FEBS Open Bio* **5**, 419-428
70. Garavaglia, S., D'Angelo, I., Emanuelli, M., Carnevali, F., Pierella, F., Magni, G., and Rizzi, M. (2002) Structure of human NMN adenylyltransferase. A key nuclear enzyme for NAD homeostasis. *J Biol Chem* **277**, 8524-8530
71. Preiss, J., and Handler, P. (1958) Biosynthesis of diphosphopyridine nucleotide. I. Identification of intermediates. *J Biol Chem* **233**, 488-492
72. Preiss, J., and Handler, P. (1958) Biosynthesis of diphosphopyridine nucleotide. II. Enzymatic aspects. *J Biol Chem* **233**, 493-500
73. Bieganowski, P., and Brenner, C. (2004) 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
74. Chowdhry, S., Zanca, C., Rajkumar, U., Koga, T., Diao, Y., Raviram, R., Liu, F., Turner, K., Yang, H., Brunk, E., Bi, J., Furnari, F., Bafna, V., Ren, B., and Mischel, P. S. (2019) NAD metabolic dependency in cancer is shaped by gene amplification and enhancer remodelling. *Nature* **569**, 570-575
75. Konno, K., Feldmann, F. M., and McDermott, W. (1967) Pyrazinamide susceptibility and amidase activity of tubercle bacilli. *Am Rev Respir Dis* **95**, 461-469
76. Seiner, D. R., Hegde, S. S., and Blanchard, J. S. (2010) Kinetics and inhibition of nicotinamidase from Mycobacterium tuberculosis. *Biochemistry* **49**, 9613-9619
77. Zhang, Y., and Mitchison, D. (2003) The curious characteristics of pyrazinamide: a review. *Int J Tuberc Lung Dis* **7**, 6-21
78. Alston, T. A., and Abeles, R. H. (1988) Substrate specificity of nicotinamide methyltransferase isolated from porcine liver. *Arch Biochem Biophys* **260**, 601-608
79. Aksoy, S., Szumlanski, C. L., and Weinshilboum, R. M. (1994) Human liver nicotinamide N-methyltransferase. cDNA cloning, expression, and biochemical characterization. *J Biol Chem* **269**, 14835-14840

80. Seifert, R., Hoshino, J., and Kroger, H. (1984) Nicotinamide methylation. Tissue distribution, developmental and neoplastic changes. *Biochim Biophys Acta* **801**, 259-264
81. Makarov, M. V., Trammell, S. A. J., and Migaud, M. E. (2019) The chemistry of the vitamin B3 metabolome. *Biochem Soc Trans* **47**, 131-147
82. Bitterman, K. J., Anderson, R. M., Cohen, H. Y., Latorre-Esteves, M., and Sinclair, D. A. (2002) Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. *J Biol Chem* **277**, 45099-45107
83. Sauve, A. A., Moir, R. D., Schramm, V. L., and Willis, I. M. (2005) Chemical activation of Sir2-dependent silencing by relief of nicotinamide inhibition. *Mol Cell* **17**, 595-601
84. Purnell, M. R., and Whish, W. J. (1980) Novel inhibitors of poly(ADP-ribose) synthetase. *Biochem J* **185**, 775-777
85. Nikiforov, A., Kulikova, V., and Ziegler, M. (2015) The human NAD metabolome: Functions, metabolism and compartmentalization. *Crit Rev Biochem Mol Biol* **50**, 284-297
86. Mori, V., Amici, A., Mazzola, F., Di Stefano, M., Conforti, L., Magni, G., Ruggieri, S., Raffaelli, N., and Orsomando, G. (2014) Metabolic profiling of alternative NAD biosynthetic routes in mouse tissues. *PLoS One* **9**, e113939
87. Burgos, E. S., Ho, M. C., Almo, S. C., and Schramm, V. L. (2009) A phosphoenzyme mimic, overlapping catalytic sites and reaction coordinate motion for human NAMPT. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 13748-13753
88. Duarte-Pereira, S., Pereira-Castro, I., Silva, S. S., Correia, M. G., Neto, C., da Costa, L. T., Amorim, A., and Silva, R. M. (2016) Extensive regulation of nicotinate phosphoribosyltransferase (NAPRT) expression in human tissues and tumors. *Oncotarget* **7**, 1973-1983
89. Revollo, J. R., Korner, A., Mills, K. F., Satoh, A., Wang, T., Garten, A., Dasgupta, B., Sasaki, Y., Wolberger, C., Townsend, R. R., Milbrandt, J., Kiess, W., and Imai, S. (2007) Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab* **6**, 363-375
90. Gazzaniga, F., Stebbins, R., Chang, S. Z., McPeek, M. A., and Brenner, C. (2009) Microbial NAD metabolism: lessons from comparative genomics. *Microbiol Mol Biol Rev* **73**, 529-541, Table of Contents
91. Gossmann, T. I., Ziegler, M., Puntervoll, P., de Figueiredo, L. F., Schuster, S., and Heiland, I. (2012) NAD(+) biosynthesis and salvage--a phylogenetic perspective. *FEBS J* **279**, 3355-3363
92. Bockwoldt, M., Houry, D., Niere, M., Gossmann, T. I., Reinartz, I., Schug, A., Ziegler, M., and Heiland, I. (2019) Identification of evolutionary and kinetic drivers of NAD-dependent signaling. *Proceedings of the National Academy of Sciences of the United States of America*
93. Revollo, J. R., Grimm, A. A., and Imai, S. (2004) The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. *J Biol Chem* **279**, 50754-50763
94. Carneiro, J., Duarte-Pereira, S., Azevedo, L., Castro, L. F., Aguiar, P., Moreira, I. S., Amorim, A., and Silva, R. M. (2013) The evolutionary portrait of metazoan NAD salvage. *PLoS One* **8**, e64674
95. Greiss, S., and Gartner, A. (2009) Sirtuin/Sir2 phylogeny, evolutionary considerations and structural conservation. *Mol Cells* **28**, 407-415
96. (!!! INVALID CITATION !!! (66)).
97. Gerdes, S. Y., Kurnasov, O. V., Shatalin, K., Polanuyer, B., Sloutsky, R., Vonstein, V., Overbeek, R., and Osterman, A. L. (2006) Comparative genomics of NAD biosynthesis in cyanobacteria. *J Bacteriol* **188**, 3012-3023
98. Leder, I. G., and Handler, P. (1951) Synthesis of nicotinamide mononucleotide by human erythrocytes in vitro. *J Biol Chem* **189**, 889-899
99. Preiss, J., and Handler, P. (1957) Enzymatic synthesis of nicotinamide mononucleotide. *J Biol Chem* **225**, 759-770

100. Samal, B., Sun, Y., Stearns, G., Xie, C., Suggs, S., and McNiece, I. (1994) Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Mol Cell Biol* **14**, 1431-1437
101. Fukuhara, A., Matsuda, M., Nishizawa, M., Segawa, K., Tanaka, M., Kishimoto, K., Matsuki, Y., Murakami, M., Ichisaka, T., Murakami, H., Watanabe, E., Takagi, T., Akiyoshi, M., Ohtsubo, T., Kihara, S., Yamashita, S., Makishima, M., Funahashi, T., Yamanaka, S., Hiramatsu, R., Matsuzawa, Y., and Shimomura, I. (2005) Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* **307**, 426-430
102. Martin, P. R., Shea, R. J., and Mulks, M. H. (2001) Identification of a plasmid-encoded gene from Haemophilus ducreyi which confers NAD independence. *J Bacteriol* **183**, 1168-1174
103. Rongvaux, A., Shea, R. J., Mulks, M. H., Gigot, D., Urbain, J., Leo, O., and Andris, F. (2002) Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *Eur J Immunol* **32**, 3225-3234
104. Burgos, E. S., Vetticatt, M. J., and Schramm, V. L. (2013) Recycling nicotinamide. The transition-state structure of human nicotinamide phosphoribosyltransferase. *Journal of the American Chemical Society* **135**, 3485-3493
105. Burgos, E. S., and Schramm, V. L. (2008) Weak coupling of ATP hydrolysis to the chemical equilibrium of human nicotinamide phosphoribosyltransferase. *Biochemistry* **47**, 11086-11096
106. Khan, J. A., Tao, X., and Tong, L. (2006) Molecular basis for the inhibition of human NMPRTase, a novel target for anticancer agents. *Nat Struct Mol Biol* **13**, 582-588
107. Buonvicino, D., Mazzola, F., Zamporlini, F., Resta, F., Ranieri, G., Camaioni, E., Muzzi, M., Zecchi, R., Pieraccini, G., Dolle, C., Calamante, M., Bartolucci, G., Ziegler, M., Stecca, B., Raffaelli, N., and Chiarugi, A. (2018) Identification of the Nicotinamide Salvage Pathway as a New Toxification Route for Antimetabolites. *Cell Chem Biol* **25**, 471-482 e477
108. Smith, J. L. (1995) Enzymes of nucleotide synthesis. *Curr Opin Struct Biol* **5**, 752-757
109. Hershey, H. V., and Taylor, M. W. (1986) Nucleotide sequence and deduced amino acid sequence of Escherichia coli adenine phosphoribosyltransferase and comparison with other analogous enzymes. *Gene* **43**, 287-293
110. Sommer, G., Garten, A., Petzold, S., Beck-Sickinger, A. G., Bluher, M., Stumvoll, M., and Fasshauer, M. (2008) Visfatin/PBEF/Nampt: structure, regulation and potential function of a novel adipokine. *Clin Sci (Lond)* **115**, 13-23
111. Wang, T., Zhang, X., Bheda, P., Revollo, J. R., Imai, S., and Wolberger, C. (2006) Structure of Nampt/PBEF/visfatin, a mammalian NAD+ biosynthetic enzyme. *Nat Struct Mol Biol* **13**, 661-662
112. Elliott, G. C., and Rechsteiner, M. C. (1982) Evidence for a physiologically active nicotinamide phosphoribosyltransferase in cultured human fibroblasts. *Biochem Biophys Res Commun* **104**, 996-1002
113. Burgos, E. S. (2011) NAMPT in regulated NAD biosynthesis and its pivotal role in human metabolism. *Current medicinal chemistry* **18**, 1947-1961
114. Hasmann, M., and Schemainda, I. (2003) FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis. *Cancer Res* **63**, 7436-7442
115. Nahimana, A., Attinger, A., Aubry, D., Greaney, P., Ireson, C., Thougaard, A. V., Tjornelund, J., Dawson, K. M., Dupuis, M., and Duchosal, M. A. (2009) The NAD biosynthesis inhibitor APO866 has potent antitumor activity against hematologic malignancies. *Blood* **113**, 3276-3286
116. Ginet, V., Puyal, J., Rummel, C., Aubry, D., Breton, C., Cloux, A. J., Majjigapu, S. R., Sordat, B., Vogel, P., Bruzzone, S., Nencioni, A., Duchosal, M. A., and Nahimana, A. (2014) A critical role of autophagy in antileukemia/lymphoma effects of APO866, an inhibitor of NAD biosynthesis. *Autophagy* **10**, 603-617

117. Garten, A., Schuster, S., Penke, M., Gorski, T., de Giorgis, T., and Kiess, W. (2015) Physiological and pathophysiological roles of NAMPT and NAD metabolism. *Nature reviews. Endocrinology* **11**, 535-546
118. Goldinger, S. M., Gobbi Bischof, S., Fink-Puches, R., Klemke, C. D., Dreno, B., Bagot, M., and Dummer, R. (2016) Efficacy and Safety of APO866 in Patients With Refractory or Relapsed Cutaneous T-Cell Lymphoma: A Phase 2 Clinical Trial. *JAMA Dermatol* **152**, 837-839
119. Zheng, X., Bauer, P., Baumeister, T., Buckmelter, A. J., Caligiuri, M., Clodfelter, K. H., Han, B., Ho, Y. C., Kley, N., Lin, J., Reynolds, D. J., Sharma, G., Smith, C. C., Wang, Z., Dragovich, P. S., Oh, A., Wang, W., Zak, M., Gunzner-Toste, J., Zhao, G., Yuen, P. W., and Bair, K. W. (2013) Structure-based identification of ureas as novel nicotinamide phosphoribosyltransferase (Nampt) inhibitors. *J Med Chem* **56**, 4921-4937
120. Gardell, S. J., Hopf, M., Khan, A., Dispagna, M., Hampton Sessions, E., Falter, R., Kapoor, N., Brooks, J., Culver, J., Petucci, C., Ma, C. T., Cohen, S. E., Tanaka, J., Burgos, E. S., Hirschi, J. S., Smith, S. R., Sergienko, E., and Pinkerton, A. B. (2019) Boosting NAD(+) with a small molecule that activates NAMPT. *Nat Commun* **10**, 3241
121. Yoon, M. J., Yoshida, M., Johnson, S., Takikawa, A., Usui, I., Tobe, K., Nakagawa, T., Yoshino, J., and Imai, S. (2015) SIRT1-Mediated eNAMPT Secretion from Adipose Tissue Regulates Hypothalamic NAD+ and Function in Mice. *Cell Metab* **21**, 706-717
122. Li, Z., Zhuang, M., Zhang, L., Zheng, X., Yang, P., and Li, Z. (2016) Acetylation modification regulates GRP78 secretion in colon cancer cells. *Sci Rep* **6**, 30406
123. Moreno-Gonzalo, O., Fernandez-Delgado, I., and Sanchez-Madrid, F. (2018) Post-translational add-ons mark the path in exosomal protein sorting. *Cell Mol Life Sci* **75**, 1-19
124. Moreno-Gonzalo, O., Villarroya-Beltri, C., and Sanchez-Madrid, F. (2014) Post-translational modifications of exosomal proteins. *Front Immunol* **5**, 383
125. Xu, G., and Jaffrey, S. R. (2013) Proteomic identification of protein ubiquitination events. *Biotechnol Genet Eng Rev* **29**, 73-109
126. Dunker, A. K., Brown, C. J., Lawson, J. D., Iakoucheva, L. M., and Obradovic, Z. (2002) Intrinsic disorder and protein function. *Biochemistry* **41**, 6573-6582
127. Dunker, A. K., Cortese, M. S., Romero, P., Iakoucheva, L. M., and Uversky, V. N. (2005) Flexible nets. The roles of intrinsic disorder in protein interaction networks. *FEBS J* **272**, 5129-5148
128. Reeves, R. (2001) Molecular biology of HMGA proteins: hubs of nuclear function. *Gene* **277**, 63-81
129. Lau, C., Niere, M., and Ziegler, M. (2009) The NMN/NaMN adenylyltransferase (NMNAT) protein family. *Front Biosci (Landmark Ed)* **14**, 410-431
130. Svoboda, P., Krizova, E., Sestakova, S., Vapenkova, K., Knejzlik, Z., Rimpelova, S., Rayova, D., Volfova, N., Krizova, I., Rumlova, M., Sykora, D., Kizek, R., Haluzik, M., Zidek, V., Zidkova, J., and Skop, V. (2019) Nuclear transport of nicotinamide phosphoribosyltransferase is cell cycle-dependent in mammalian cells, and its inhibition slows cell growth. *J Biol Chem* **294**, 8676-8689
131. Kitani, T., Okuno, S., and Fujisawa, H. (2003) Growth phase-dependent changes in the subcellular localization of pre-B-cell colony-enhancing factor. *FEBS Lett* **544**, 74-78
132. Kaffman, A., and O'Shea, E. K. (1999) Regulation of nuclear localization: a key to a door. *Annu Rev Cell Dev Biol* **15**, 291-339
133. Poon, I. K., and Jans, D. A. (2005) Regulation of nuclear transport: central role in development and transformation? *Traffic* **6**, 173-186
134. Madison, D. L., Yaciuk, P., Kwok, R. P., and Lundblad, J. R. (2002) Acetylation of the adenovirus-transforming protein E1A determines nuclear localization by disrupting association with importin-alpha. *J Biol Chem* **277**, 38755-38763
135. Dietschy, T., Shevelev, I., Pena-Diaz, J., Huhn, D., Kuenzle, S., Mak, R., Miah, M. F., Hess, D., Fey, M., Hottiger, M. O., Janscak, P., and Stagljar, I. (2009) p300-mediated acetylation of the Rothmund-Thomson-syndrome gene product RECQL4 regulates its subcellular localization. *J Cell Sci* **122**, 1258-1267

136. di Bari, M. G., Ciuffini, L., Mingardi, M., Testi, R., Soddu, S., and Barila, D. (2006) c-Abl acetylation by histone acetyltransferases regulates its nuclear-cytoplasmic localization. *EMBO Rep* **7**, 727-733
137. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations (Rikova K (2012); CST Curation Set: 16031)). *Nucleic Acids Res* **43**, D512-520
138. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations (Rikova K (2012); CST Curation Set: 16032)). *Nucleic Acids Res* **43**, D512-520
139. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations (Rikova K (2013); CST Curation Set: 18854)). *Nucleic Acids Res* **43**, D512-520
140. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations (Rikova K (2013); CST Curation Set: 20216)). *Nucleic Acids Res* **43**, D512-520
141. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations (Rikova K (2013); CST Curation Set: 20219)). *Nucleic Acids Res* **43**, D512-520
142. Grolla, A. A., Miggiano, R., Di Marino, D., Bianchi, M., Gori, A., Orsomando, G., Gaudino, F., Galli, U., Del Grosso, E., Mazzola, F., Angeletti, C., Guarneri, M., Torretta, S., Calabro, M., Boumya, S., Fan, X., Colombo, G., Travelli, C., Rocchio, F., Aronica, E., Wohlschlegel, J. A., Deaglio, S., Rizzi, M., Genazzani, A. A., and Garavaglia, S. (2020) A nicotinamide phosphoribosyltransferase-GAPDH interaction sustains the stress-induced NMN/NAD(+) salvage pathway in the nucleus. *J Biol Chem*
143. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations (Guo A (2010); CST Curation Set: 10265)). *Nucleic Acids Res* **43**, D512-520
144. Kokkinidis, M., Glykos, N. M., and Fadouloglou, V. E. (2012) Protein flexibility and enzymatic catalysis. *Adv Protein Chem Struct Biol* **87**, 181-218
145. Mukherjee, J., and Gupta, M. N. (2015) Increasing importance of protein flexibility in designing biocatalytic processes. *Biotechnol Rep (Amst)* **6**, 119-123
146. Varley, P. G., and Pain, R. H. (1991) Relation between stability, dynamics and enzyme activity in 3-phosphoglycerate kinases from yeast and Thermus thermophilus. *J Mol Biol* **220**, 531-538
147. Papaleo, E., Saladino, G., Lambrughi, M., Lindorff-Larsen, K., Gervasio, F. L., and Nussinov, R. (2016) The Role of Protein Loops and Linkers in Conformational Dynamics and Allostery. *Chem Rev* **116**, 6391-6423
148. Kurkcuoglu, Z., Bakan, A., Kocaman, D., Bahar, I., and Doruker, P. (2012) Coupling between catalytic loop motions and enzyme global dynamics. *PLoS Comput Biol* **8**, e1002705
149. Folcarelli, S., Battistoni, A., Falconi, M., O'Neill, P., Rotilio, G., and Desideri, A. (1998) Conserved enzyme-substrate electrostatic attraction in prokaryotic Cu,Zn superoxide dismutases. *Biochem Biophys Res Commun* **244**, 908-911
150. Batra, J., Szabo, A., Caulfield, T. R., Soares, A. S., Sahin-Toth, M., and Radisky, E. S. (2013) Long-range electrostatic complementarity governs substrate recognition by human chymotrypsin C, a key regulator of digestive enzyme activation. *J Biol Chem* **288**, 9848-9859
151. Xue, Y., Liu, Z., Cao, J., Ma, Q., Gao, X., Wang, Q., Jin, C., Zhou, Y., Wen, L., and Ren, J. (2011) GPS 2.1: enhanced prediction of kinase-specific phosphorylation sites with an algorithm of motif length selection. *Protein Eng Des Sel* **24**, 255-260
152. Blom, N., Gammeltoft, S., and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* **294**, 1351-1362

153. Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* **4**, 1633-1649
154. Zhao, Q., Xie, Y., Zheng, Y., Jiang, S., Liu, W., Mu, W., Liu, Z., Zhao, Y., Xue, Y., and Ren, J. (2014) GPS-SUMO: a tool for the prediction of sumoylation sites and SUMO-interaction motifs. *Nucleic Acids Res* **42**, W325-330
155. Rawson, S., Bisson, C., Hurdiss, D. L., Fazal, A., McPhillie, M. J., Sedelnikova, S. E., Baker, P. J., Rice, D. W., and Muench, S. P. (2018) Elucidating the structural basis for differing enzyme inhibitor potency by cryo-EM. *Proceedings of the National Academy of Sciences of the United States of America* **115**, 1795-1800
156. Chen, J. W., Romero, P., Uversky, V. N., and Dunker, A. K. (2006) Conservation of intrinsic disorder in protein domains and families: II. functions of conserved disorder. *J Proteome Res* **5**, 888-898
157. Chen, J. W., Romero, P., Uversky, V. N., and Dunker, A. K. (2006) Conservation of intrinsic disorder in protein domains and families: I. A database of conserved predicted disordered regions. *J Proteome Res* **5**, 879-887
158. Uversky, V. N. (2013) A decade and a half of protein intrinsic disorder: biology still waits for physics. *Protein Sci* **22**, 693-724
159. Smith, J. L., Zaluzec, E. J., Wery, J. P., Niu, L., Switzer, R. L., Zalkin, H., and Satow, Y. (1994) Structure of the allosteric regulatory enzyme of purine biosynthesis. *Science* **264**, 1427-1433
160. Fisher, G., Thomson, C. M., Stroek, R., Czekster, C. M., Hirschi, J. S., and da Silva, R. G. (2018) Allosteric Activation Shifts the Rate-Limiting Step in a Short-Form ATP Phosphoribosyltransferase. *Biochemistry* **57**, 4357-4367
161. Smith, L. D., and Gholson, R. K. (1969) Allosteric properties of bovine liver nicotinate phosphoribosyltransferase. *J Biol Chem* **244**, 68-71
162. Niedel, J., and Dietrich, L. S. (1973) Nicotinate phosphoribosyltransferase of human erythrocytes. Purification and properties. *J Biol Chem* **248**, 3500-3505
163. Mager, P. P., and Walther, H. (1996) A hydrophilic omega-loop (Tyr181 to Tyr188) in the nonsubstrate binding area of HIV-1 reverse transcriptase. *Drug Des Discov* **14**, 225-239
164. Krishna, M. M., Lin, Y., Rumbley, J. N., and Englander, S. W. (2003) Cooperative omega loops in cytochrome c: role in folding and function. *J Mol Biol* **331**, 29-36
165. Papaleo, E., Casiraghi, N., Arrigoni, A., Vanoni, M., Coccetti, P., and De Gioia, L. (2012) Loop 7 of E2 enzymes: an ancestral conserved functional motif involved in the E2-mediated steps of the ubiquitination cascade. *PLoS One* **7**, e40786
166. English, C. A., Sherman, W., Meng, W., and Gierasch, L. M. (2017) The Hsp70 interdomain linker is a dynamic switch that enables allosteric communication between two structured domains. *J Biol Chem* **292**, 14765-14774
167. Liu, J., and Nussinov, R. (2010) Molecular dynamics reveal the essential role of linker motions in the function of cullin-RING E3 ligases. *J Mol Biol* **396**, 1508-1523
168. Akimoto, M., Selvaratnam, R., McNicholl, E. T., Verma, G., Taylor, S. S., and Melacini, G. (2013) Signaling through dynamic linkers as revealed by PKA. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 14231-14236
169. Knowles, D. G., Lee, J., Taneva, S. G., and Cornell, R. B. (2019) Remodeling of the interdomain allosteric linker upon membrane binding of CCTalpha pulls its active site close to the membrane surface. *J Biol Chem* **294**, 15531-15543
170. Bloom, C. R., Choi, W. E., Brzovic, P. S., Ha, J. J., Huang, S. T., Kaarsholm, N. C., and Dunn, M. F. (1995) Ligand binding to wild-type and E-B13Q mutant insulins: a three-state allosteric model system showing half-site reactivity. *J Mol Biol* **245**, 324-330
171. Biemann, H. P., and Koshland, D. E., Jr. (1994) Aspartate receptors of Escherichia coli and Salmonella typhimurium bind ligand with negative and half-of-the-sites cooperativity. *Biochemistry* **33**, 629-634

172. Wu, P., Cisneros, G. A., Hu, H., Chaudret, R., Hu, X., and Yang, W. (2012) Catalytic mechanism of 4-oxalocrotonate tautomerase: significances of protein-protein interactions on proton transfer pathways. *J Phys Chem B* **116**, 6889-6897
173. Galassi, L., Di Stefano, M., Brunetti, L., Orsomando, G., Amici, A., Ruggieri, S., and Magni, G. (2012) Characterization of human nicotinate phosphoribosyltransferase: Kinetic studies, structure prediction and functional analysis by site-directed mutagenesis. *Biochimie* **94**, 300-309
174. Rongvaux, A., Andris, F., Van Gool, F., and Leo, O. (2003) Reconstructing eukaryotic NAD metabolism. *Bioessays* **25**, 683-690
175. Simpson, G. G. (1950) The Meaning of Evolution.

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Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

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ISBN: 9788230852989 (print)
9788230844106 (PDF)