Università degli Studi del Piemonte Orientale

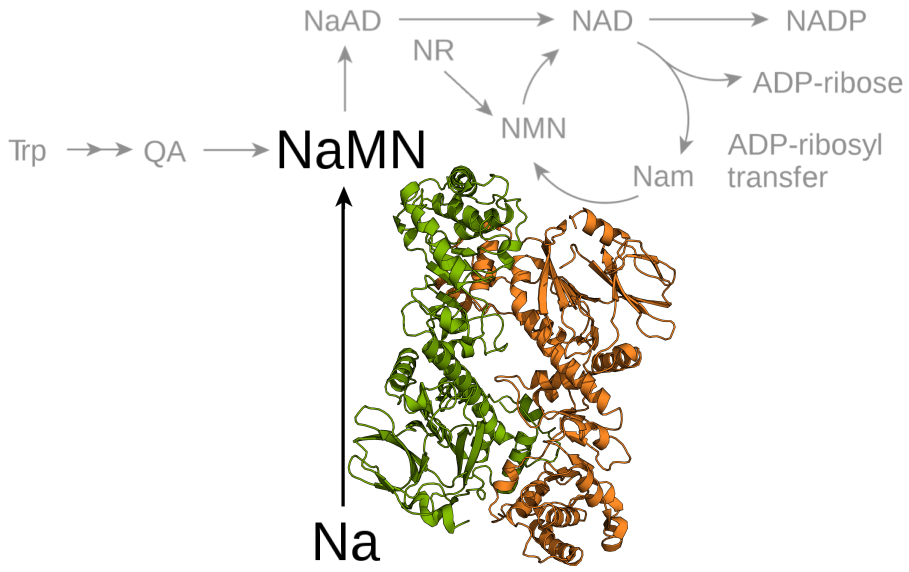
"Amedeo Avogadro"

Dipartimento di Scienze del Farmaco

Dottorato di Ricerca in Biotecnologie Farmaceutiche ed Alimentari

XXVII ciclo a.a. 2011-2014

Biochemical and structural studies on human enzymes involved in NAD homeostasis



Ada Serena Marletta

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Biochemical and structural studies on human enzymes involved in NAD homeostasis

Ada Serena Marletta

Supervised by Dr. Silvia Garavaglia

PhD program co-ordinator Prof. Menico Rizzi

A Ivana e Salvatore

"Il viaggio da Kamakura a Kyoto dura dodici giorni:
se viaggi per undici giorni e ti fermi quando ne manca uno solo,
come puoi ammirare la luna sopra la capitale?"
N.D.

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Chapter 1

Introduction

1.0 The Chemistry of NAD(P) and its physiological roles

Nicotinamide adenine dinucleotide (NAD) and its phosphorylated counterpart (NADP) are ubiquitous dinucleotides, since they consist of two nucleotides joined through their phosphate groups. In particular, one nucleotide contains an adenine base and the other nicotinamide, namely adenosine monophosphate (AMP) and nicotinamide mononucleotide (NMN), respectively. Because of the presence of a pyridine ring on the NMN moiety, due to the fact that nicotinamide is a derivative of pyridine, NAD and NADP are also referred as pyridine nucleotides **(Figure 1A)**. The presence of an additional phosphate group at the 2' position on the ribose of the AMP moiety represents the chemical difference between NAD and NADP. Both these molecules exist in the form of a pair comprising an oxidized and a reduced form, NAD+/NADH and NADP+/NADPH, and are termed redox couples.
It is usual to refer to the NAD+/NADH and NADP+/NADPH couples as NAD and NADP, respectively; all these species are collectively indicated as NAD(P). The chemical difference between the two species in each redox couple depends on the transfer of a hydride ion, consisting of two electrons and a proton, at the level of the nicotinamide ring constituting the reactive portion of NAD(P)+ **(Figure 1B)**. This transfer is driven by numerous hydride transfer enzymes or oxidoreductases, which interconvert either NAD+ and NADH or NADP+ and NADPH to reduce or oxidize small-molecule metabolites in the biological systems. On the basis of their capacity to support the activity of enzymes catalysing oxidation-reduction reactions, as hydride acceptors and donors, NAD and NADP are defined coenzymes.

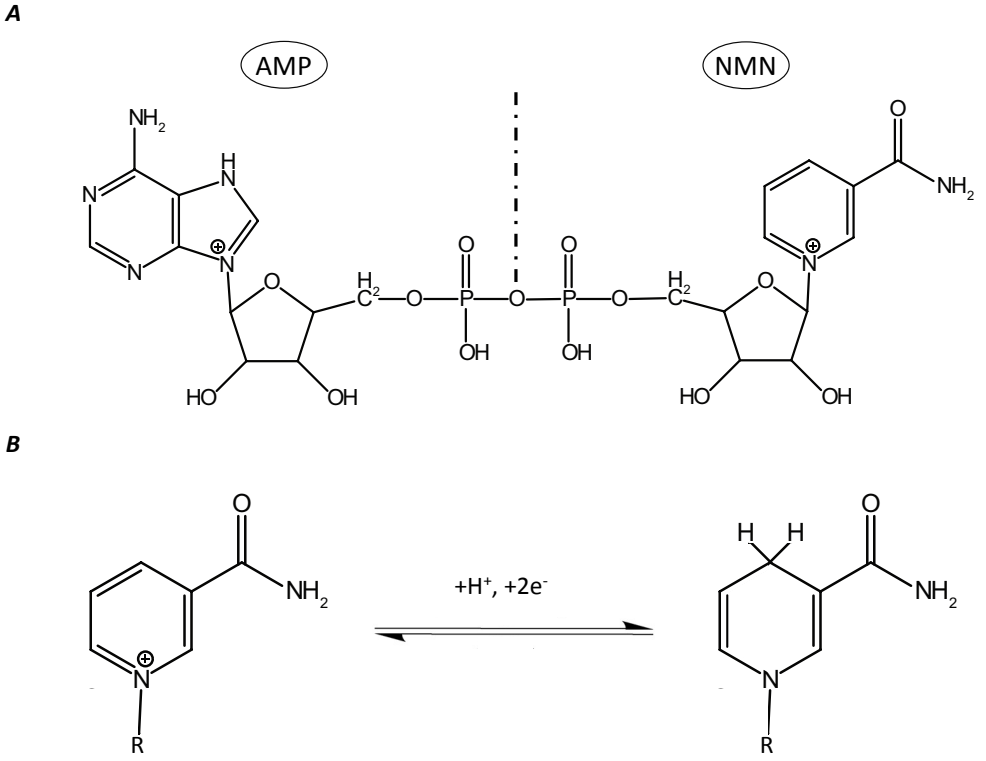


Figure 1.A. Chemical structure of NAD+. Instead of a hydrogen atom, NADP+ has a phosphate group at the 2' position on the ribose of its AMP moiety. **B**. **Bi-directional redox reaction between NAD+ and NADH.**

Moreover, it is now well established that pyridine nucleotides and the balance between their oxidized and reduced forms play a wide variety of pivotal roles in cellular functions as important interfaces, beyond their coenzymatic activity. These include maintenance of redox status, cell survival and death, ion channel regulation, and cell signalling under normal and pathological conditions (1). In the role of signal transducer, NAD(P)+ is a substrate of many enzymes and need to be constantly resynthesized in the cell.

1.0.1 NAD(P)-the coenzyme

Since the beginning of the last century, seminal discoveries have identified pyridine nucleotides as the major redox carriers in all organisms. Indeed, it is beyond doubt that NAD(P) holds a key position in cellular metabolism and energy production due to its coenzymatic activity. The function of NAD as coenzyme was firstly discovered by Otto Warburg, in 1936. On the basis of his work on alcoholic fermentation, he discovered the capability of NAD, at that time known as "von Euler's cozymase", to transfer hydrogen from one molecule to another. We have to credit Warburg also with the discovery of NADP, the "cozymase II", as another coenzyme with similar properties (2, 3). In the cellular metabolism, the NAD+/NADH couple primarily drives oxidation reactions (catabolic reactions), while the NADP+/NADPH couple drives reductive reactions (anabolic reactions). Fatty acid β-oxidation and biosynthesis are an example of metabolic pathways where NAD+/NADH and NADP+/NADPH are involved as coenzymes, respectively. To understand the reason why the two redox couples are generally implicated in those distinct branches of cellular metabolism, it has to be considered their redox potentials (4). The intrinsic tendency of a chemical species to acquire electrons is expressed by its reductive potential, also known as redox potential, that is measured in volt (V); the more positive the redox potential is, greater the electron affinity of the considered species will be. Both in the cytosol and the mitochondrial matrix, the NADP redox potential is higher than the NAD one **(Figure 2)**; that means that the ratio NADPH/NADP+ is much greater than NADH/NAD+ ratio in the mitochondrial matrix. One process maintaining this disequilibrium is the mitochondrial proton motive force-dependent transhydrogenase (5). Moreover, it has long been recognized that the reductive potential of NAD is much higher than that in the cytosol, corresponding to a 40-fold difference in the NAD+/NADH ratio in the two compartments **(Figure 2)**.

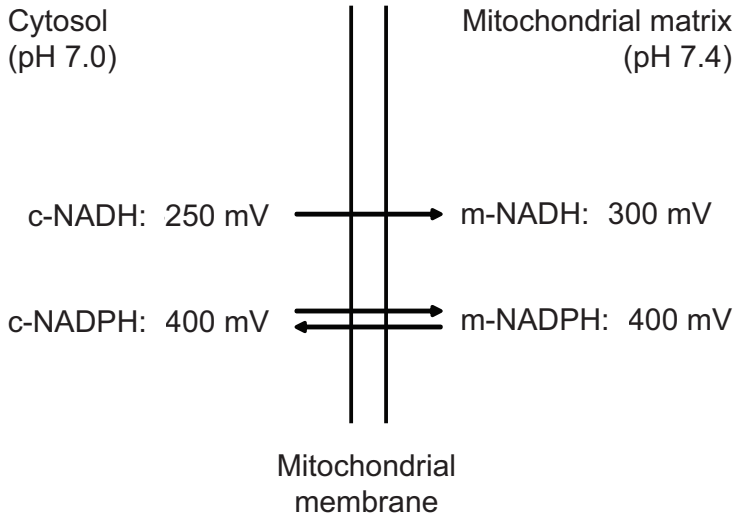


Figure 2. **Redox potentials of the mitochondrial and cytosolic NAD(H) and NADP(H) systems in the liver.** c, cytosolic; m, mitochondrial. (modified from ref. 4).

The redox state of NAD and NADP, their different distribution in distinct cell types and tissues, together with the well-established presence of intracellular binding proteins modulating the free concentration of NAD(P) (4), all these factors contribute in determining the redox state of the cell, that represents itself an important regulatory mechanism. Indeed, the expression of several genes is linked to the redox state of pyridine nucleotides via NAD+/NADH-binding proteins that act as redox sensors (6). For example, transcriptional activity can be reciprocally regulated by direct binding of either NADH or NAD+ to transcriptional co-repressor C-terminal binding protein (7). Pyridine nucleotides have been shown to serve as electron carriers to synthesize adenosine triphosphate (ATP) in mitochondria via the electron transport chain (ETC) and oxidative phosphorylation. The participation of NAD(P) in electron transfer reactions, does not result in a net consumption of the nucleotides. Consequently, except for cell divisions, the

constant requirement for pyridine nucleotide resynthesis does not arise from their function as coenzymes, but rather from their involvement in signalling reactions (8).

1.0.2 *NAD(P)+- the substrate*

Nowadays, it is well assessed that pyridine nucleotides, besides their vital functions as electron carriers, play important roles as mediators in a plethora of fundamental cellular processes. Since many years, it has been discovered an astonishing range of enzymatic activities where NAD(P)+ is not involved as a coenzyme but rather as a substrate. These NAD(P)+-consuming enzymes get the energy to proceed in the catalysis of their reactions by the cleavage of the N-glycosidic bond between the nicotinamide ring and the ribose of the NMN moiety of NAD(P)+. Indeed, nicotinamide (Nam) and ADP-ribose (ADPr) are the common products of all these reactions; Nam can be recycled entering the NAD+ salvage pathway while the ADPr is transferred to an acceptor molecule or can undergo a cyclization (see below) **(Figure 3)**. NAD(P)+-consuming enzymes drive essentially post-translational protein modification and messenger molecule synthesis reactions that are involved in important functions in metabolism, signalling transduction, cell survival and death as well as in gene expression regulation and aging. It is important to note that all the conversions require the oxidized form of NAD(P)+. The reduced form is not a substrate for these signalling reactions (8). Three protein families that consume NAD(P)+ in signalling reactions have been characterized on a molecular level: ADP-ribosyltransferases (ARTs and PARPs), Sirtuins (SIRTs), and NAD+ glycohydrolases (NADases) (6). NAD+-dependent ADP-ribosylation is an important post-translational reversible protein modification that regulates diverse biological processes including DNA-repair, transcriptional regulation, telomere dynamics, energy metabolism and apoptosis (10, 11).

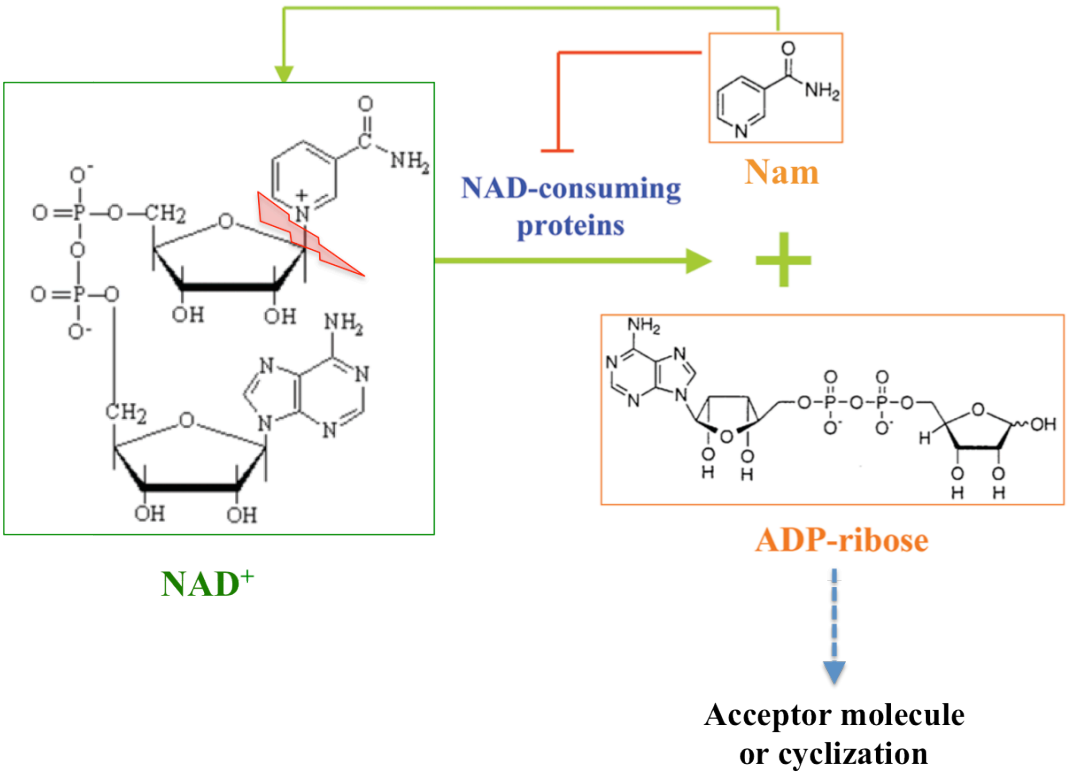


Figure 3. **Consumption of NAD+ driven by NAD(P)+ dependent enzymes.** All the reactions catalysed by NAD+-consuming proteins use NAD(P)+ as a donor of ADPr, while generating nicotinamide (Nam) as a side product. Nicotinamide has the product inhibition effect on the enzymatic activities of most of the NAD+-consuming proteins, probably by competing for the NAD+ binding pocket (modified from ref .9).

ADP-ribosylation enzymes (ARTs) can be distinguished in mono- and poly-ADP-ribosyltransferases and catalyse, respectively, the transfer of one or more ADP-ribose moieties from NAD+ onto specific aminoacid side chains in target protein under release of nicotinamide **(Figure 4)**. In mammals, the ART family is the largest and, perhaps, most versatile among the NAD+-converting protein families (12), and that has generated some confusion about a common shared nomenclature (13).

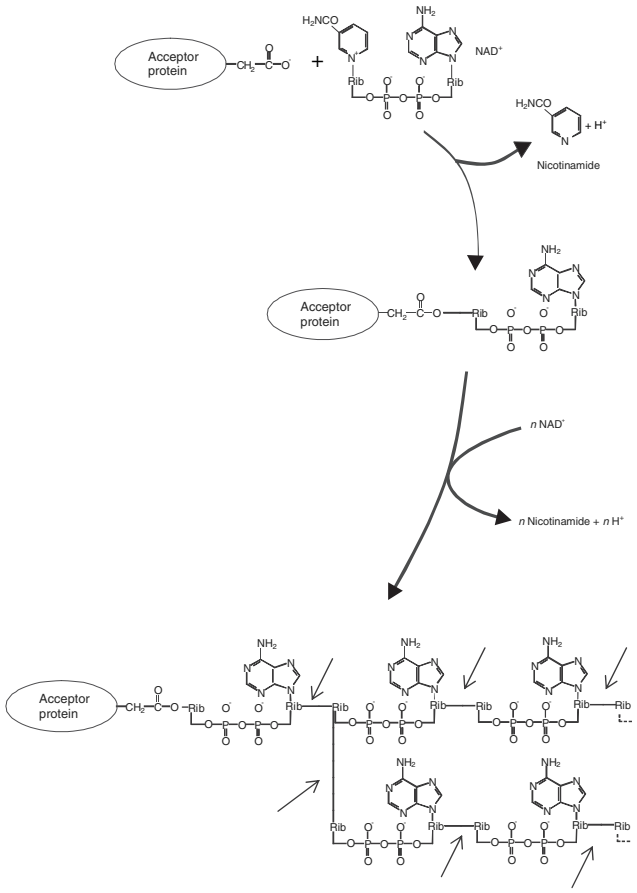


Figure 4. ADP-ribosylation reaction. Mono-ADP-ribosyltransferases catalyse the transfer of one unit of NAD+; Poly-ADP-ribosyltransferases catalyse an adduct elongation, giving rise to polymers of up to about 200-ADP-ribosyl units. Arrows indicate the site of hydrolysis catalysed by PARG (modified from ref. 11).

Sequence and structure homology searches have identified 22 human genes encoding proteins with ADP-ribosylation activity that have been grouped into three major families: the extracellular membrane-associated ADP-ribosyltransferases (ecto-ARTs), a single member family of NAD+-dependent tRNA 2'-phosphotransferases and the poly-ADP-ribose polymerases (PARPs) (13).

Surprisingly, all identified ecto-ARTs are mono-ADP-ribosyltransferases (2) and their activity in the extracellular compartment provides a complex regulatory mechanism for cell communication (13). Mono-ADP-ribosylation (MARylation) was originally identified as the catalityc activity of several bacterial toxins such as diphtheria, cholera and pertussis toxins, and its physiological roles are related to immune response and inflammation, transcription regulation, cell adhesion, signal and energy metabolism (14). Many functions of Poly-ADP-ribosylation (PARylation) may be explained considering the multiple roles played by PARP-1, the most studied human poly-ADP-ribosyltransferase, now considered a moonlighting protein that serves the cells both in peace (resting condition) and in war (DNA damage) (15). After the discovery of PARP-1 in the early 1960s, many other enzymes with poly ADP-ribosyltransferase activity had been discovered, highlighting a huge number of conditions inducing ADP-ribose polymer (PAR) formation (11). Both mono- and poly- ADP-ribosylation are dynamic processes because of the presence of other enzymes, ADP-ribosyl hydrolases (ARHs) and Poly-ADP-ribose glycohydrolases (PARGs), which remove mono-ADP ribose from the target and hydrolyse the O-glycosidic ribose-ribose 1-2' bonds within PAR, respectively (16). Mono-ADP-ribosyltransferase activity was also detected in some sirtuins such as human mitochondrial SIRT4 and nuclear SIRT6 (17). Sirtuins are a family of evolutionally conserved class III histone deacylases that consume one molecule of NAD+ during each deacylation cicle (18). Even though sirtuins have been recognised mostly as deacetylases, recent studies have shown that Sirt5 is endowed of demalonylase and desuccinylase activities (19). Chemically, deacylation primarily occurs on N-ε-lysine residues and tipically regulates protein function (17). For example, sirtuin catalysed NAD+-consuming deacetylation takes place on an acetyl-lysine and results in the production of deacetylated lysine, nicotinamide and 2'-O-acetyl-ADP-ribose (OAADPr) **(Figure 5)**.

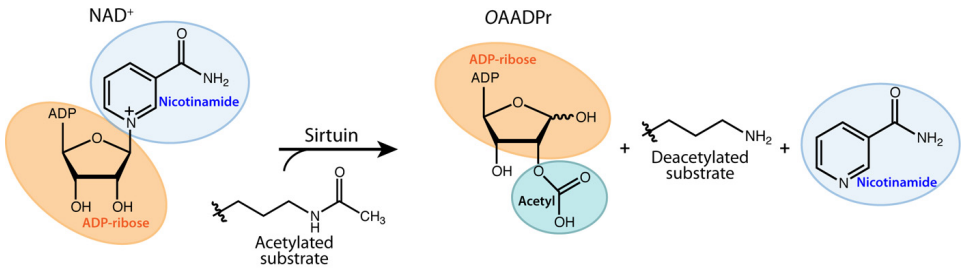


Figure 5. Schematic representation of a deacetylation reaction catalysed by sirtuins (modified from ref. 20).

In particular, OAADPr undergoes a non-enzymatic transesterification reaction and exists in solution as a mixture of 2' and 3' OAADPr (20). Moreover, OAADPr has been implicated as a signalling molecule and it can also be the substrate of macrodomain family enzymes that may function in the regulation of OAADPr cellular levels, hydrolysing the 2 or 3-ester bond to generate ADPr and acetate as products (16, 21). The mammalian family of sirtuins consists of seven members, Sirt1-7, and the number of their reported histone and non-histone protein targets is continually increasing. Therefore, it is not surprising that sirtuins have important roles in many biological processes such as regulation of chromatin structure and gene expression, oxidative stress reduction, glucose homeostasis and cell survival (4). The versatile functions of sirtuins are supported by their diverse cellular location allowing cells to sense changes in energy levels in the nucleus, cytoplasm, and mitochondrion (18), but it is not clear whether the link between intracellular NAD+ levels and sirtuin activity is correlative or causal (4). Alongside its consumption by sirtuins and ARTs, NAD(P)+ is also a substrate for NAD glycohydrolases enzymes, responsible for the generation of second messengers associated with calcium signalling, a universal signal trasduction mechanism involved in a wide range of physiological functions. The two major Ca^{2+} mobilising NAD(P)+ derivatives are the cyclic ADP ribose (cADPr) and the Nicotinic acid Adenine dinucleotide phosphate (NAADP) and, in mammals, their

formation is catalysed by a single enzyme, CD38 (and its Janus CD157). cADPr and NAADP are structurally and functionally distinct and thus, it is surprising that they can be synthesized by the same enzyme. It has been shown how the different reactions occur at distinct pH, with the synthesis of NAADP at acidic pH, and that one of cADPr at neutral pH **(Figure 6)**.

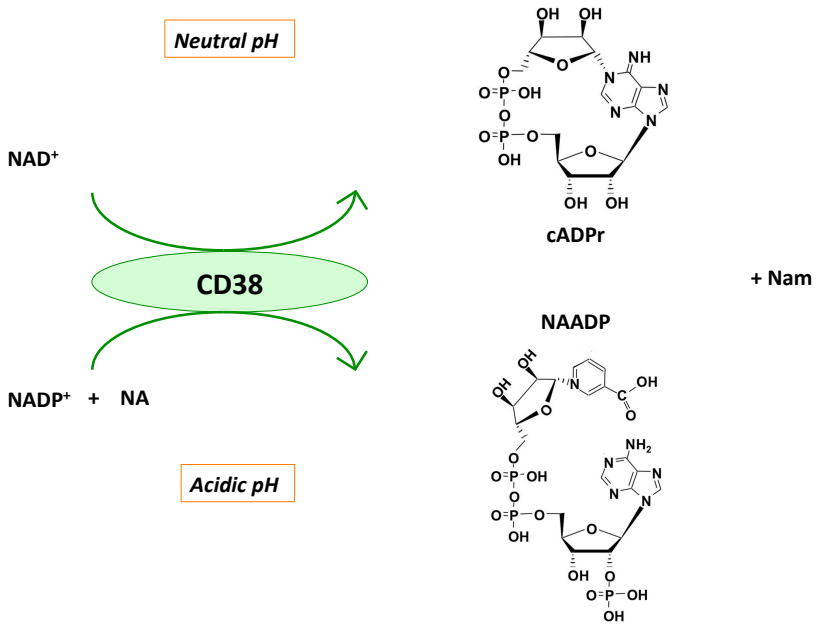


Figure 6. Generation of second messengers NAADP and cADPr by CD38. The synthesis of cADPr occurs at neutral pH, whereas that one of NAADP occurs at acidic pH.

Many structural and site-directed mutagenesis studies have elucidated the molecular basis of the multifunctionality of CD38. Even though sharing the same activity, cADPr and NAADP mobilise different intracellular Ca^{2+} stores by binding distinct targets. Indeed, cADPr binds the ryanodine receptor and mobilises the calcium store in the endoplasmic reticulum (ER), while NAADP mobilises the endolysosomes by binding the lysosomal two-pore channels (TPCs). Though

separated, these Ca^{2+} stores can interact through different mechanisms that have been proposed (22). In mammalian cells, even OAADPr and ADPr can modulate calcium influx through the transient receptor potential malastatin-related channel 2 (TRPM2), a non selective cation channel that is stimulated by oxidative and nitrative stress (23). By mediating the Ca^{2+} signalling in the cells, adenine dinucleotides second messengers participate in the coordination of many important cellular processes. For example, Ca^{2+} signalling is fundamental in the activation of the T-lymphocytes, so being essential for a functional immune response (24). All these NAD(P)+-consuming reactions call for a continuous replenishment of the pyridine nucleotides. NAD(P)+ biosynthesis can be accomplished through distinct pathways that notably vary in prokaryotes and eukaryotes. For the scope of this thesis, I will concentrate on the biosynthesis of NAD(P)+ in humans.

1.1 Biosynthesis of NAD(P)+ in humans

In human cells, NAD+ biosynthesis is based on several different extracellular precursors **(Figure 7)**. The catabolism of tryptophan (Trp) supports NAD+ generation by the formation of quinolinic acid (QA) in the kynurenine pathway and all reactions from Trp to NAD+ are commonly referred as *de novo* pathway. Other pathways, known as salvage pathways, initiate NAD+ biosynthesis starting from the vitamin precursors, namely nicotinamide (Nam), nicotinic acid (Na), Nicotinamide riboside (NR) and nicotinic acid riboside (NaR), or recycle NAD degradation products (25). Although the molecular identities of the human enzymes involved in NAD(P)+ synthesis have been established, information regarding their subcellular distribution and regulation is scarce or inconclusive (26).

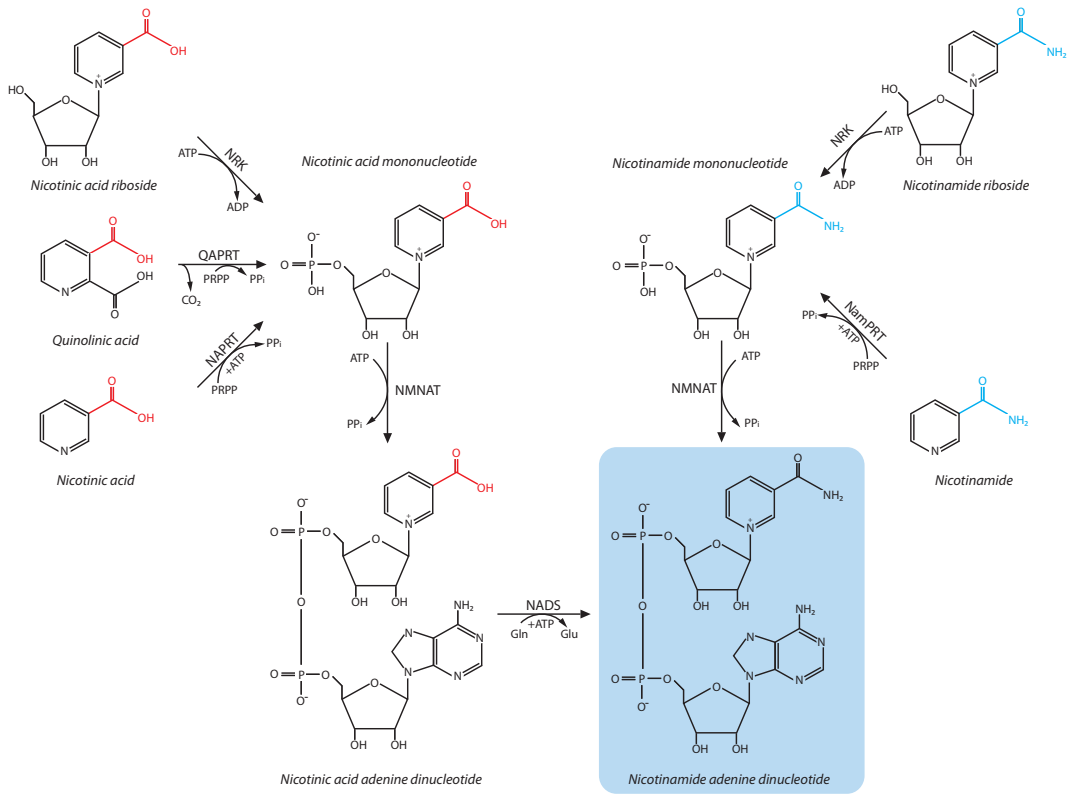


Figure 7. Overview of NAD+ biosynthesis in humans (26).

1.1.1 De novo pathway

Tryptophan is the *de novo* precursor of NAD+ in all vertebrates (3) and is taken up from the diet (27). In particular, tryptophan serves as a NAD+ precursor when degraded to quinolinic acid (QA) in the kynurenine pathway **(Figure 8)**. The first rate-limiting step in *de novo* NAD+ biosynthesis is the conversion of tryptophan to N-formylkynurenine by either indoleamine 2,3, dioxygenase (IDO) or tryptophan 2,3, dioxygenase (TDO), both catalysing the indole ring cleavage at the C2-C3 bond, and requiring molecular oxygen. These two enzymes show distinct substrate specificity and are differently expressed in the various tissues and organs.

Indeed, TDO is highly expressed in the liver, the primary site of L-tryptophan catabolism, while IDO is ubiquitously expressed in extrahepatic tissues with the lung, spleen, small intestine and placenta having the highest activity (4, 28).

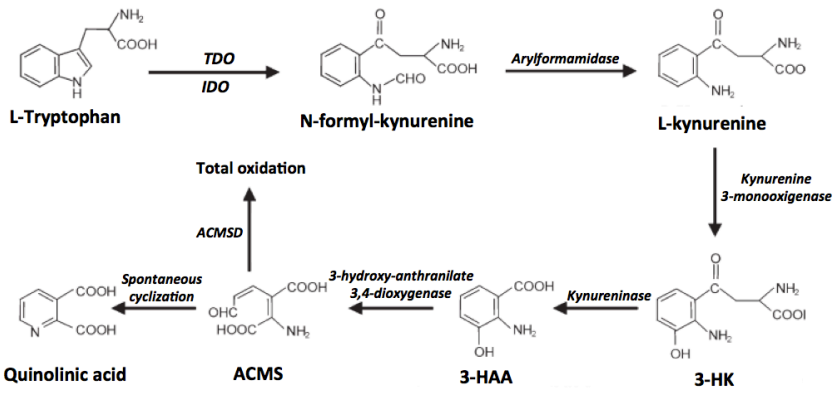


Figure 8. Reaction pathway for the quinolinic acid synthesis in humans. Abbreviation used: TDO, tryptophan 2,3-dioxygenase; IDO, indoleamine 2,3-dioxygenase; 3-HK, 3-hydroxy-L-kynurenine; 3-HAA, 3-hydroxy-anthranilic acid; ACMS, a-amino-b-carboxy-muconate-e-semialdehyde; ACMSD, α-amino-β-carboxymuconate-e-semialdehyde decarboxylase (modified from ref. 29).

Moreover, TDO and IDO functions have been associated with different physiological and pathological conditions. For example, IDO is involved in the immuno-regulating system (29). The three-dimensional structures of both these two heme-containing dioxygenases have been solved, providing the structural basis to understand the chemistry of their mechanism of catalysis (30, 31). Briefly, N-formylkynurenine is subsequently converted in four individual steps to the unstable α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMS), which can undergo either enzymatic conversion directed to total oxidation or non enzymatic cyclisation to quinolinic acid (4, 32). Interestingly, kynurenine 3-monooxigenase reaction product, 3-hydroxykynurenine (3-HK), induces an H_2O_2-neurotoxicity in

the central nervous system, and thus this enzyme has become an attractive pharmacological target (29). Although human kynurenine 3-monooxigenase crystallization was not achieved, *Saccharomyces cerevisiae* orthologue structure was determined (sharing 38% of sequence identity with the human enzyme) and validated as a template for structure-based drug design (33). The reaction representing the entry point into NAD+ biosynthesis from aminoacid catabolism is catalysed by QAPRTase and converts QA to nicotinic acid mononucleotide (NAMN) **(Figure 7)**. This conversion represents the second rate-limiting step in the *de novo* pathway and consists in QA phosphoribosylation, using 5-phophoribosyl-pyrophosphate (PRPP) as co-substrate, followed by its essentially irreversible decarboxylation (25). Determination of the human QAPRTase structure helped to establish the correct substrate binding mechanism with QA binding occurring first, and PRPP immediately after. Moreover, structural analysis has shown how the active site is formed at the dimer interface and it is located within the α/β barrel domain, whose composition ascribes QAPRTase to the type II phosphoribosyl-transferases (34, 35) **(Figure 9)**. As in the case of kynurenine 3-monooxigenase, also the product of QAPRTase reaction, QA, is neurotoxic and its imbalace has been observed in various neurophatological conditions. Thus, by shuttling QA to NAD+ biosynthesis, QAPRTase exerts also a neuroprotective effect (25). Finally, NAMN formed by QAPRTase converges to the Preiss-Handler pathway leading to NAD+, as described in the next session 1.1.2. In contrast to the other two phosphoribosyltransferases involved in NAD+ biosynthesis in humans, NMPRTase and NaPRTase, QAPRTase does not require ATP (25). Even though its importance has been stressed by the human disease pellagra, tryptophan alone is insufficient to support physiological NAD+ concentration (26) in a large majority of mammalian organs.

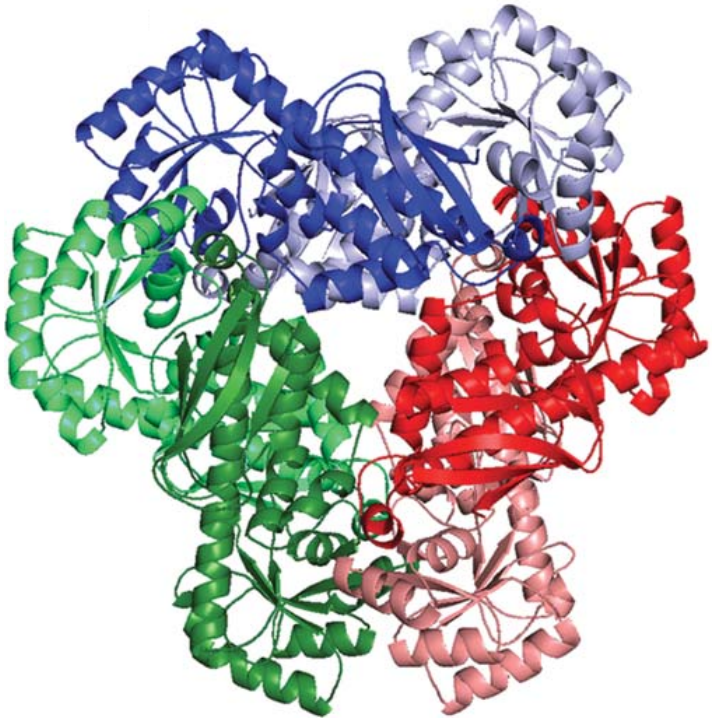


Figure 9. **Quaternary structure of human quinolinic acid phosphoribosyltransferase (hQAPRTase)**. (35). Human QAPRTase exists and functions as a hexamer in solution.

1.1.2 Salvage pathways

Although NAD+ can be synthesized *de novo* from tryptophan, it is assumed that the main source of NAD+ is from salvage pathways, which require the uptake of other NAD+ precursors from diet (4). Na and Nam are considered salvageable NAD+ precursors and were identified as the "anti-black tongue factor" in dogs with pellagra symptoms in late 1930s. They are collectively termed niacin, despite the fact that the biosynthetic pathways through which Na and Nam are used by cells are not the same. In 1958, Preiss and Handler discovered the existence of an alternate pathway of NAD+ biosynthesis from nicotinic acid in human

erythrocytes. In particular, they showed how incubation of nicotinic acid-C14 with human erythrocytes resulted in synthesis of two radioactive nucleotides identified as nicotinic acid mononucleotide (NaMN) and nicotinic acid dinucleotide (NAAD), respectively. They also showed that NAAD-C14 formation *in vivo* occurred before NAD-C14 formation, concluding that the last step of NAD+ synthesis from nicotinic acid consisted of the amidation of NAAD into NAD+ (36). Immediately after, Preiss and Handler identified the three enzymes required to accomplish synthesis of NAD+ from Na (37). The first rate-limiting reaction of this three-step pathway, now known as Preiss-Handler pathway, is catalysed by Nicotinic acid phosphoribosyltransferase (NaPRTase). It catalyses the conversion of Na to NAMN using PRPP as phosphoribosyl donor and releasing pyrophosphate (PPi). The human NaPRTase is matter of interest of this thesis and will be abundantly discussed in Chapter 3. The second step in the Preiss-Handler pathway is the conversion of NaMN to NAAD in presence of ATP, catalysed by one of the nicotinamide mononucleotide adenylyltransferases isoforms, NMNAT1, NMNAT2 or NMNAT3, identified in humans (38-40). NaMN deriving from the *de novo* pathway enters the Preiss-Handler pathway at this point. NMNAT activity represents the single universal common reaction in all NAD+ biosynthetic pathways **(Figure 7)**, thus, it has been immediately recognized as an attractive target for the development of antibacterial drugs. The expression of the three human isoforms of NMNAT is ascribed to different cellular compartments, with NMNAT1 being a nuclear enzyme, NMNAT2 and NMNAT3 localising to the Golgi apparatus and the mitochondria, respectively (41). The crystal structures of human NMNAT1 (42) and NMNAT3 (43) have been solved, while only structural models are available so far for NMNAT2. Interestingly, the human enzymes display a unique dual specificity being able to recognize with the same efficiency both NaMN and NMN as substrates. The ATP-dependent amidation of NAAD to NAD+ represents the final step in the Preiss-Handler pathway and it is catalysed by NAD synthetase (NADS). As for the NMNAT reaction, also this step is shared

with the *de novo* biosynthesis. The human NADS uses glutamine as amide donor, and it is composed of two protein domains. The C-terminal domain is responsible of the amide transfer onto NAAD, whereas the N-terminal domain catalyses the glutamine hydrolysis. The structural determination of *Mycobacterium tuberculosis* NADS, another glutamine-dependent NADS, revealed the existence of a tunnel of communication between the two domains, of about 40 Å, that permits the transfer of the amide group generated in the N-terminal domain towards the C-terminal domain (25, 44). The other niacin-derived molecule Nam is the product of all NAD+-consuming reactions and it is saved distinctly from Na. In vertebrates, the intracellular Nam salvage pathway depends on a nicotinamide phosphoribosyl-transferase (NMPRTase), which entered the literature with the names pre-B cell colony enhancing factor (PBEF) (45) and Visfatin (46). Intracellularly, NMPRTase catalyses the phosphoribosyl transfer from PRPP onto Nam and yields NMN and pyrophosphate. Whether its extracellular counterpart PBEF/Visfatin is endowed of phosphoribosyltransferase activity is still matter of debate (47, 48), though it was reported that PRPP and NMN are virtually absent in mouse plasma (48). NMPRTase possesses a facultative ATPase activity that allows the production of NMN at products/substrates ratios thermodynamically forbidden in the absence of ATP. Indeed, by coupling ATP hydrolysis to NMN synthesis, the catalytic efficiency of the system is improved 1100-fold and substrate affinity dramatically increased for both substrates. Moreover, NMPRTase undergoes feedback inhibition by both NAD+ and NADH (49). As a growth factor, a cytokine and an enzyme, NMPRTase plays pleiotropic physiological functions, as suggested by its ubiquitous tissue distribution, and its dysregulation is implicated in several human diseases, including cancer. Indeed, NMPRTase overexpression has been found in many tumour cells and several studies have indicated that NMPRTase may be an attractive diagnostic and drug target for cancer therapy (50, 51). Several potent NMPRTase inhibitors, such as FK866, have been identified (52). Human NMPRTase structure has been determined (53) as well as the molecular basis of its

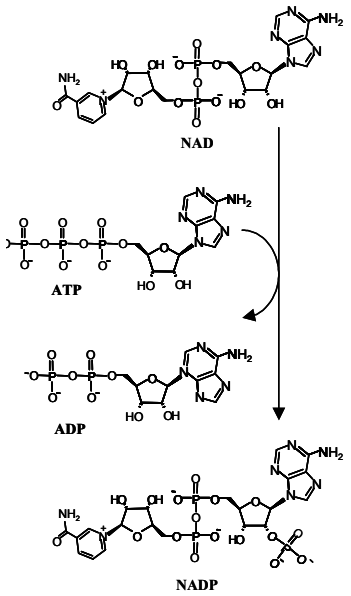
inhibition by FK866 (54). After the NMPRTase reaction, NMN is converted to NAD+ by NMNAT. Ten years ago, nicotinamide riboside (NR) was discovered as an additional salvageable NAD+ precursor vitamin. By observing that NR was already known to be a NAD+ precursor in bacteria such as *Haemophilus influenzae,* Bieganowsky and Brenner demonstrated how NR could also function as precursor of the pyridine nucleotide in *Saccharomyces cerevisiae*, through a previously unknown route to NAD+. In this pivotal study, Bieganowsky and Brenner employed a genetic approach based on the use of mutant yeast strains, and noticed that NR supplementation was capable to rescue the growth of mutants carrying the lethal deletion of QNS1. The latter is the yeast orthologue of NADS, catalysing the final NAD+ biosynthetic step, common to all pathways known until then in *S.cerevisiae*. At the same time, the molecular identity of the conserved *NRK* genes, encoding for the enzymes responsible for the phosphorylation of NR to NMN, was also identified (55). In humans, there are two isoforms of NRK, namely NRK1 and NRK2, that show both high specificity for phosphorylation of NR and the anticancer drug tiazofurin (55). Nevertheless, NRK1 can utilise either ATP and GTP as phosphodonor, while NRK2 is an ATP-specific kinase (56). Human NRK1 and NRK2 could use as substrate also the deamidated form of NR, Nicotinic acid riboside (NaR), that was demonstrated to be utilised in yeast as a NAD+ precursor in a novel biosynthetic pathway that depends on NRK and NADS (56). The structures of NRK1 in complex with substrates and products have been determined (56, 57) while NRK2 structure has yet to be solved. In summary, in human cells NaR and NR are converted to NAD+ in a three-step and two-step pathway, respectively **(Figure 7)**. In the first step, NRK converts NaR to NaMN and NR to NMN. Subsequently, NaMN enters the Preiss-Handler pathway while NMN is directly deamidated to NAD+ by NMNAT. A second NRK-indipendent NR salvage pathway has been described in yeast (58), even if it has yet to be investigated in mammalian systems. In conclusion, all NAD+ salvage pathways as well as the *de novo* pathway after QA synthesis, share a common scheme towards

NAD+ formation, that consists first in the formation of a pyridine mononucleotide and then in the dinucleotide formation. Indeed, from five different precursors (QA, Nam, NA, NR and NaR) we assist at the synthesis of two intermediates, NAMN and NMN, that are subsequently converted, in a common step, to NAAD and NAD+, respectively. Finally, as previously discussed, NAAD undergoes an additional step of amidation to form NAD+ (25).

1.1.3 NADP+ biosynthesis

Some of the cellular NAD+ is converted into NADP+ by NAD Kinase (NADK), that catalyses the transfer of a phosphate group from ATP to the 2' position of the adenosine ribose of NAD+, thus forming NADP+ and ADP (59) **(Figure 10A)**. This reaction represents the only known way to generate NADP+ in all living organisms (29). NADK plays a key role in the regulation of the cellular redox state, by modulating the levels of NAD and NADP, and provides the substrate for many important metabolic reactions and for the formation of the second messenger NAADP. Surprisingly, despite the fundamental role of NADK, only a single isoform of NADK has been found in mammals so far. Lerner and coworkers identified the human NADK cDNA in 2001, immediately after the identification of the protein sequences of *Micrococcus flavus* and *Mycobacterium tuberculosis* NADKs (60, 61).

A



B

Physical and Kinetic Properties of Human NAD Kinase

Subunit molecular mass (calculated)	49,228 Da
Subunit composition (total molecular mass)	Homotetramer (200 kDa)
$K_{M_{ATP}}$	3.3 mM
$K_{M_{NAD}}$	0.54 mM
V_{max}	6.7 μmol (min $\times$ mg protein)$^{-1}$
Temperature optimum	55°C
pH optimum	7.5 (7.0–8.0)

Figure 10. A. NAD Kinase-catalysed reaction (59). **B. Physical and Kinetic Properties of human NAD Kinase** (61).

The human gene was shown to be expressed mostly in every tissue but not in skeletal muscle. The recombinant human enzyme was expressed in *E. coli* and was shown to be highly selective for the substrates NAD+ and ATP, showing Km values of 0.54 and 3.33 mM, respectively **(Figure 10B)**. It requires a divalent cation to function but is not calmodulin/Ca^{2+} dependent, in contrast with the enzyme purified from neutrophils that was shown to be dependent from these effectors (59, 61). Also on the basis of this difference, further studies have been encouraged to exclude the existence of other NADK isoforms. As for its primary sequence being determined on the basis of the precedent work on the bacterial *M.tuberculosis* enzyme, also the first three-dimensional structure that was determined has been the *M.tuberculosis* NADK (62). The interest for the *M.tuberculosis* NADK was especially due to the fact that this enzyme is essential for the growth of the multidrug-resistant pathogen, thus representing an attractive

target for novel antitubercular agents. Nowadays, a pdb entry (3PFN) for the human enzyme has been deposited in the Protein Data Bank, but no descriptions have been reported so far. The comparison of the human and *M.tuberculosis* NADK structures could be useful in the rational design for inhibitors of the pathogen enzyme.

1.2 Systemic features of NAD homeostasis

Not only the multiple NAD+ biosynthetic pathways and the breathtaking number of NAD(P)+-consuming reactions, but also the compartmentation of both enzymes and NAD metabolites, and their transport among the organelles and between the extracellular and intracellular spaces (NAD influx/efflux), critically influence the maintenance of NAD homeostasis in human cells. Moreover, the availability of NAD(P) may depend on the type of cell and its contingent conditions such as, metabolic and redox state, as well as stress and other environmental stimuli, and it plays a pivotal role in the determination of the pathophysiological functions of the cell (6). It is therefore not surprising that NAD homeostasis must be tightly regulated (63) even though, the mechanisms through which all these regulation determinants influence each other still arise challenging questions. The compartmentation of NAD(P) is reflected by a distinct spatial organization of the multitude of NAD(P)+-dependent signalling pathways (6) **(Figure 11)**. To better understand the presence of the distinct NAD pools, Nikiforov and co-workers have elegantly determined the subcellular localization of NAD+ biosynthetic enzymes. They used a new detector system, based on the addressing of the catalytic domain of PARP1 (PARP1cd) to the mitochondrial matrix, to monitor the organellar changes in NAD content through the immunodetectable formation of PAR. (26). Although NAD+ is synthesized mainly in the cytosol, nucleus and mitochondria, it

has been detected also in vescicular and extracellular compartments (6). Indeed, distinct subcellular NAD pools have been identified also in the endoplasmic reticulum, Golgi complex and peroxisomes (64, 65). All these NAD pools are subjected to variations within the cells and their relative differences can vary in a cell type-specific manner (66). The NAD pool in mitochondria has received particular attention (26, 67, 68) because of the crucial functions for cellular energy metabolism played by these organelles and their dysfunction being associated with a large number of diseases. Mitochondrial NAD pool appears to be sufficiently robust to preserve cell viability and ATP levels and there is a growing body of evidence suggesting that mitochondria are endowed of their own NAD+ biosynthetic machinery (67). Indeed, a mitochondrial isoform of NMN adenylyltransferase, NMNAT3 has been identified (69) and localised to the mitochondrial matrix (26). Furthermore, the same study that has assigned NMNAT3 to the mitochondria, has also shown how NMN is the cytoplasmic precursor of mitochondrial NAD, solving the long standing problem of NAD generation in mitochondria (26).

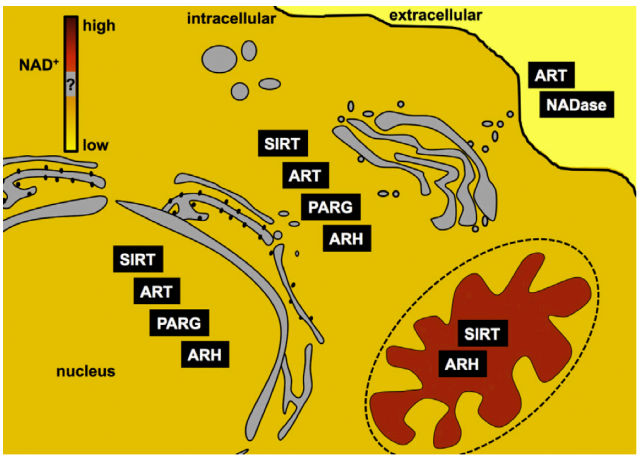


Figure 11. Compartmentation of NAD+ and NAD+ converting enzymes (6). The concentration of NAD+ is high in mitochondria (~ 400 μM) intermediate in the nucleus and cytosol (~ 100 μM) and low (<1 μM) in the extracellular space.

Another field that has elicited intensive investigation regards the extracellular NAD "reservoire" and how the extracellular NAD+ precursors and intermediates may serve for intracellular NAD+ biosynthesis. This topic includes the unravelling of the complex transport system of NAD metabolites inside and among the cells. Vitamin B3 NAD+ precursors Na e Nam are provided with the diet and enter the bloodstream for distribution to tissues (70). NR has been detected in cow milk (55) and it is presumed to be translocated from the extracellular to the intracellular compartment by a nicotinamide riboside transporter (Nrt), currently unidentified in mammals (71). NMN levels have been detected in mouse plasma (47) but its formation by the extracellular form of NMPRTase (eNAMPT) is still matter of debate (47, 48) and its transport through the plasma membrane has not been elucidated so far. Extracellular NAD+ has been detected in plasma and fluids (72, 73) at low concentration **(Figure 11)** and it can cross the plasma membrane through Cx43 hemichannels (74). Although its uptake has been postulated in some human cell lines (73), other studies support the idea that nucleosides, but not nucleotides can go through the plasma membrane, unless cell-type specific transport systems are present (26). This last hypothesis is consistent with the generally accepted idea that plasma membrane is impermeable to phosphorylated molecules. At the end of the last century, it had already been described a multitude of ecto-enzymes hydrolysing extracellular ATP and other nucleotides in order to inactivate intercellular signalling via extracellular nucleotides (e.g. purinergic signalling) and recycle the hydrolysis products (75). These enzymes are expressed differently on the plasma membrane of various human cells and tissues and include a family of ecto-phosphodiesterases/pyrophosphatases possessing a surprisingly broad substrate specificity, being capable of hydrolysing also NAD+ to AMP and NMN (76, 77). The multifunctional ecto-enzyme CD38 can hydrolyse not only its second messenger products, NAADP and cADPr, but also ADPr, ADPr phosphate (22) and NMN (78). After the hydrolysis of NMN by CD38 into Nam and 5'-phosphoribose, Nam can thus enter the cells through the plasma membrane. Also

the presence of a lymphocyte surface protein, CD73, endowed with a 5'-nucleotidase activity had been known for a long time (79). Indeed, this glycosylphosphatidylinositol (GPI)-anchored protein was well known to be responsible of the hydrolysis of nucleoside 5'-monophosphates, mainly AMP, to their respective nucleosides and Pi. It was only recently, after the determination of the crystal structure of the *Haemophilus influenzae* NAD nucleotidase (NadN) and the identification of CD73 as the human orthologue of this bacterial enzyme, that other features of CD73 have been explored (80). Indeed, Garavaglia and co-workers demonstrated how CD73 could also hydrolyse NAD+ and NMN, thus disclosing a possible novel function of this enzyme in systemic NAD metabolism. In 2012, the crystal structure of the soluble form of CD73 (without the GPI anchor) has been determined indicating how a prominent domain motion may control the enzymatic substrate specificity (81). The potential novel role played by CD73 in a hypothesized extracellular NAD+ biosynthetic cycles in vertebrates, as delineated by Belenky in 2006 (3) **(Figure 12)**, has found positive feedbacks in the experimental results of many studies (26, 80, 82). Firstly, it was shown how, when Nam salvage pathway was blocked in human cell lines, extracellular NMN utilisation as NAD+ precursor was prevented by the use of a competitive substrate (CMP) for the dephosphorylation reaction driven by the external 5'-nucleotidase. Thus, it was established that NMN (as well as the other nucleotides) has to be firstly converted to its corresponding nucleoside NR to serve as precursor for NAD+ biosynthesis (26). Grozio and collaborators demonstrated that CD73 enables, while CD38 impairs, the utilization of extracellular NMN for NAD+ biosynthesis by converting it into NR and Nam, respectively (82). Investigations to measure NAD and its precursors and metabolites (NAD metabolome) may address the opening questions on the routes for NAD uptake in different human cells and provide a framework of what a particular cell or tissue is doing. So far, the major challenges to determine NAD pools content have been represented by the nature of the methods themselves, requiring cell disruption and thus possibly affecting the

organellar concentrations, and by their robustness and reproducibility that should take into account the high complexity of the samples. The PARAPLAY method (64) as well as new HPLC-MS methods (83) seem to bypass some of these challenges and permit a more reliable and comprehensive analysis of NAD metabolome.

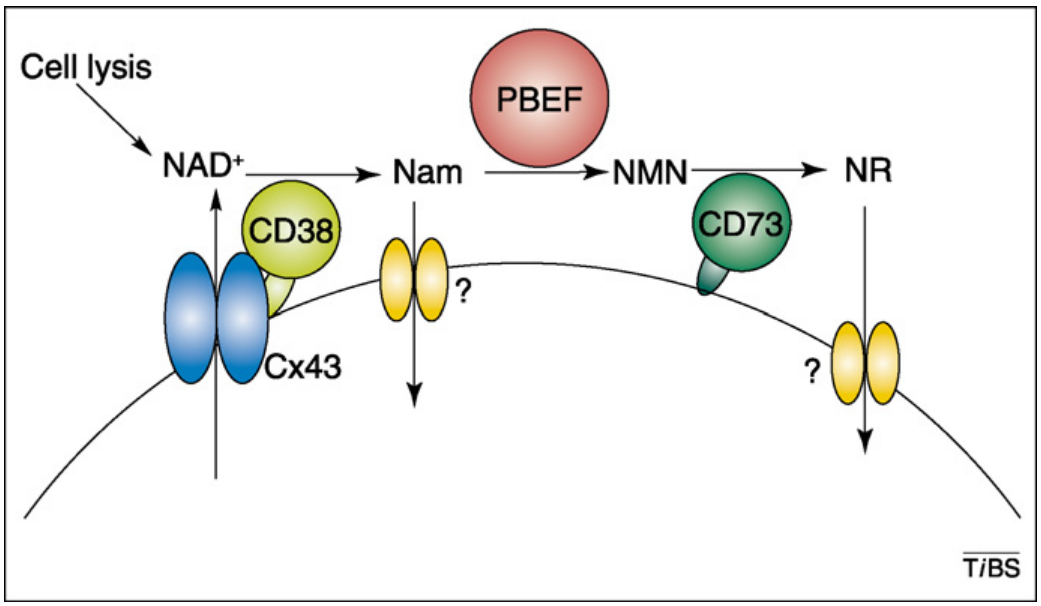


Figure 12. A potential extracellular NAD+-cycle in vertebrate as delineated by Belenky et al. in 2006 (3).

In conclusion, NAD homeostasis in human cells should be perceived as a dynamic state that is influenced by many intracellular and external factors finely interconnected, and regulated in response of the contingent physiological or pathological conditions of the cell of a determined tissue. Maintaining NAD homeostasis is essential for proper cellular function and aberrant NAD metabolism has been implicated in a number of metabolic- and age-associated diseases (84), including cancer. For example, there are different results supporting a relationship

between the NAD+-dependent sirtuins and cancer (18) and it is now well assessed that NMPRTase overexpression constitutes a potential biomarker for some cancer treatments (85). A look more and more in depth on the molecular basis of function and regulation of each of the metabolic and signalling processes discussed so far where NAD(P) plays the title role, without losing sight of the overall vision of the whole, so could offer important opportunities for the development of new therapies to maintain health and treat these various diseases.

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Chapter 2

Outline of the thesis

Outline of the thesis

NAD(P) is an essential electron carrier and a key molecule of signalling pathways. In bioenergetic pathways, NAD(P) serves as a coenzyme and it is reversibly converted between its oxidized (NAD(P)+) and reduced (NAD(P)H) states. The interchange within the redox couples is fundamental in determining the redox state of the cell, but do not require pyridine nucleotide consumption. In the guise of signal transducer, NAD(P)+ is involved in a variety of pivotal cellular processes, such as gene expression, calcium homeostasis, intercellular communication, cell death and aging. In this wide panorama of processes where pyridine nucleotide involvement goes beyond its coenzymatic activity, NAD(P)+ is used as a substrate and, therefore, consumed. In order to supply the cell with the pyridine nucleotides consumed in the signalling reactions and to meet its circumstantial needs, different pathways are responsible for NAD+ biosynthesis starting from several precursors in humans. Indeed, NAD+ biosynthesis is accomplished through either the *de novo* pathway from tryptophan or any of the salvage pathways that utilise the vitamin precursors of NAD+, namely nicotinamide (Nam), nicotinic acid (Na) and nicotinamide riboside (NR).
Nicotinate phosphoribosyltransferase (NaPRTase) is the enzyme responsible for the synthesis of NAD+ from Na. It catalyses the first rate-limiting reaction of the ubiquitous three-step Preiss-Handler pathway where Na is converted to nicotinate mononucleotide (NaMN) using 5-phosphoribosyl-1-pyrophosphate (PRPP) as donor of the 5-phosphoribose.

In the first part of my PhD dissertation, described in Chapter 3, I report on the structural determination of the human NaPRTase. We determined its three dimensional structure at 2.9 Å resolution in its free form. Both our structural analysis and molecular docking studies confirm that the minimal functional unit in human NaPRTase consists of a dimer. Indeed, the active site is formed at the dimer

interface with residues belonging to both monomers participating in ligand recognition and stabilisation. Moreover, overall structural analysis of hNaPRTase reveals that this enzyme belongs to the type II phosphoribosyltransferase subfamily. Structural comparisons with hQAPRTase and hNMPRTase, two other important phosphoribosyltransferases involved in NAD+ biosynthesis, show a conserved molecular architecture, but also highlight several peculiar features of hNaPRTase.

NAD(P)+-consuming reactions are compartimentalised and distinct NAD pools exist, whose relative content may change depending on the type of tissue considered as well as the pathophysiological conditions of the cell. Therefore, in human cells, NAD homeostasis has a dynamic and systemic nature that is believed to be subject of a tight regulation, and its maintenance is essential for proper cellular functions. Besides NAD+ biosyntesis, also the availability of different NAD+ precursors and the mechanisms regulating their uptake from the extracellular space critically contribute to the NAD homeostasis maintenance. Several transporters and ecto-enzymes, differently distributed on the plasma membrane of the various human cells, mediate the internalisation of NAD metabolites. CD73 is a glycosilphosphatidylinositol (GPI)-linked cell surface human enzyme involved in NAD homeostasis through its 5'-nucleotidase activity. Indeed, CD73 mainly catalyses the dephosphorylation of extracellular purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to their corresponding membrane-permeable nucleosides, but it can also process both NAD+ and NMN, with implications for uptake of these extracellular nucleotides.

In the second part of this thesis, discussed in chapter 4, I present a biochemical investigation on the uptake mechanisms of nicotinamide mononucleotide (NMN) and NR in human hepatocarcinoma cell lines. We worked with wild type and knockdown CD73 HepG2 cells and fed them with double-labelled NR and NMN in

order to investigate their cellular uptake. Through the use of chemical biology tools and LC-MS^2, our preliminary results confirm, in a direct manner, the dependency of extracellular NMN upon CD73 activity to be used as intracellular source of NAD+ in human hepatocytes. Moreover, this study offers a complete framework of the relationships among NAD metabolites regardless the extracellular precursor utilised by cells, demonstrating the utility of stable labelling experiments coupled to LC-MS/MS analysis to elucidate metabolic relationships.

In summary, by determining the three-dimensional structure of hNaPRTase, a pivotal enzyme involved in NAD+ biosynthesis, and confirming the availability of extracellular NAD+ precursors to the intracellular pool, the results discussed in this thesis will contribute to our understanding of the mechanisms involved in cellular NAD homeostasis.

Chapter 3

The crystal structure of human Nicotinic Acid Phosphoribosyltransferase: a key enzyme in Preiss-Handler pathway

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ABSTRACT

Human nicotinic acid phosphoribosyltransferase (EC 2.4.2.11) (hNaPRTase) is the rate-limiting enzyme in the three-step Preiss-Handler pathway for the biosynthesis of NAD+. It catalyses the conversion of nicotinic acid (Na) to nicotinic acid mononucleotide (NaMN), using 5-phosphoribosyl-1-pyrophosphate (PRPP) as phosphoribosyl donor and releasing pyrophosphate (PPi). Several studies have underlined NaPRTase importance for NAD homeostasis in mammals, but no crystallographic data have been available so far for higher eukaryotes.

Here, the three-dimensional structure of a recombinant hNaPRTase is reported. The human enzyme was overexpressed in *Escherichia coli* and its crystal structure was solved by means of molecular replacement at a resolution of 2.9 Å in its free state. Our structural data reveal that hNaPRTase functions as a dimer. Indeed, the active site formation occurs at the dimer interface and requires the participation, on each monomer, of an irregular α /β barrel that ascribes hNaPRTase to the Type II phosphoribosyltransferase subfamily. Furthermore, due to the absence of co-crystallized ligands within hNaPRTase, we performed molecular docking simulations to better understand the catalytic mechanism of the enzyme. We identified residues involved in the recognition and stabilisation of several ligands, and confirm their belonging to both monomers. Interestingly, among all available NaPRTase structures, we found that hNaPRTase is more similar to *E. faecalis* NaPRTase. We postulated a possible adaptation mechanism for the commensal bacterium to survive in the human small intestine, where hNaPRTase is highly expressed. Moreover, structural comparisons of hNaPRTase with the other two human Type II phosphoribosyltransferases involved in NAD+ biosynthesis, hQAPRTase and hNMPRTase, reveal that the three enzymes show a similar overall structure in accordance with their common function. Nevertheless, they also present important structural differences. In particular, as previously reported for *Ta*NaPRTase, we show that hNaPRTase active site lacks of a tunnel that, in hNMPRTase, represents the binding site of its potent inhibitor FK866, currently used in clinical trials as antitumoral agent.

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) and its phosphorylated form (NADP) are essential and ubiquitous coenzymes, which play a fundamental role in cellular metabolism. Beyond its pivotal role as a redox cofactor in energy transduction and cellular metabolism, NAD(P) is also intimately involved in signalling pathways. Indeed, in the last years NAD(P) has emerged as a major topic with a wealth of biochemical investigations that confirmed its central role in a plethora of physiological and pathological conditions (1, 2). The NAD(P) derivatives, nicotinic acid adenine dinucleotide phosphate (NaADP) and cyclic ADP-ribose, are likewise among the most potent calcium-mobilising agents. NAD+ is the substrate for poly(ADP-ribosyl)ation, a vitally important post-translational modification occurring within the nuclei of higher eukaryotes by poly(ADP-ribose) polymerase. Also, NAD+ is the substrate for mono-ADP-ribosyltransferase, an enzyme that transfers a single ADP-ribose unit from NAD+ onto target proteins in both mammalian and prokaryotic cells. Moreover, overexpression of the NAD+ biosynthetic pathway enzymes, were shown to extend yeast cell lifespan, a phenomenon linked to the action of NAD+-dependent catalysis of protein deacetylation (3-7). Severe altering of NAD homeostasis features a number of pathological conditions ranging from neurological disorders, cancer and infectious diseases, and is also observed in the process of ageing (8)**.** Consequently, all these NAD+-degrading reactions clearly necessitate permanent regeneration of the cofactor (9). Based on the literature data, it is possible to identify four different substrates that can be used as a source of the pyridine ring in the NAD+ biosynthesis: Quinolinic Acid (QA) in the *de novo* pathway; Nicotinic Acid (Na) and Nicotinamide (Nam) in the salvage pathways; Nicotinamide Riboside (NamR) in the "Preiss-Handler-independent" pathway (2). The three precursor QA, Na and Nam can be transferred onto 5-phosphoribosyl-1-pyrophosphate (PRPP) by the

respective phosphoribosyltransferase. The resulting, Nicotinic Acid Mononucleotide (NaMN) and Nicotinamide Mononucleotide (NMN), are converted in their corresponding dinucleotides: Nicotinic Acid Dinucleotide (NaAD) and NAD+ by Nicotinamide Mononucleotide Adenylyltransferase (NMNAT) (10). Finally NaAD is amidated to NAD+, by NAD synthase **(Figure 1)** (11, 12).

Although Nam has been thought to represent the main precursor of the salvage synthesis to keep cellular contents of NAD+ constant in mammals (13), the supplementation of Nam does not seem so effective in elevating cellular NAD content beyond the basal concentrations (14). Indeed, Nam levels are much higher than Na levels throughout mammalian living cells, but some tissues preferentially use Na for NAD+ synthesis (15). Effectively, Na was found to be more successful than Nam in increasing NAD+ levels in heart, kidney (16), and red blood cells (17). Therefore, both precursors appear relevant for NAD+ biosynthesis, possibly with distinct and complementary roles in different cells. Moreover, in a recent work it was demonstrated that Na added to stress-induced damaged human cells, reversed the H_2O_2-cytotoxicity in a dose dependent manner. In contrast, the addition of corresponding concentration of Nam to the culture medium did not protect the cell from the stress (18).

In mammals, Na is mainly extracellular and derives directly from the diet, and indirectly from dietary Nam by a gut flora Nam deamidase. As shows in **Figure 1**, Na is the substrate of a Nicotinate Phosphoribosyltransferase (NaPRTase, EC 2.4.2.11), originally named NaMN pyrophosphorylase, which catalyses the synthesis of NaMN from Na and PRPP. Handler first identified the enzyme NaPRTase in humans together with the different ability of Na and Nam to elevate NAD+ levels in red blood cells. The enzyme has also been partially characterised from different sources: prokaryotes, archaea, and eukaryotes indicating the ubiquitous nature of the biosynthetic pathway from Na to NAD+, the so-called “Preiss-Handler pathway” (19-22). The hNaPRTase significance for NAD cellular

homeostasis in mammals has been reinforced by the indication that in the human embryonic kidney cells (HEK293) the addition of Na, but not Nam, markedly elevates NAD+ levels. Moreover, there is evidence that hNaPRTase appears to be more expressed in those tissues where Na is the preferential source for NAD+ biosynthesis (18). This Na effect could be ascribed to the finding that NaPRTase, unlike NamPRTase, is not subject to feedback inhibition by NAD+ (18). The ability of orally administered Na to increase cellular NAD+ levels via hNaPRTase may also account for some of its vitamin effects (17). Indeed, this awareness suggested novel applications for the treatment of conditions associated with cellular NAD+ depletion, such as in photodamaged skin (23). Furthermore, Na represents one of the oldest lipid-modifying drugs, showing a unique anti-lipolytic effect (24). This action is mediated by its interaction with GPR109A, a Na-receptor on the plasma membranes of adipocytes (25, 26). The investigation on the possible modulation of blood Na levels by hNaPRTase in treated patients, based on the enzyme characteristics could optimize current pharmacological applications of the vitamin in this important metabolic contest. Like bacterial enzymes, human NaPRTase is strictly specific for Na as a substrate. In contrast, its activity is not affected by the presence of NaMN, ADP, NAD+ and NaAD+ (24), and consequently the lack of inhibition by NAD+ supports the ability of Na to elevate NAD+ levels via the "Preiss-Handler pathway". Otherwise, with respect to ATP requirement, NaPRTases have been grouped in three distinct classes: strictly ATP-dependent, strictly ATP-independent, or independent but stimulated by ATP (11). In this contest, ATP is cleaved to ADP and Pi stoichiometrically with respect to NaMN formation (28, 29), and the activation of NaPRTase by ATP is conformationally coupled to the product formation *via* a phosphoenzyme intermediate (30, 31). Indeed, it was demonstrated that NaPRTase showed to be autophosphorylated in *S. typhimurium* (32) and the activity of the human NaPRTase mutant H213A resulted not stimulated but rather inhibited by ATP. This inhibition was found to be non-competitive with respect to Na but competitive with

respect to PRPP consistent with the ATP occupancy of the PRPP binding site as also demonstrated in *S. typhimurium* NaPRTase (33). In particular, human NaPRTase has a dimeric organisation and ATP utilisation might promote allosteric interactions between subunits. Such ATP-driven conformational change can also be inferred by the observation that, in microbial NaPRTases, ATP binding protects from proteolysis (34) and heat inactivation (35). In addition, the effect of inorganic Pi on human NaPRTase activity results in an increasing of the K_{cat} value of about 25-fold without altering substrate affinity (33). Besides ATP and Pi, several central compounds of cellular metabolism appear to affect human NaPRTase. The enzyme is indeed activated by DHAP and pyruvate and inhibited by CoA (and its derivatives acetyl-, glutaryl- and succinyl-CoA), G3P, phosphoenolpyruvate, and fructose 1,6-bisphosphate, leading to hypothesize a link between NAD+ synthesis and glucose or/and fatty acid metabolism (33).

Here we report the crystal structure of human Nicotinic Acid Phosphoribosyltransferase at 2.9 Å resolution in its free state. Our data can contribute to shed more light on the catalysis mechanism of this enzyme involved in NAD cellular homeostasis and complete the structural bases of the salvage pathway enzymes of NAD+ biosynthesis. All these structural data may be used to assist the structure-based rational design of enzyme inhibitors of potential medical interest.

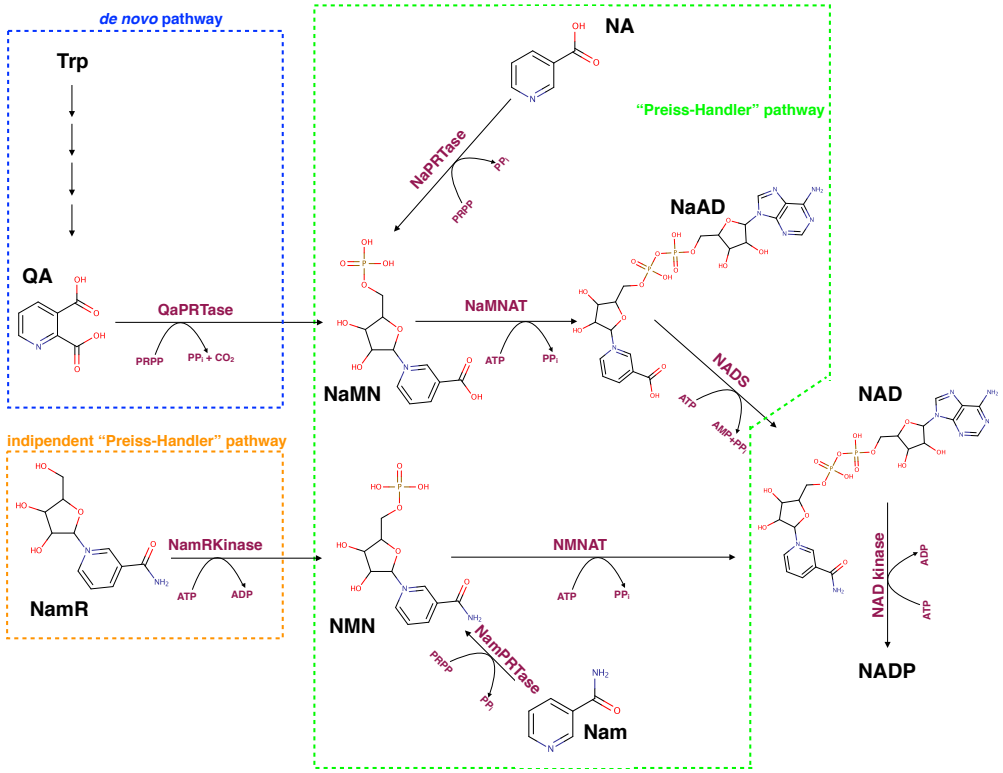

Figure 1. NAD(P)+ biosynthesis in humans. NAD+ biosynthesis initiates from four different sources of the pyridine ring, namely QA, Na, Nam and NamR, through three distinct pathways. The *de novo* pathway (blue dotted line) allows NAD biosynthesis starting from QA derived from Trp; Na and Nam are processed to NAD+ through the Preiss-Handler pathway enzymes (green dotted line), although NamPRTase does not belong to this pathway. The Preiss-Handler independent pathway (orange dotted line) is responsible of the pyridine nucleotide synthesis starting from NamR. Finally, some of the cellular NAD can be converted into NADP by NAD kinase (EC 2.7.1.23). QaPRTase, quinolinic acid phosphoribosyltransferase (EC.2.4.2.19); NaPRTase, nicotinic acid phophoribosyltransferase (EC 2.4.2.11); NamPRTase, nicotinamide phosphoribosyltransferase (EC 2.4.2.12); NamRKinase, nicotinamide riboside kinase (EC 2.7.1.22); NMNAT/NaMNAT, nicotinamide mononucleotide adenylyltransferase (EC 2.7.7.1); NADS, NAD+ synthetase (EC6.3.5.1).

RESULTS AND DISCUSSIONS

Overall Quality of the model

The three-dimensional structure of hNaPRTase has been solved by molecular replacement and refined at a resolution of 2.9 Å. The crystal asymmetric unit contains a dimer of hNaPRTase and a total of 98 solvent molecules. The two protein molecules have been designated as monomers A and B (**Figure 2)**. Monomer A contains 503 residues out of 538 and no electron density is present for the following regions: first 15 residues at the N-terminus, residues 379-389, residues 412-415, and last five residues at the C-terminus. Monomer B contains 501 residues out of 538 and no density is present for the same regions as in monomer A except for the lacking region 408-414 and for the last four C-terminal residues. The stereochemistry of the model has been assessed with the program PROCHECK (36). 80% of protein residues falls in the most favoured regions of the Ramachandran plot with no outliers.

Overall structure of human NaPRTase monomer

Human NaPRTase monomer folds into 17 α-helices, 24 β-strands, and the connecting loops organized in two domains: an A domain characterized by an open-faced sandwich and an irregular α/β barrel, domain B (**Figure 3A)**. The Domain A is composed of residues provided by both the N- and C-terminal regions (residues 16-127 and 390-534). It consists of 7 α-helices disposed like a shield and delimiting the open space where 15 β-strands form 4 antiparallel β-sheets, showing a specular two-by-two disposition. Indeed, the seven-stranded fully antiparallel β-sheet (β1, β3, β5, β15, β16, β22 and β23) and the four-stranded partially

antiparallel β-sheet (β17, β18, β21 and β24) face each other as the other two smaller β-sheets do (β2, β4 and β19, β20). The longest helix α5, with 8 turns (residues 128-159) connects the two domains and participates in both of them. The second domain (residues 160-378) contains a six-stranded α/β core attesting the belonging of hNaPRTase to the Type II Phosphoribosyltransferases (PRTases) subfamily (**Figure 3B)**. The monomers in the crystal have essentially the same conformation, with r.m.s. deviation of about 0.5 Å for their equivalent Cα atoms.

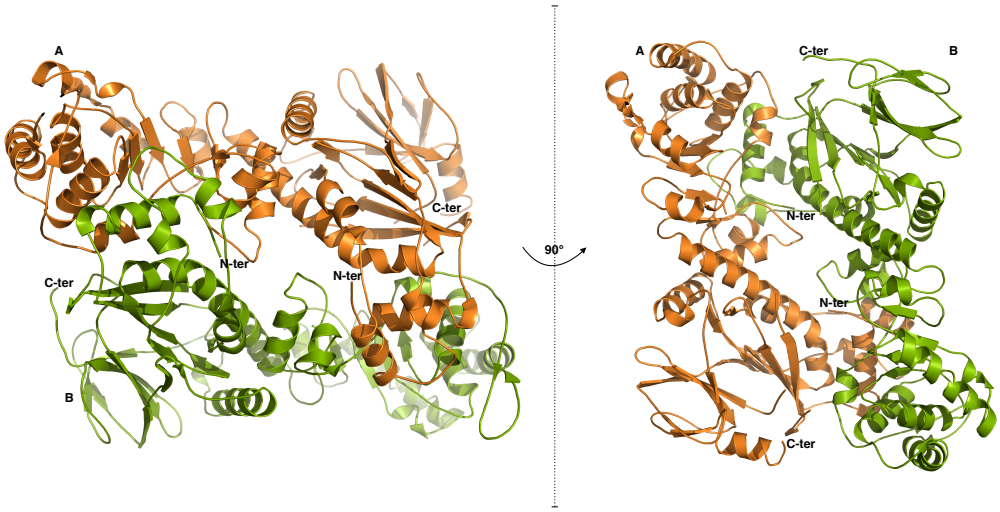

Figure 2. The crystal structure of human NaPRTase. The enzyme has a dimeric structure and monomers A and B are coloured in orange and green, respectively. Two different orientations of the dimer, showing the head-to-tail arrangement of the monomers, are represented. N-ter, N-terminus; C-ter, C-terminus.

The dimer of human NaPRTase

hNaPRTase has a predicted molecular weight of 58 kDa and it has been reported to show a native molecular mass of about 87,000 Da after gel filtration (37, 33). Consistently, our size exclusion data are in accordance with previous analyses (data not shown). The native crystal structure of hNaPRTase reveals the presence of an intimately associated dimer in the asymmetric unit with 3,000 $Å^2$ of the surface area of each monomer buried at the dimer interface. The two monomers are arranged head to tail with the N-terminal domain in one monomer contacting the α/β barrel in the other monomer (**Figure 2)**. Therefore, the available structural data together with the conservation in hNaPRTase of some residues participating in the formation of the active site at the dimeric interface in both *Ta*NaPRTase and hNMPRTase, as shown by their structural superposition, confirm that the minimal functional unit in the hNaPRTase enzyme consists of a dimer. Moreover, it has been shown how the major interaction at the dimeric interface in *Thermoplasma acidophilum* NaPRTase is ionic rather than hydrophobic, thus suggesting that the oligomeric state could be modulated by different factors such as pH, salt or protein concentration influencing these ionic interactions (38). The 3D analysis of the surface interaction between hNaPRTase monomers A and B confirmed that the main interactions are ionic.

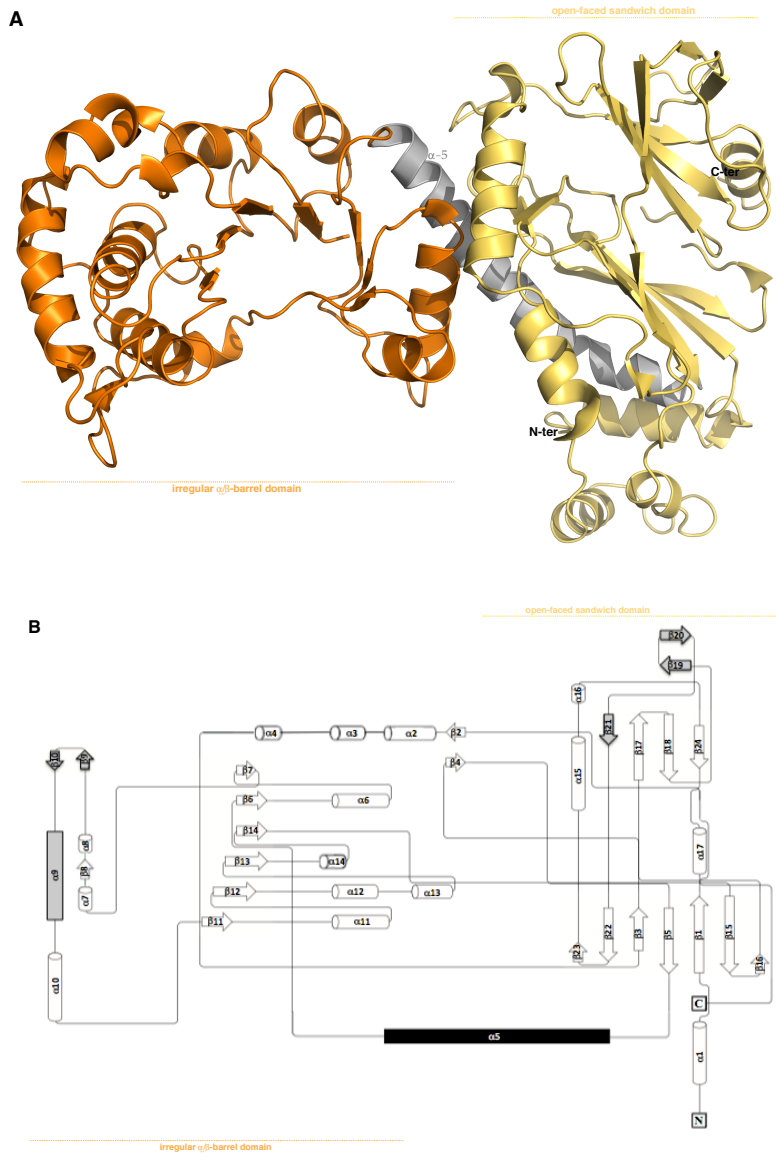

Figure 3. Protein fold of hNaPRTase monomer. A. Ribbon representation of the overall structure of hNaPRTase monomer. Each monomer consists of two domains: the domain A, characterized by an open-faced sandwich, is shown in yellow, while the domain B, formed by an irregular α/β barrel, is coloured orange. The two domains are connected by a long α-helix, α5, coloured in grey. **B.** Topology diagram of hNaPRTase. Arrows represent β-strands and cylinders α-helices. Grey β-strands and α-helices form two structural regions, composed by β9, β10 and α9 and β19, β20 and β21 respectively, that appear to be univocally present only in mammal NaPRTases. Long helix α5, connecting the two domains, is shown in black.

Molecular docking studies

Due to the absence of hNaPRTase substrates co-crystallized within the protein, we decided to perform a molecular docking simulation in order to better understand the molecular mechanism of the enzyme. The functional dimeric model of hNaPRTase was first re-built using the X-ray crystal structure and subsequently refined by molecular dynamics simulations. Na, NaMN, Na plus PRPP, and ATP were docked in the generated model.

Na. Docking pose of Na revealed that the nicotinic ring is stabilized in the active site by a π-π stacking interaction with Tyr21'. Arg318 results engaged in an ionic interaction with the carboxyl group of Na, while Leu170, Gly209 and Leu211 are involved in Van der Waals interactions with pyridine ring (**Figure 4A)**. The ligand pose is in accord with the Na bound to *E. faecalis* NaPRT (PDB ID: 2F7F).

NaMN. The binding mode of nicotinic ring is in accordance with Na binding. A hydroxyl group of the sugar moiety is involved in a hydrogen bond with Arg318 and the phosphate group displays an ionic interaction with Arg171 (**Figure 4B)**.

Na plus PRPP. The docked pose of Na results quite similar to that previously obtained for Na alone. The PRPP docked pose results stabilised by hydrogen bonds with Arg318 and Ser214, and by ionic interactions with Arg171 and Lys396' (**Figure 4C)**. Arg171, Arg318 and Ser214 correspond to the same conserved residues that in *E. faecalis* NaPRTase (PDB ID: 2F7F) represent the aminoacids involved in the binding of diphosphate.

ATP. The binding mode is similar to PRPP, since the γ-phosphate group shows an ionic interaction with Lys396' while the β group is involved in hydrogen bonds with His213 (backbone amide) and Ser214. The adenine moiety of ATP is involved in a π-polar interaction with Arg318 (**Figure 4D)**.

Molecular docking studies confirm the participation of both monomers of hNaPRTase in the formation of the catalytic site. Our observations, based on docking analysis on the X-ray structure of hNaPRTase, suggest a new hypothesis

on how the enzyme binds its substrates and products that differs from the scenario coming from molecular docking studies performed on the structural predicted hNaPRTase monomer (33).

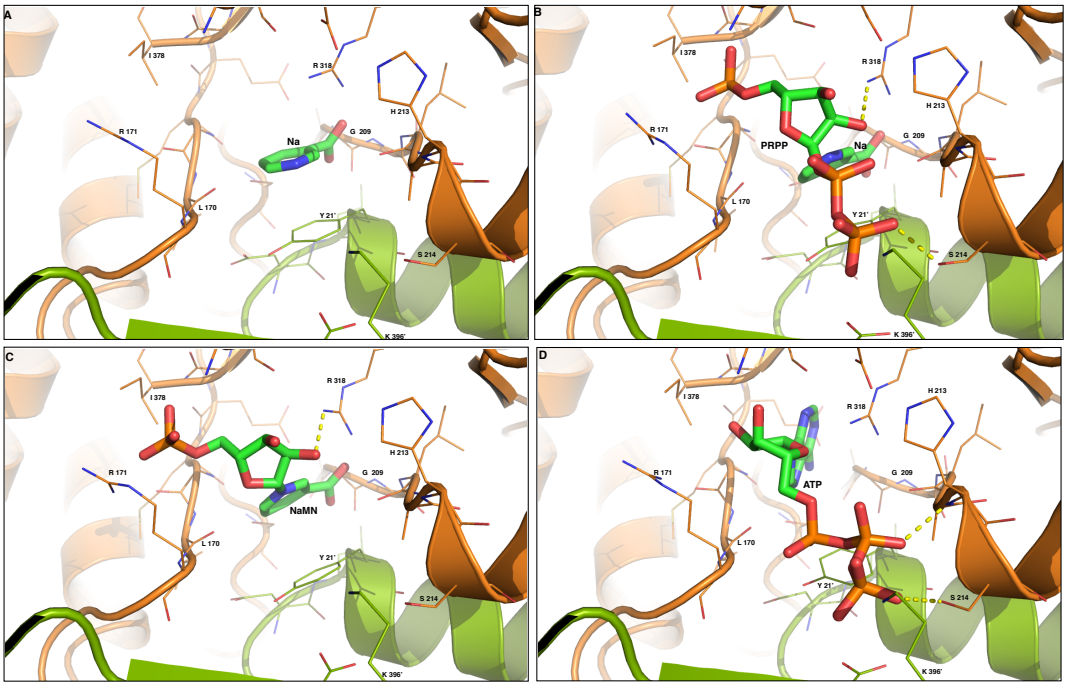

Figure 4. Active site of hNaPRTase at the dimeric interface in complex with different ligands as predicted by molecular docking studies. Ribbon representation of the active site formed at the dimeric interface, between the α/β barrel domain of monomer A, coloured in orange, and the α1 helix of monomer B, shown in green. Side chains of residues participating in the coordination of ligands (A: Na; B: NaMN; C: Na and PRPP; D: ATP) are represented as thin sticks and their identity is indicated, whereas ligands are depicted as green sticks. Hydrogen bonds are shown as yellow dotted lines.

Comparison with hNMPRTase and hQAPRTase

Despite sharing very limited sequence similarity, hNaPRTase shows a molecular architecture that closely resembles those ones firstly described for hNMPRTase (39) and hQAPRTase (**Figure 5)** (40). This fact is in accordance with their

common function, consisting in the transfer of the phosphoribosyl group from PRPP onto their respective substrates. A DALI search shows that the highest similarity is found with hNMPRTase with Z-value of 22.3 (rmsd of 3.8 Å). hQAPRTase is the smallest in size among the three, according to the hypothesis that is the most ancient (41). Contrarily, hNaPRTase contains 46 and 241 more amino acid residues than hNMPRTase and hQAPRTase, respectively. The structural superposition of the three enzymes highlights how some of the additional residues in hNaPRTase form two structural regions that, from a Blastp research, appear to be univocally present only in mammal NaPRTases. The region between amino acids 223-264 cointains two β-strands (β9, β10) and one a-helix (α9) that extend the borders of the α/β barrel domain and contribute to contact the other monomer. Indeed, the side chain of Asp227 is hydrogen bonded to the side chain of Arg31 belonging to the other monomer. The second unique region (447-472) of hNaPRTase encompasses three β-strands (β19, β20 and β21) that stand at the bottom of the open-faced sandwich (**Figure 3B; Figure 5)**.

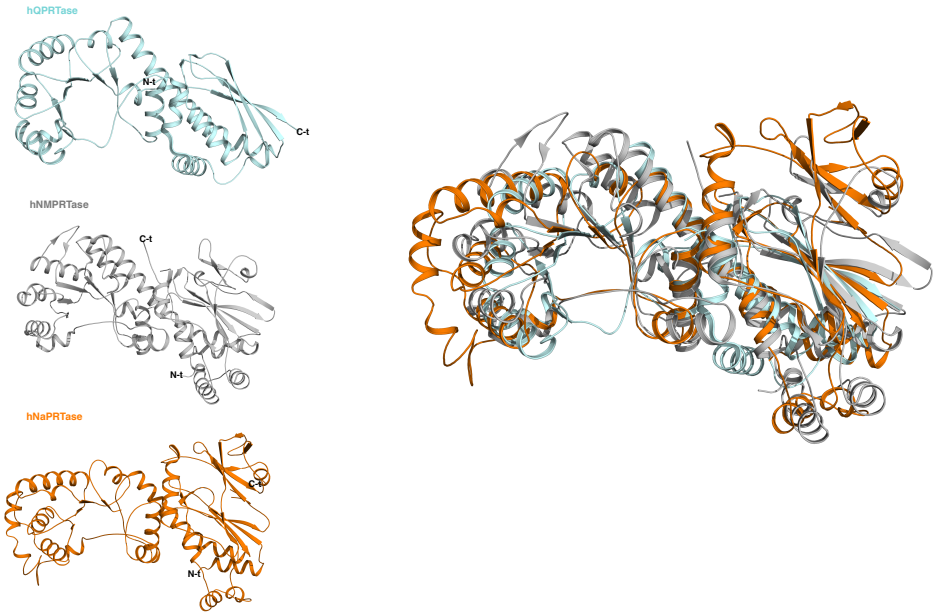

Figure 5. **Structural comparison of the three Type II phosphoribosyltransferases involved in NAD biosynthesis**. Ribbon representations of human quinolic acid phosphoribosyltransferase (hQAPRTase), nicotinamide phosphoribosyltransferase (hNMPRTase) and nicotinic acid phosphoribosyltransferase (hNaPRTase) are shown separately (*left panel*) coloured in light blue, grey and orange, respectively. The structural superposition of the three enzymes is also represented (*right panel*).

Comparison with NaPRTases of other species

The structures of NaPRTase monomers of gram-negative bacteria *A. tumefaciens*, *Y. pestis*, *P. gingivalis*, *V. cholerae* and of the unicellular eukaryote *S. cerevisiae* are very similar. It is quite surprising that the structure of the human NaPRTase is more similar to the *E. faecalis* NaPRTase than to the NaPRTase of the eukaryotic *S. cerevisiae*. It could be hypothesized that this is due to the fact that *E. faecalis* is a commensal bacterium inhabiting the gastrointestinal tract of humans and other mammals. Moreover, it has been already established that the small intestine is one of the tissue where Na is preferred as source of NAD, so that this can represents an example of adaptation of the bacterium to survive.

Lack of inhibition by FK866

As previously described in literature, the potent antitumoral agent FK866, bounds at the center of the parallel β-sheet in the α/β barrel domain, in a tunnel at the dimer interface of hNMPRTase (42). In particular, in hNMPRTase there are two small β-strands (region 240-245) that close the circumference outlined by the parallel β-sheet at the α/β core; in hNaPRTase, the residues in corresponding region (206-211) are oriented closer to the center of the α/β barrel, so determining a smaller diameter of the entrance of the tunnel site in hNaPRTase, compared to hNMPRTase. This different disposition is due to the presence of an insertion of 10 residues in hNMPRTase that is not present in hNaPRTase as well as in *Ta*NaPRTase (42). Moreover, the orientation of the whole β-sheet forming the binding site, compared to that of hNMPRTase, brings the central β-strands β13 and β14 to be closer to the center of the barrel of about 4.5 Å compared to those of hNMPRTase, with also the loop connecting α14 and β14 sterically preventing the binding of the specific FK866 and other antitumoral inhibitors for hNMPRTase (**Figure 6).** The fact that hNaPRTase prevents, in both normal and tumoral cells, the formation of Reactive oxygen species (ROS) induced by hNMPRTase inhibitors (43), support the envisaged possibility to co-treat with nicotinic acid those tumours where hNaPRTase is poorly expressed, in order to increase the efficacy of a therapy based on hNMPRTase inhibitors (44). Indeed, the administration of nicotinic acid protected non-tumour cells from toxic effects resulting from depletion of NAD caused by hNMPRTase inhibition (43). Therefore, while strong inhibition of hNMPRTase is the key point for high toxicity toward tumour cells, no inhibition should be envisaged for hNaPRTase in order to reduce the toxic effect of the anticancer drug.

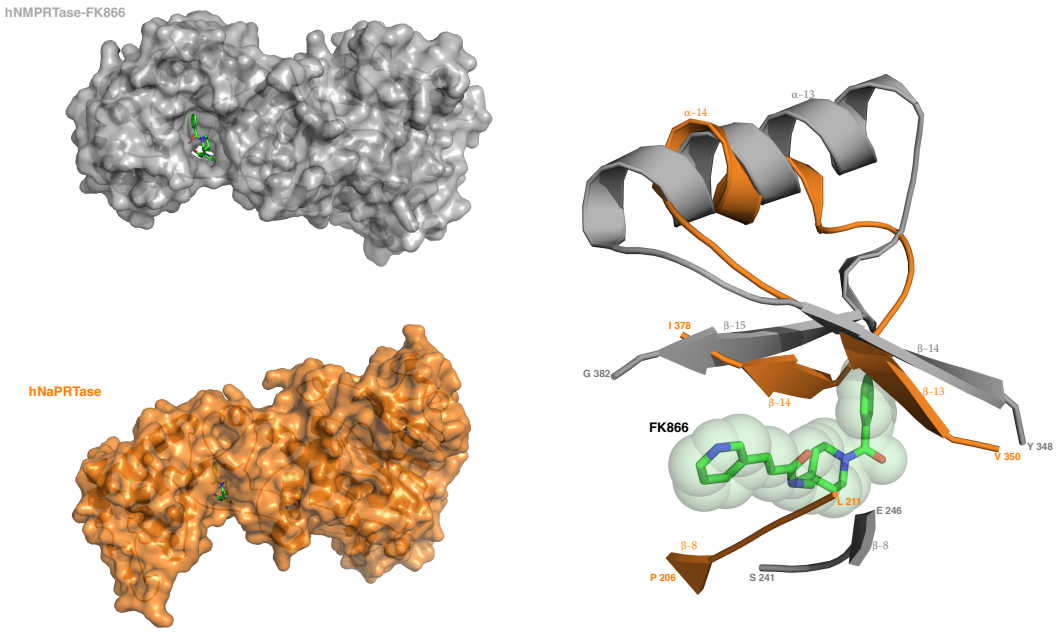

Figure 6. Structural comparison between hNMPRTase and hNaPRTase at the FK866-binding site. Human NMPRTase structure in complex with its inhibitor FK866 (PDB ID: 2GVJ) is coloured in grey and hNaPRTase is coloured in orange. Structural superposition between these two structures poses in evidence the absence, in hNaPRTase, of the tunnel where FK866 binds in hNMPRTase. Buried surface areas of hNMPRTase (*upper left panel*) and hNaPRTase (*lower left panel*) monomers are represented and FK866 is shown as green stick. A ribbon represented close-up view (*right panel*) of the superposed secondary structure elements sterically hampering (in hNaPRTase) or permitting (in hNMPRTase) the binding of FK866**.** Light green spheres represent steric hindrance of FK866.

CONCLUSIONS

NAD+ salvage pathways have a prominent role in cellular NAD metabolism and homeostasis. Pyridine nucleotide availability has been shown to be implicated in a large number of physiological and pathological conditions. Preiss-Handler pathway is responsible for the synthesis of NAD+ starting from Na, and NaPRTase represents the first enzyme involved in this ubiquitous salvage route. Beyond its role in NAD+ biosynthesis, it was hypothesized that, in humans, NaPRTase could be potentially linked to other cellular metabolic pathways. Indeed, it has previously been shown that several metabolites related to glucose or fatty acid metabolism can influence hNaPRTase activity, thus a pleiotropic role for this enzyme could not be excluded (33). Moreover, the substrate of NaPRTase, Na, is known to have antidyslipidaemic properties and it has been used as a lipid-modifying drug (24). More recently, also the anti-oxidant functions of Na have been explored (45-47). Although mechanisms underlying the antilypolitic effects of Na are not completely understood, it has been shown that Na beneficial properties in protecting against H_2O_2-citotoxicity depend on its intracellular metabolism by NaPRTase (18, 43, 48). Several studies have evidenced how hNaPRTase expression levels vary consistently among populations and in different pathological conditions (44, 49-51). With respect to cancer, evaluation of the structural impact of the numerous hNaPRTase mutations found in tumours where the enzyme is poorly expressed, could help in predicting the usefulness of a potential co-treatment of these tumours, with Na and hNMPRTase inhibitors (49), towards a personalised medical approach. In this scenario, our structural and molecular docking results, contribute to a better understanding of hNaPRTase molecular mechanism, allowing the study of the structure-function relationships.

EXPERIMENTAL PROCEDURES

Enzyme expression and purification

The procedure adopted for the bacterial overexpression and purification of the recombinant human NaPRTase has been here reported. The pET32b vector (Novagen) plasmid containing the *orf* for human Nicotinic Acid Phosphoribosyltransferase was replicated in *Escherichia coli* TOP10, sequence-verified, and transformed into *E. coli* BL21 (33). In order to have a long term storage of the expressing-vector, *E.coli* BL21 glycerol stocks were prepared by adding 50% glycerol to the BL21 cell culture and store them at -80°C. The bacterial growth was started by scraping some frozen bacteria then, dissolving them into 10 ml 2XTY medium aliquots supplemented with 100 mg/ml Ampicillin. Bacteria were grown overnight at 37°C in a slow shake. The cells were then diluted (1/40) in the same medium and cultured at 30°C until the optical density at 600 nm (OD_{600}) reached 0.6 to 0.8. Expression was induced by addition of isopropyl-D-thiogalactoside (IPTG) to final concentration of 0.8 mM, and the temperature was decreased to 20°C. Cells were harvested by centrifugation after 16 h of induction. Induced cells were collected by centrifugation and were resuspended in 1/50 original volume with 100 mM KH_2PO_4, pH 8, 300 mM KCl, 10 mM imidazole, 1 mM β-mercaptoethanol and 250 units of benzonase nuclease. Plasma membranes of cells were distrupted by applying a high pressure through the use of the French Press twice at 1.5 KBar. Protease inhibitor cocktail was added (100 ml per 40 ml extraction Buffer) to the crude extract that was clarified by 1h centrifugation at 18,000 rpm. The supernatant was purified by a His-tag affinity chromatography and a subsequent size-exclusion chromatography as follows. In particular, in the first purification step, the solution containing the soluble fraction of human enzyme was batch-mixed for 30 min with a Qiagen Ni-NTA resin (0.1 ml per ml extract),

previously equilibrated with the buffer, then packed into a chromatographic column. The flow-through and the 30mM imidazole buffer wash were discarded. The recombinant protein was eluted by 250 and 500 mM imidazole. Protein concentration was determined by the Bradford protein assay. Active, SDS-PAGE homogeneous fractions were pooled, centrifuged and loaded on a Sephacryl S200 16/60 column in a FPLC system. Elution Buffer contained 50 mM Hepes/KOH buffer, pH 7.5, and 300 mM KCl and the flow rate was 0.2 ml/min. Eluted fractions were analysed by SDS-PAGE and used for crystallization trials. All steps were performed at 4 °C and with 100 ml/40 ml Protease Inhibitor cocktail to preserve the protein stability.

Crystallization and Structure Determination

Crystals of hNaPRTase were obtained by means of the vapour diffusion technique in sitting drops. 4 ml of a protein solution at a concentration of 15 mg/ml, preincubated with 1 mM Acetyl-CoA and 5 mM DTT, were mixed with an equal volume of a reservoir solution containing 0.1 M Sodium cacodylate pH 6.5 and 1.7 M Sodium acetate, and equilibrated against 1.2 ml of the reservoir solution, at 4°C. Crystals grew to a maximum dimension of 0.1 x 0.018 x 0.015 mm in about 5 days. For X-ray data collection, crystals were quickly equilibrated in a solution containing the crystallization buffer and 20% glycerol as the cryo-protectant and flash frozen at 100 K under a stream of liquid nitrogen. Data up to 2.9 Å resolution were collected at the beamline ID23 EH1 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Analysis of the diffraction data set collected allowed us to assign the crystal to the orthorhombic $P2_12_12_1$ space group with cell dimensions a = 88.01 Å b = 101.09 Å and c = 125.14 Å containing two molecules per asymmetric unit with a corresponding solvent content of 48%. Data were processed using the program package XDS (52) and the CCP4 suite of program

(53) were used for scaling. The structure determination of hNaPRTase was carried out by means of the molecular replacement technique using the coordinates of a monomer of *Enterococcus faecalis* NaPRTase as the search model (Protein Data Bank ID code 2F7F). Firstly, an improvement of the quality of the search model was carried out by using information contained in the sequence alignment between the hNaPRTase and the *E. faecalis* NaPRTase through the program SCULPTOR (54). The new procedure Phenix.mr_rosetta (55), that combines crystallographic and structure-modeling algorithms from Phenix and Rosetta, respectively, was used to automatically determine the hNaPRTase structure. Phenix.mr_rosetta returned a unique molecular replacement solution with an LLG of 44.56 and TFZ=7.4. The initial model was subjected to iterative cycles of crystallographic refinement with the program PHENIX (56), alternated with manual graphic session for model building using the program Coot (57). 5% randomly chosen reflections were excluded from refinement of the structure and used for the Free R factor calculation (58). The program ARP/wARP (59) was used for adding solvent molecules. When the R-factors dropped to a value of R factor and Free R factor of 0.21 and 0.28, with ideal geometry at 2.9 Å resolution, we performed an inspection of the electron density in the enzyme active site. Data collection and refinement statistics are given in **Table I**.

Molecular Modelling

A molecular dynamics simulation was performed using the GROMACS simulation package v.4.5.3 (60) with the standard GROMOS96 force field. The X-ray crystal structure of hNaPRTase was energy-minimized *in vacuo*, using 1000 steps of steepest descent in order to remove distorted geometries, and it was embedded in a solvent box. The next step was a 2 ns simulation with position restraints to the protein, followed by a 500 ps unrestrained simulation. The last 500 ps of the simulation were used to compute an average structure for hNaPRTase, which, after minimization with 1000 steps of steepest descent, represents the final model. After aminoacids' stereochemistry has been assessed with the program PROCHECK (52), the model was used for docking simulations. The ligands tested for docking were FK866, Na, NaMN, Na plus PRPP and ATP. Ligand structures were built from a SMILES string and were minimized using Omega2 (61). The docking simulations were performed using FRED, and using the default settings (62). Each ligand-NAPRT complex was subsequent subjected to a molecular structure optimization using SZYBKI [(63) SZYBKI 1.7.0] in order to refine the molecular structure using the Merck Molecular Force Field with solvent effect.

Deposition

The atomic coordinates and structure factors of hNaPRTase have been deposited with the Protein Data Bank (www.rcsb.org) with the accession code XXX.

Illustrations

Figures were generated by using the program PyMOL (64).

Data Collection	
Resolution (Å)	2.9
Observations	110729
Unique reflections	25261 (2464)
R-merge (%)	11.4 (37)
R-pim (%)	6.1 (20.5)
Mean (I)/sd (I)	10.0 (3.7)
Multiplicity	4.4 (4.2)
Completeness (%)	99.7 (99.3)
Refinement	
N° of protein atoms	7576
N° of solvent molecules	98
R-work (%)	21.40
R-free (%)	28.98
Rmsd Bond lengths (Å)	0.011
Rmsd Bond angles (°)	1.69
Mean B-factor main chain (Å2)	36.90
Mean B-factor side chain (Å2)	42.67
Mean B-factor solvent (Å2)	33.60

Table I. Data collection and refinement statistics. Values in parentheses are for the highest resolution shell.

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Chapter 4

Unpublished results

Extracellular NMN sustains Intracellular NAD+ Biosynthesis through CD73-mediated Nicotinamide Riboside Production

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Unpublished results.

ABSTRACT

All NAD+ extracellular precursors and metabolites contribute to NAD+ intracellular biosynthesis. However, in order to accomplish this function, cells must first take them up. Previous studies have already established that, besides nicotinamide and nicotinic acid, only riboside precursors NR and NaR can be

transported through the plasma membrane, while mono- and dinucleotide precursors should undergo a conversion into their respective nucleosides to sustain NAD+ biosynthesis inside cells. Ecto-enzyme CD73 mediates extracellular NMN uptake through its 5'-nucleotidase activity by converting it into permeant NR.
Our studies aim to investigate the metabolic fate of extracellular NMN and NR in human hepatocarcinoma cells. Based on the employment of labelled extracellular precursors to feed wild type and CD73 knockdown HepG2 cells, we confirm, in a direct manner, that extracellular NMN strictly depends on CD73 activity to sustain NAD+ biosynthesis. Moreover, our studies strongly suggest that NR is preferred to NMN as extracellular precursor in hepatocytes and demonstrate the effectiveness of a combined approach, based on the usage of chemical biology tools and LC-MS^2 analysis, to investigate NAD+ metabolome.

INTRODUCTION

NAD+ vitamin precursors and NAD+ metabolites are assumed everyday through diet. In order to be available for intracellular NAD+ biosynthesis, all these pyridine nucleotide building blocks should be first internalised by the cells. Both nicotinamide (Nam) and nicotinic acid (Na) are absorbed from the alimentary canal, enter the bloodstream for distribution to tissues and finally cells through several import mechanisms (1). Nicotinamide riboside (NR), an additional salvageable NAD+ precursor entering NAD+ biosynthesis via Nicotinamide riboside kinase (Nrks), has been detected in cow milk (2) and it is presumed to be translocated from the extracellular to the intracellular compartment by a nicotinamide riboside transporter (Nrt), currently unidentified in mammals (3). Although nucleoside and nucleobase transport systems have been extensively

investigated (4), some other aspects of NAD+ and its nucleotide metabolite uptake remain matter of interest and have elicited further investigations. Moreover, the import/export mechanisms of either salvageable precursors and NAD+ metabolites among cells account also for the signalling functions of NAD+. Indeed, several ecto-enzymes hydrolysing extracellular NAD+, ATP and other nucleotides, in order to generate signalling molecules or inactivate intercellular signalling via extracellular nucleotides, have been described (5-7). These enzymes are expressed differently on the plasma membrane of various human cells and tissues (6). Even several members of the NAD+-consuming ADP-ribosyltransferase (ART) family are expressed on the outer side of the plasma membrane and necessitate of extracellular NAD+ to function (8). Extracellular NAD+ has been detected in plasma and fluids at low concentrations (9-10) and mounting evidence indicates that NAD+ can also be released from cells in a regulated fashion. For example, it was demonstrated that NAD+ can cross the plasma membrane through Cx43 hemichannels to sustain CD38 catalytic activity and second messenger formation in fibroblasts (11). Although NAD+ uptake has been postulated in some human cell lines (10), other studies showed that nucleosides, but not nucleotides can go through the plasma membrane, unless cell-type specific transport systems are present (12). In particular, several works have contributed to finally demonstrate the dependency of extracellular nicotinamide mononucleotide (NMN) upon CD73 activity to serve as intracellular NAD+ precursor. CD73 is a glycosylphosphatidylinositol (GPI)-anchored protein, known for a long time to be responsible of the hydrolysis of nucleoside 5'-monophosphates, mainly AMP, to their respective nucleosides and Pi (13). Already in 2006, Belenky and Brenner hypothesized the dephosphorylation of extracellular NMN to NR by the surface protein CD73, as a step of a potential extracellular NAD+- cycle in vertebrates (14). It was only recently, however, that the ability of CD73 to hydrolyse NMN and NAD+ through its 5'-nucleotidase activity was demonstrated, thus disclosing its possible involvement in systemic NAD metabolism (15). In particular, the enzyme

appeared highly active with NMN as substrate, producing NR and Pi. In another study, based on the employment of a new detection system (16) to monitor changes in NAD content within mitochondria, the fate of NMN as extracellular precursor for NAD+ biosynthesis was followed (12). Indeed, NMN uptake was prevented when either a competitive substrate for the dephosphorylation by the external 5'-nucleotidase or plasma membrane nucleoside transporters inhibitors were used, thus proving the necessity of its extracellular conversion to its corresponding nucleoside NR to serve as intracellular NAD+ precursor (12). Finally, the contribute of CD73 in sustaining NAD+ biosynthesis was determined, demonstrating that this ecto-enzyme enables the utilisation of extracellular NMN as NAD+ precursor by converting it into NR, in FK866-treated tumour cells (17).
In this study we employed double labelled NMN and NR precursors to feed wild type and knockdown CD73 hepatocarcinoma cell lines, and a recently reported LC-MS^2 method (18), to analyse NAD+ metabolites inside and outside fed cells. Our results confirm in a direct manner, by means of chemical biology tools, that extracellular NMN uptake is dependent on CD73 activity converting it into permeant NR. Finally, we show how a combined approach based on labelling experiments coupled to LC-MS/MS analysis permits a reliable and comprehensive measurement of NAD+ metabolome.

EXPERIMENTAL PROCEDURES

Materials

All chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich, unless otherwise stated.

Knockdown of CD73

Stable knockdown (KD) CD73 HepG2 cell lines were obtained and provided by Dr. Sobol's group from the Department of Pharmacology and Chemical Biology, University of Pittsburgh, USA. Five different viruses expressing shRNA directed against CD73 mRNA were designed *ad hoc,* and wild type (wt) HepG2 cells were transfected with them. Moreover, a control KD cell line, transfected with a scrambled (scrb) shRNA not interfering with CD73 expression, was obtained.

Cell cultures

HepG2 (human hepatocarcinoma) cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humified atmosphere with 5% CO_2. KD CD73 HepG2 cells were grown as stated for wt HepG2 cells with the addition of puromycin (1 μg/ml) in the media.

qPCR Analyses

Total RNA was extracted from all KD CD73 HepG2 cell lines using the RNeasy Mini Kit (Qiagen). Quality and quantity of RNA were analysed using a NanoDrop spectrophotometer (ThermoFisher). cDNA (0.8 μg) was synthesized by using the iScript cDNA synthesis Kit (Bio-Rad). PCR primers were designed by using Primer 3 software and their sequences were as follows: human CD73, 5'-CTCCTCTCAATCATGGCGCT-3' (forward) and 5'-TGGATTCCATTGTTGCG-TTCA-3' (reverse); human GAPDH, 5'-GAGTCAACGGATTTGGTGT-3'

(forward) and 5'-GACAAGCTTCCCGTTCTCAG-3' (reverse); human NRK1, 5'-GGATGGAAAGCGCAAGACAC-3' (forward) and 5'-GGAGGCTGATAGAC-CCTTGTATTAAT-3' (reverse); human NRK2, 5'-TCCTGACCGTCCCGT-ATGAA-3' (forward) and 5'-CCAGGTAGACCACTTCCACAC-3' (reverse). qPCR were performed using the iQ SYBR Green Supermix (Bio-Rad) on the CFX96 Real-Time PCR System (Bio-Rad). Statistical analyses of the qPCR were obtained by applying the $2^{-\Delta\Delta}C_t$ method, which calculated relative changes in gene expression of the target normalised to GAPDH. Experiments were repeated twice in duplicate.

Western Blot Analyses

70% confluent cells ($3x10^6$) were washed twice with cold PBS, collected and centrifuged at 1000 rpm for 5 min at 24 °C. Cell pellets were lysed in cold RIPA Buffer (Thermo Scientific) (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Total, pellet and supernatant fractions representing 20% of the total amount of the cells were prepared, resuspended in LDS Sample Buffer (Life-Technologies) containing 8% β-mercaptoethanol, loaded onto 10% SDS-PAGE (Bio-Rad), then elettrophoretically separated and transferred to Immun-Blot PVDF membranes (Bio-Rad). Membranes were blocked with 5% non fat dry milk in TBST buffer (1X TBS, 0.1% Tween 20) for 1 h at room temperature and incubated with the following antibodies: anti-CD73 (ab124725, Abcam Inc., Cambridge, MA) and anti-GAPDH (Abcam). Anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, Danvers, MA) was the secondary antibody. Western blots were developed with the ECL-Plus Kit (GE Healtcare), according to the manufacturer's instructions. Band detection and quantitation were performed using the ChemiDoc XRS^+ System (Bio-Rad) and the Image Lab software (Bio-Rad).

Labelled precursor synthesis

Synthesis of ^{18}O labelled NR, ^{18}O labelled NMN and deuterated (D),^{13}C double labelled NR was performed by Dr. Migaud's group, School of Pharmacy, Queen's University Belfast, UK.

Double labelled NMN Synthesis

Synthesis of D,^{13}C NMN was achieved enzymatically by using the corresponding double labelled nucleoside D,^{13}C NR as substrate of human NRK2. Twenty enzymatic reactions were set up at the following conditions: 20 mM Hepes sodium pH 7.5, 100 mM NaCl, 5 mM MgCl2, 500 μM D,^{13}C NR, 500 μM ATP and 10 μg NRK2, in a total volume of 500 μl each at 37°C for 1h. Reaction aliquots were stopped by adding 20 mM EDTA and boiling for 2 min at 100°C, then pooled and passed through a 3000 MWCO Amicon (Merck Millipore) to eliminate the 26 kDa NRK2. Product formation was tested by LC/MS^2. The reaction volume was fractionated, injected onto a PrincetonSPHER60 SAX 5 μm (250 x 4.6 mm) column in a HPLC system (Varian Prostar 210). The flow rate was 1 ml/min and the elution conditions were: 8 min at 100% buffer A (10 mM KH_2PO_4), 30 s at up to 100% buffer B (750 mM KH_2PO_4) and hold at 100% buffer B for 8 min; then the gradient returned to 100% buffer A in 30 s and maintained for 6 min. D,^{13}C NMN peaks were collected, pooled and loaded on a lyophilizer (VirTis) in order to obtain the precursor powder to be resuspend in the most suitable volume for label enrichment experiments.

Label enrichment experiments using single labelled ^{18}O NMN or ^{18}O NR in wild type HepG2 cells

Wt HepG2 cells were grown in 150 x 20 mm Petri dishes as specified above. When cells reached ≈ 70-80% confluency, complete media was replaced with nicotinamide-free media without FBS, and cells were left in these conditions for 24 h. Subsequently, wt HepG2 cells were supplemented with either 10 μM ^{18}O NR or ^{18}O NMN. Media and cells were collected before the addition of the compounds (0 h), and at the following timepoints: 30 min, 2 h, 4 h, 7 h and 24 h. For each timepoint, the corresponding cell pellet was washed with PBS, and media was filtered with a sterile 0.2 micron syringe filter, before both being frozen in liquid nitrogen and stored at -80°C. All chosen timepoints were tested in duplicate. Quantification of NAD+ metabolites was determined by LC-MS/MS as described in (18).

Label enrichment experiments with D,^{13}C NR on scrb and KD3 CD73 HepG2 cells

The protocol followed to perform label enrichment experiments on KD CD73 HepG2 cell lines was the same used for wt HepG2 cells; KD3 CD73 and scrb HepG2 were supplemented with D,^{13}C NR. Every timepoint was tested once in two temporally distinct experiments (biological duplicates).

RESULTS AND DISCUSSIONS

CD73, NRK1 and NRK2 expression levels in knockdown CD73 HepG2 cell lines

By means of qPCR experiments, we determined CD73 mRNA expression levels in KD1, KD2, KD3, KD4 and KD5 CD73 HepG2 cells. Our data indicated that KD3 and KD4 were the most suitable knockdown cell lines to be used for the label enrichment experiments with double labelled precursors D,^{13}C NR and D,^{13}C NMN. In particular, repeated cDNA synthesis and qPCR experiments on the positive control *scrb* and on KD3 and KD4 HepG2 cell lines, showed that CD73 expression was 70% depleted in both knockdown mutants **(Figure 1)**. Western blot analyses confirmed a lower CD73 expression in KD3 and KD4 HepG2 cell lines also at protein level, and the presence of both membrane-bound and cytoplasmic CD73 in HepG2 cells, even though CD73 membrane-bound version was predominant (data not shown).

We performed a parallel qPCR experiment to assess which isoform of Nicotinamide riboside kinase (NRK) is predominant in HepG2 cells. We found that NRK1 is expressed about 400 times more than NRK2 **(Table 1)**; this fact is relevant in order to obtain a transient NRK1 knockdown HepG2 cell line to be used as a further control in label enrichment experiments. Indeed, by blocking the enzymatic activity of the predominant NRK1, cells lose their capacity to use extracellular double labelled D,^{13}C NR as precursor for the synthesis of NAD+. This transient NRK1 knockdown would be achievable by designing an anti-Nrk1 shRNA and transforming HepG2 cells with it. Nevertheless, HepG2 cells are known in literature to show a low percentage of transformation efficiency (even around 10%), but this problem could be overcome through the use of different

reverse-transfection protocols and so, transient NRK1 knockdown HepG2 cells is planned to be obtained in the future.

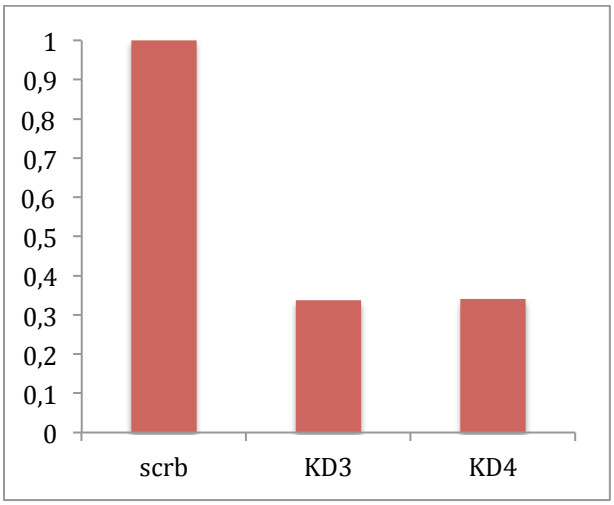

Figure 1. CD73 mRNA expression levels in *scrb*, KD3 and KD4 HepG2 cell line.

	HepG2 cells						
	Measure 1	*Measure 2*	*Measure 3*	*Measure 4*	*Measure 5*	*Measure 6*	*Avg M*
Ct GAPDH	18.38	20.03	17.22	19.99	16.87	18.86	18.56
Ct NRK1	30.32	30.37	28.61	29.08	28.36	28.19	29.16
Ct NRK2	n.d	37.61	36.86	n.d	38.14	38.12	37.68
ΔCt NRK1 = 10.6 ; ΔCt NRK2 = 19.1							
$\Delta\Delta Ct = 8.52$ $2^{-\Delta\Delta Ct} = 0.0027$							

Table 1. qPCR values showing expression levels of GAPDH, NRK1 and NRK2. GAPDH is an housekeeping gene in human cells and its expression level is monitored in order to normalise NRK1 and NRK2 expression level. Cycle threshold number (Ct) represents the number of cycles needed to reach a set threshold fluorescent signal level in qPCR experiments. The $2^{-\Delta\Delta Ct}$ method is used and it shows how NRK2 is expressed 1/0.0027 times less than NRK1 (≈ 400 fold less).

D,^{13}C NMN Synthesis

For the labelled enrichment experiments on knockdown CD73 HepG2 cells we decided to use new double labelled precursors in order to prevent the loosing of the label, so obtaining more robust results. Indeed, D,^{13}C NR and NMN carry a label on both their nicotinamide and ribosyl moieties **(Figure 2)**. LC-MS^2 analysis after the enzymatic reaction to synthesize D,^{13}C NMN from D,^{13}C NR, evidenced how D,^{13}C NMN production occurred, and 95% of it was double labelled **(Figure 3)**.
Ion Exchange chromatogram in HPLC system showed that the method used was efficient to separate D,^{13}C NMN from other components in the mixture reaction. However, chromatogram peak area integration highlighted how D,^{13}C NMN synthesis protocol needed to be optimized because not all the D,^{13}C NR was converted in the corresponding product (not shown). Unfortunately, the amount of D,^{13}C NMN obtained following the reported protocol was not sufficient to perform label enrichment experiments on KD CD73 HepG2 cells.

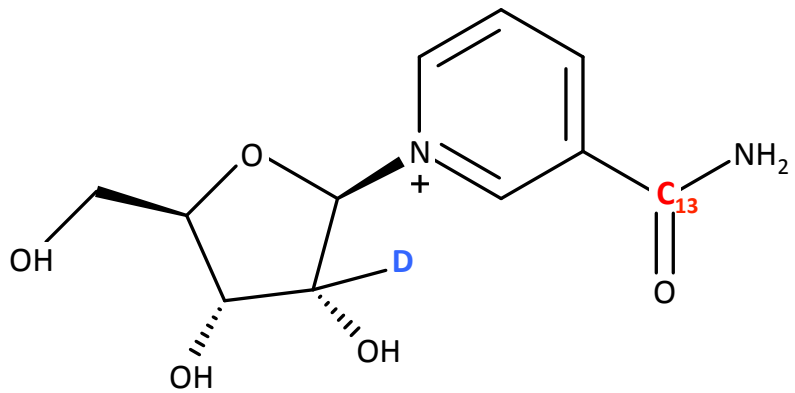

Figure 2. Structure of D,^{13}C NR. Labelled atoms D and C_{13} are coloured in blue and red, respectively

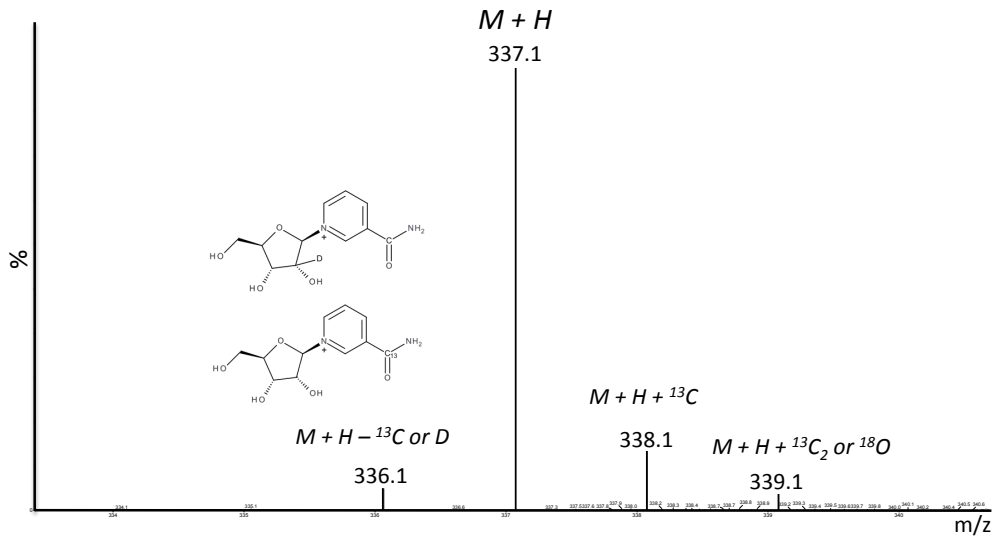

Figure 3. Fragmentation patterns in the mass spectrum of D,^{13}C NMN. Single labelled peak (336.1 m/z) represents the 5% of double labelled peak (337.1).

Label enrichment experiments

Both extracellular NMN and NR support intracellular NAD+ biosynthesis (12). However, not every cell is capable of converting each NAD+ precursor to NAD+ at all times. Indeed, precursors are differentially utilised in a tissue- and cell-specific manner (1). Our results from label enrichment experiments on wt HepG2 cells show that NR is faster than NMN in entering cells, strongly suggesting that NR is preferred to NMN as extracellular precursor in HepG2 cells **(Figure 4)**. When cells were threated with ^{18}O NR, all analysed NAD+ metabolites showed a higher intracellular percentage of the labelled portion within 24 h, compared to what was observed in ^{18}O NMN threated cells. Moreover, the different velocity in NMN and NR internalisation and the detection of NR, already after 30 min, in the media of ^{18}O NMN threated wt HepG2 cells, support the already proved need for NMN to be firstly processed to NR in order to serve as intracellular NAD+ biosynthetic precursor (12). Indeed, in the last 5 years, different studies have explored the roles

of the human ecto-enzymes, CD38 and CD73, in providing ectocellular precursors for NAD+ biosynthesis (12, 15, 17). In particular, these works have already shown that extracellular NMN appears to depend upon CD73 or, via Nam, CD38 activities to be used as NAD+ precursor. Our label enrichment experiments on knockdown CD73 HepG2 cells are aimed to confirm, in a direct manner, the dependency of extracellular NMN, as intracellular source of NAD+, upon CD73 activity. As expected, our results on KD3 CD73 HepG2 cell line supplemented with D,^{13}C NR, showed that NR internalization as well as its contribution in NAD+ biosynthesis are not affected by the scarcity of CD73 (data not shown). Label enrichment experiments with D,^{13}C NMN on KD CD73 HepG2 cells are currently on-going. Our expectation is to see that, when CD73 is expressed at low levels, the entrance of NMN into the cells does not occur or occur at a severely reduced rate compared to what is observed in wild type cells.

These experiments also reveal the relationship of the metabolites regardless of the precursor **(Figure 4)**. Enrichment of NAD+ is linear with NMN, which is in great agreement with the fact that NMN is the direct biosynthetic intermediate to NAD+. Nam enrichment occurs at a slower rate than NAD+ and NMN, which suggests two important biological relationships: i) NR is not greatly hydrolysed intracellularly to Nam and appears to mostly shunt to NAD+ biosynthesis. ii) Most Nam observed in the cell is by-product of NAD+-consuming enzymes. NADP enrichment lags behind NAD+ and NMN as well, suggesting that the NADP(H) pool is segregated from the NAD pool.

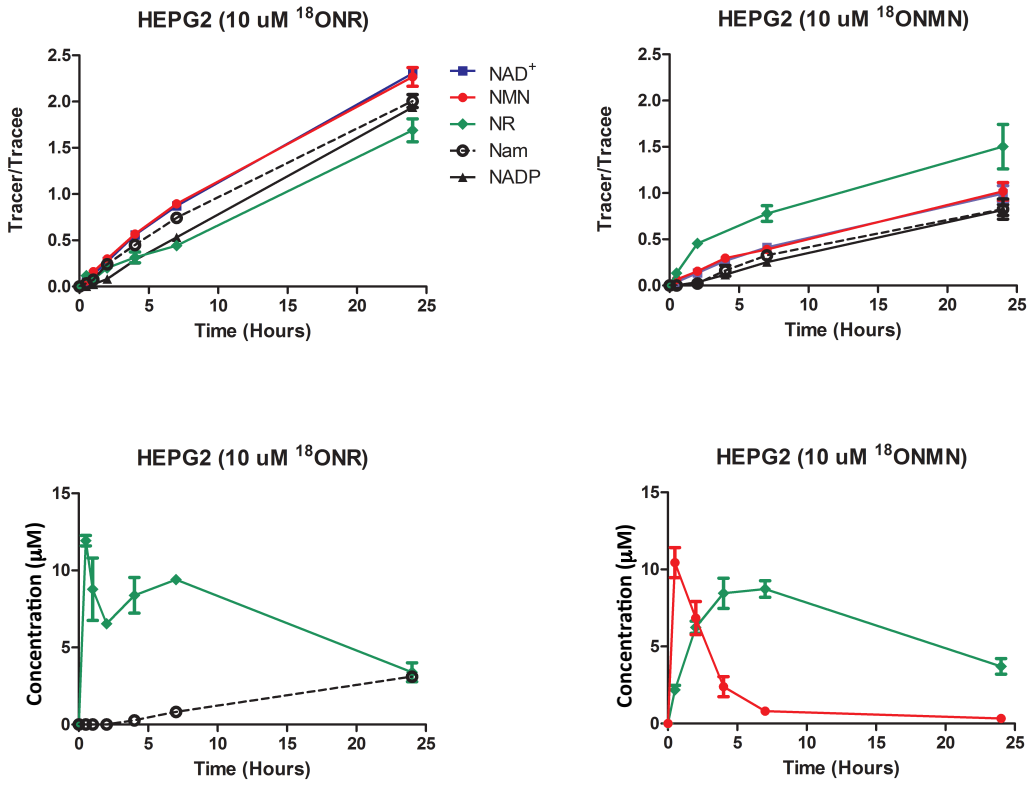

Figure 4. LC-MS Quantification of NAD(P)+ metabolites after label enrichment experiments on wt HepG2 cells. Analysis of pellets (above) and media (below) of cells treated with ^{18}O NR and ^{18}O NMN are reported on the right and on the left, respectively. The LC-MS^2 analysis of cell pellets content was based on the measurement over time of the ratio tracer/tracee for each NAD+ metabolite, where ^{18}0-form constitutes the tracer and ^{16}0-form is the tracee. Analyses of the culture media calculated the change in concentration of the residual corresponding labelled precursor over time.

CONCLUSIONS

In literature, other works have already shown that NMN uptake depends upon CD73 or, via Nam, CD38 activities. In particular, it has been demonstrated how extracellular NMN can serve as intracellular NAD+ precursor only after being

converted, on the outer side of the plasma membrane, to NR by CD73. In our studies we employed double labelled precursors NMN and NR and followed their intracellular fate by LC-MS^2 analysis. Through this combined approach based on chemical biology tools we confirmed, in a direct manner, that extracellular NMN sustains NAD+ biosynthesis through CD73-mediated NR formation. Moreover, our data strongly suggest that NR on its own is more efficacious than NMN as a precursor in HepG2 cells. If potentially extended to other cellular lines, this study may also elucidate cellular preferences for distinct extracellular precursors on the basis of the tissue or cell type considered. Not of secondary importance, our work will also demonstrate the utility of stable labelling experiments coupled to LC-MS/MS for use in elucidating metabolic relationships.

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Chapter 5

Conclusions

In the last 20 years, a breathtaking number of studies have evidenced the pleiotropic role of NAD(P) in all organisms. This ubiquitous molecule is a fundamental coenzyme as well as a substrate for a plethora of enzymes involved in pivotal cellular processes. Indeed, NAD(P) stands at the center of many metabolic processes through its function as hydride transporter, and it governs a large number of signalling reactions during which it is used as substrate and, therefore, consumed. NAD+ biosynthesis is accomplished through different pathways and important differences are present among organisms. In humans, NAD+ biosynthesis can start from five different precursors, all contributing to replenish cellular vital pyridine nucleotide supplies, even though some tissues prefer to use one precursor more than the others. Due to NAD(P) essential roles in energy metabolism and signalling transduction, its biosynthesis and homeostasis should be tightly regulated. In mammals, NAD+ biosynthesis is not a closed, cell-autonomous system, and intercellular communication determines that NAD homeostasis is a systemic and dynamic process influenced by both the pathophysiological conditions of the cell and the external environment.

During my PhD, I investigated two aspects involved in NAD homeostasis, namely NAD+ biosynthesis and extracellular NAD+ metabolite and precursor uptake, both contributing to maintain this fundamental state of balance. NAD+ biosynthetic pathways occur intracellularly while the uptake of NAD+ precusors takes place on the outer surface of the plasma membrane. Thus, giving a glance at what happens either inside and outside cells, our lines of research highlight the systemic nature of NAD homeostasis.

My PhD research activity mainly focused on the structural characterisation of the recombinant human Nicotinic acid Phosphoribosyltransferase (hNaPRTase). hNaPRTase catalyses the first rate-limiting reaction of the three-step Preiss-Handler pathway, responsible for the synthesis of NAD+ from nicotinic acid (Na).

The importance of this NAD+ biosynthetic pathway, as well as the other salvage pathways, is underlined by the fact that tryptophan contribution to maintain cellular NAD+ levels has been shown to seem negligible in the large majority of human organs. Some tissues preferentially use the Preiss-Handler pathway for NAD+ biosynthesis, as attested by the fact that Na was found to be more effective than nicotinamide (Nam) in increasing NAD+ levels in red blood cells, kidney and heart. Indeed, in these tissues hNaPRTase was found to be expressed at high levels. Previous studies have already focused on the biochemical characterisation of human NaPRTase as well as other NaPRTases from different organisms, elucidating some peculiar features of this pivotal enzyme. hNaPRTase is strictly specific for Na as substrate and it is not subject to feedback inhibition by NAD+. The lack of inhibition by the end product of the pathway together with the demonstrated fact that hNaPRTase activity is stimulated by physiological concentrations of Pi, suggested that this enzyme could be permanently active in the cell. Moreover, it has been shown how, in some organisms, NaPRTase activity can be stimulated by ATP through the formation of a phosphoenzyme intermediate, thus representing an example for a new energy-coupling mechanism of enzyme activation. Nevertheless, biochemical studies could not be supported by a structural characterisation of the human enzyme because no crystallographic data have been available so far.

On the basis of a fruitful collaboration with Prof. Orsomando from the Department of Molecular Pathology and Innovative Therapies of the Università Politecnica delle Marche (Ancona), who provided us with the cloning vector containing the cDNA coding for *human* NaPRTase, we overexpressed the recombinant protein in *Escherichia coli* and solved its crystal structure at 2.9 Å in its free state by means of molecular replacement. The analysis of hNaPRTase crystal structure allowed us to determine its molecular architecture, thus contributing to better understand the molecular basis of its functions. hNaPRTase consists of two domains: an open-faced sandwich and an irregular α/β barrel,

connected by a long α-helix. The α/β core is formed by a six-stranded β-sheet that attests hNaPRTase belonging to Type II phosphoribosyltransferase subfamily. The analysis of the interactions between the two monomers found in the crystal asymmetric unit, together with our size exclusion data, permitted us to determine that hNaPRTase minimal functional unit is a dimer. The active site formation occurs at the dimeric interface and involves the irregular α/β barrel on one monomer, and the N-terminal α1-helix on the other one. In order to identify residues involved in ligand coordination and stabilisation in the active site, we performed molecular docking studies that confirmed how dimer formation is essential for hNaPRTase to function. Moreover, structural comparisons with other available NaPRTase structures have evidenced how, surprisingly, the human enzyme resembles more to the NaPRTase of *Enterococcus faecalis* than to the enzyme of the eukaryotic *Saccaromyces cerevisiae*. On the basis of this observation and the fact that *E. faecalis* is a commensal bacterium living in the mammalian gastrointestinal tract, we speculated that this similarity could represent an example of an adaptive mechanism exploited by the bacterium to survive inside the host. Moreover, we compared hNaPRTase structure with the human nicotinamide phosphoribosyltransferase (hNMPRTase) and quinolinic acid phosphoribosyl-transferase (hQAPRTase) structures. Despite sharing very limited sequence similarity, these three enzymes showed a resembling molecular architecture, consistently with their common function. However, we noticed that some residues in hNaPRTase do not superpose with any secondary element in either hNMPRTase or hQAPRTase, and form two distinct regions that, from an *in silico* analysis, appeared to be univocally present in mammalian NaPRTases. Finally, the superposition between the structures of hNaPRTase and hNMPRTase in complex with its potent inhibitor FK866, allowed us to evidence how hNaPRTase does not present a tunnel in its active site that constitutes the binding site of the antitumoral agent in hNMPRTase. As already shown in *Thermoplasma acidophilum* NaPRTase, this difference is due to the lack in NaPRTase of 10 residues, instead

present in hNMPRTase. As a consequence, some secondary structure elements in hNaPRTase are disposed in a way that sterically prevents FK866 as well as other hNMPRTase inhibitors from binding to hNaPRTase. Given that tumoral cells have increased metabolism needs and call for a higher level of NAD+, the blockage of its biosynthesis has assumed a therapeutic relevance in cancer treatment. hNMPRTase is overexpressed in many tumours and its inhibition by FK866 and other inhibitors leads to a severe decrease in NAD+ levels, followed by ATP shortage and cell death. However, the redundant nature of NAD+ biosynthesis, consisting in several pathways starting from different precursors but ending with the formation of the same product, should be considered to evaluate the efficacy of cancer treatment with hNMPRTase inhibitors. Unlike hNMPRTase, hNAPRTase expression was found to be more varied in tumoral tissues and, therefore, could not represent a potential target for chemotherapeutics aiming to decrease NAD+ levels in cancer cells. Nevertheless, some tumours have shown high and low or absent expression levels for hNMPRTase and hNaPRTase, respectively. For the treatment of these tumours, it has been recently disclosed the possibility of increasing the therapeutic potential of hNMPRTase inhibitors by co-treating with nicotinic acid. In particular, the increment in hNMPRTase inhibitor efficacy mediated by Na co-treatment consists in diminishing their toxicity for non tumoral tissues. Indeed, human NaPRTase is not expressed in these cancers and therefore tumoral cells can not synthesize NAD+ starting from Na. On the other hand, hNaPRTase can rescue normal cells from NAD+ depletion caused by hNMPRTase inhibition. Moreover, it has been proved how hNaPRTase can counteract ROS formation induced in tumoral cells by hNMPRTase inhibitors, thus rendering NaPRTase-deficient tumours even more suitable for co-treatment with nicotinic acid. Finally, other studies have shown that Na possesses potent antioxidant properties and that its intracellular metabolism by hNaPRTase is essential for Na to counteract H_2O_2–induced cytotoxicity. In this context, we noticed that no inhibition should be

envisaged for hNaPRTase in order to reduce the toxic effect of the anticancer drugs.

In summary, our structural studies of human NaPRTase contribute to our understanding of its mechanism of action and highlight, through the comparisons with other NaPRTases and other enzymes involved in NAD+ biosynthesis, structural common features but also hNaPRTase peculiar characteristics. In the future, we are planning to obtain point mutated hNaPRTase variants in the residues participating in the active site formation in order to validate their importance in enzyme activity. Finally, another potential future goal could be to explore the molecular basis of the strict hNaPRTase substrate specificity for Na, through a protein engineering-based reverse approach aiming to convert hNaPRTase into hNMPRTase.

In collaboration with the research group directed by Professor Brenner at the Department of Biochemistry of the University of Iowa (USA), we investigated on the cellular uptake mechanisms of two extracellular NAD+ metabolites, namely nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR). Previous studies have shown the existence of an extracellular NAD+ pool as well as the presence of NAD+-consuming enzymes on the outer surface of the plasma membrane. Moreover, the major source of NAD+ precursors and metabolites is represented by diet and, lately, other studies have underlined the potential nutraceutical properties of NAD+ vitamin precursors, deriving essentially from their capacity to boost NAD+ levels. Some of the aspects related to the modalities used by the cells to take up extracellular NAD+ metabolites remain unclear. For example, it has been shown how extracellular NR can sustain intracellular NAD+ biosynthesis without undergoing any enzymatic conversion in the extracellular compartment, but nowadays the molecular identity of its transporter through the plasma membrane is still unknown in mammals. Several works have contributed to finally demonstrate the dependency of extracellular NMN upon the enzymatic

activity of the surface protein CD73 to serve as intracellular NAD+ precursor. CD73 is a cell surface enzyme, linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor in its C-terminal domain, and catalyses the dephosphorylation of extracellular purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to their corresponding membrane permeable nucleosides. In a previous study from our laboratory, it was reported that CD73 is also capable of processing both NAD+ and NMN through its 5'-nucleotidase activity. In particular, the enzyme appeared highly active with respect to NMN as a substrate, producing NR and Pi. Recently, Grozio and collaborators finally demonstrated the role of CD73 in sustaining intracellular NAD+ biosynthesis by converting extracellular NMN into NR, in FK866-treated tumour cells.

In this context, we set ourselves a double objective: to confirm in a direct manner the close dependence of extracellular NMN upon CD73, and to explore hepatocytes preferences in terms of extracellular precursors for intracellular NAD+ biosynthesis. To address these purposes, we used CD73 knockdown hepatocarcinoma cell lines (HepG2), provided by Dr. Sobol's group of the University of Pittsburgh (USA), and chemical biology tools provided by Dr. Migaud's group of the Queen's University Belfast (UK). First, we selected, from a panel of CD73 knockdown HepG2 cell lines, those ones showing the lowest CD73 expression, at both the transcriptional and translational levels. Indeed, we grew wild type and CD73 knockdown HepG2 cells in the absence of nicotinamide for 24 h, and then fed them with either singly or doubly labelled NMN and NR, respectively. We finally analysed the metabolic fate of the labelled extracellular precursors by LC-MS^2, following intracellular NAD+ metabolome variations over time. Our results from label enrichment experiments on wild type cells showed that NR is faster than NMN in entering cells, strongly suggesting that NR is preferred to NMN as extracellular precursor in human hepatocytes. Moreover, the detection of NR in the media of cells treated with labelled NMN, is in accordance with the necessity for NMN to be dephosphorylated by CD73 before being internalised by

the cell. As expected, label enrichment experiments on the selected CD73 knockdown cells supplemented with double labelled NR showed that its uptake is not affected by CD73 low levels of expression. Label experiments on CD73 knockdown cells with double labelled NMN and the optimization of its production protocol via NRK, are ongoing. Nevertheless, we are tempted to say that our last results will confirm the dependency of NMN upon CD73, as previously reported in literature. In the near future, we aim to obtain a transient NRK1 knockdown HepG2 cell line to be used in label enrichment experiments. We have already determined that NRK1 is the most prominent nicotinamide riboside kinase isoform to be expressed in hepatocarcinoma cells. Therefore, when NRK1 expression is impaired, we expect to see that NAD+ rescue will be prevented for both double labelled NMN and NR. Thus, the employment of this transient knockdown cell line could offer a further direct evidence of how usage of extracellular NMN, as a precursor for intracellular NAD+ biosynthesis, can occur only after its CD73-mediated dephosphorylation to NR. Our studies represent also a demonstration of the effectiveness of a combined approach, based on the usage of chemical biology tools and LC-MS^2 analysis, to investigate NAD+ metabolome.

List of publications

- **Recombinant production of eight human cytosolic aminotransferases and assessment of their potential involvement in glyoxylate metabolism.** Donini S, Ferrari M, Fedeli C, Faini M, Lamberto I, **Marletta AS**, Mellini L, Panini M, Percudani R, Pollegioni L, Caldinelli L, Petrucco S, Peracchi A. *Biochem Journal*. 2009 Aug; 422(2): 265-72.

- **Pancreatic transduction by helper-dependent adenoviral vectors via intraductal delivery**. Morró M, Teichenne J, Jimenez V, Kratzer R, **Marletta S**, Maggioni L, Mallol C, Ruberte J, Kochanek S, Bosch F, Ayuso E. *Human Gene Therapy*. 2014 Sep; 25(9): 824-36.

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Un sorriso

"Non costa nulla e produce molto, arricchisce chi lo riceve
senza impoverire chi lo dona.
Non dura che un istante, ma nel ricordo può essere eterno.
E' il segno indelebile di un'amicizia profonda.
Nessuno è così ricco da poterne fare a meno
e nessuno così povero da non meritarlo.
Un sorriso dà riposo alla stanchezza e allo scoraggiamento;
rinnova il coraggio, nella tristezza è consolazione.
Un sorriso è un bene che ha valore nell'istante in cui si dona.
Se incontrerai chi il sorriso a te non dona, sii generoso e dà il tuo,
perché nessuno ha tanto bisogno di sorriso
come chi non sa darlo."
-Kahlil Gibran-

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