Structural and Functional Properties of NAD Kinase, a Key Enzyme in NADP Biosynthesis

Giulio Magni*, Giuseppe Orsomando and Nadia Raffaelli

Istituto di Biotecnologie Biochimiche, Università Politecnica delle Marche, Via Ranieri, 60131 Ancona, Italy

Abstract: NAD kinase is an essential enzyme, which plays a key role in cellular energy and signal transduction systems. In this report, the recent studies on the features of bacterial and human NAD kinases are summarized. They include detailed kinetic and structural analyses and highlight important differences, which could be exploited for the design of novel selective antimicrobial drugs.

Key Words: NADP biosynthesis, pyridine dinucleotides, recombinant protein, antimicrobial agents, kinetic analysis.

INTRODUCTION

The first pyridine dinucleotide to be isolated and chemically characterized is NADP, the phosphorylated form of NAD. In the 30s, Warburg and Christian isolated and purified a cofactor termed "Coferment II" involved in the reaction catalyzed by the enzyme glucose-6-phosphate dehydrogenase [1]. These authors quickly determined that NADP contained two bases, three phosphates and two riboses [1]. The two bases were identified as adenine and nicotinamide, respectively, and the three phosphates were erroneously thought to be linked through phosphoanhydric bonds. Only twenty years later, Kornberg and Pricer determined the correct structure, with the third phosphate on the 2' position of the adenylic acid moiety [2].

NADP is a vital dual function cofactor that is intimately involved in both energy and signal transduction [3]. The cofactor NADP is indeed a key molecule in most reductive biosynthetic reactions and it is an important constituent of cellular defence mechanism against oxidative stress [4-7]. In living organisms it is synthesized by the enzyme NAD kinase (EC 2.7.1.23), which phosphorylates NAD at the 2' position of the adenosine ribose, in the presence of Mg^{+2} and ATP as the phosphoryl donor (Fig. **1**). To our knowledge this reaction represents the only route leading to NADP formation (Fig. **2**). NAD kinase is ubiquitously distributed and it is recognized of crucial importance for NADP-dependent biosynthetic pathways in the cell. Indeed the enzyme plays a key role in the regulation of the cellular redox state in that it modulates the levels of NAD and NADP, which, through their conversion to the respective reduced forms, determine the physiological redox potential (Fig. **2**). Furthermore, as shown in Fig. (**2**), NADP serves also as the substrate for nicotinate adenine dinucleotide phosphate (NaADP) formation, a known second messenger involved in Ca^{+2} mobilization [8-13].

The last review describing the properties of this enzyme appeared in the literature 20 years ago [14]. Since then, many reports have been published on the kinetic, regulatory and, very recently, structural properties of NAD kinase from

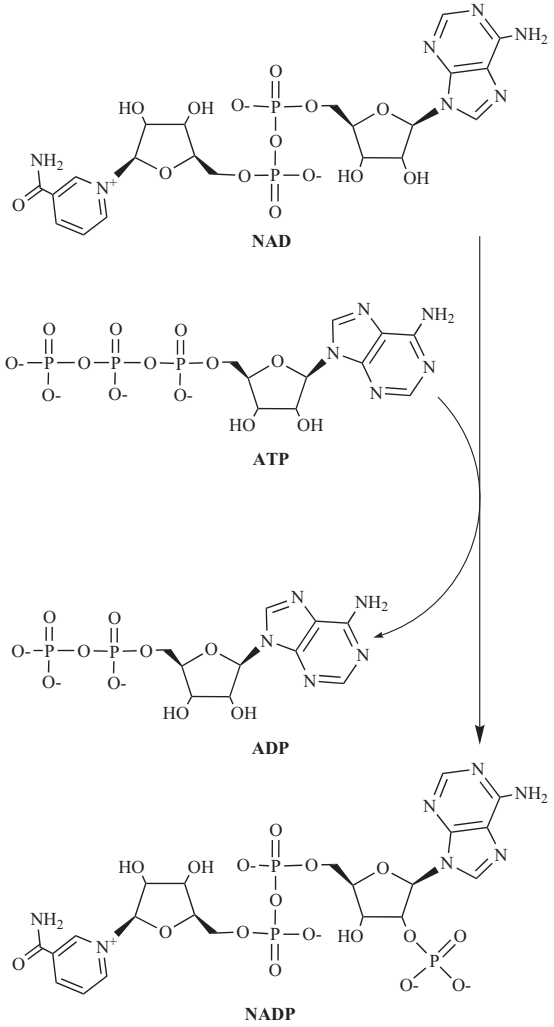

Fig. (1). NAD kinase-catalyzed reaction.

*Address correspondence to this author at the Istituto di Biotecnologie Biochimiche, Università Politecnica delle Marche, *Via* Ranieri, 60131 Ancona, Italy; Tel: +390712204678; Fax: +390712204677;
E-mail: magnig@univpm.it

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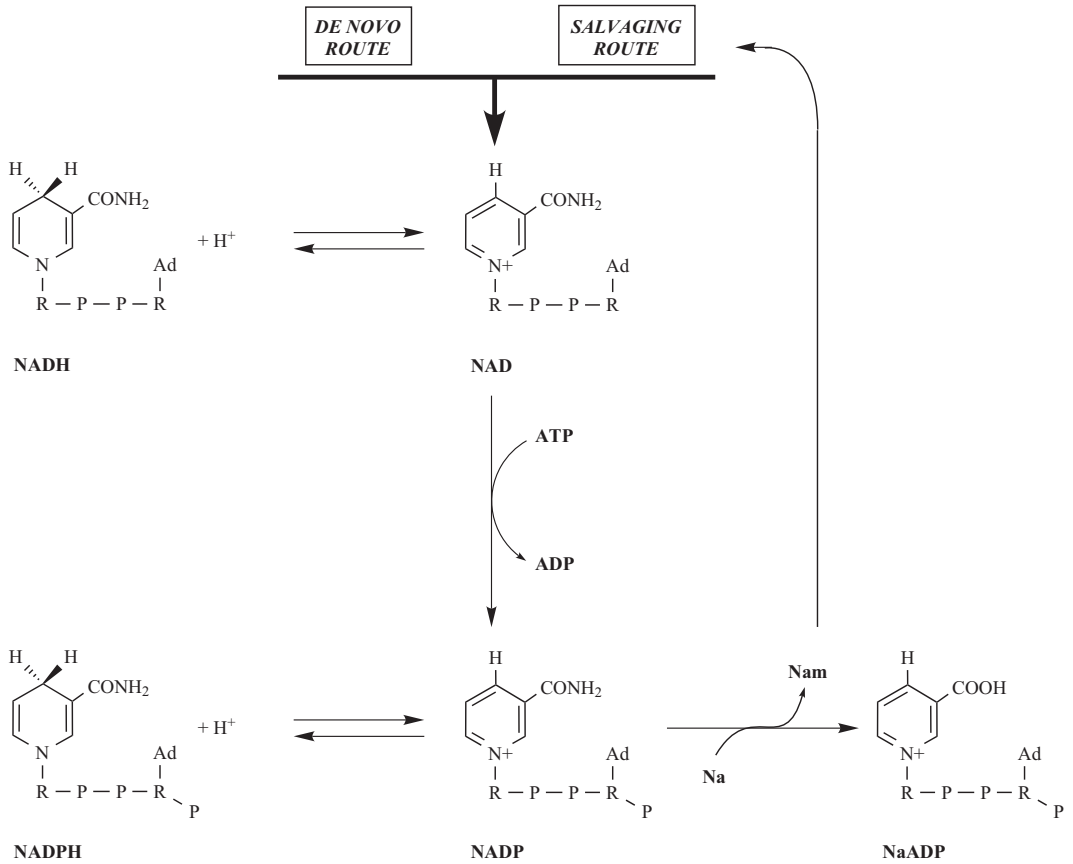

Fig. (2). Schematic overview of NADP metabolism. Na, nicotinic acid; Nam, nicotinamide; NaADP, nicotinate adenine dinucleotide phosphate; Ad, adenine.

several organisms ranging from prokaryote to humans. Owing to the specific mission of this journal, as well as to the great number of data available in the literature, this review will be limited to the description of various NAD kinases from microbial and human sources. In the yeast cell several NAD(H) kinase isoforms have been identified, differing both in cellular compartimentalization and substrate specificity [15-20], even though the essentiality of the enzyme in this microorganism has not been clearly demonstrated [20]. For this reason no description of yeast NAD kinase will be included in this report. On the contrary, NAD kinase was recently shown to be essential in *Bacillus subtilis* [21] and *Mycobacterium tuberculosis* [22], confirming the early proposal of its relevance as a novel antibacterial drug target [23]. Therefore the features of the various enzymatic proteins will be illustrated and discussed keeping in mind that their distinctive structural, molecular and functional properties could be exploited in the designing of effective enzyme inhibitors as selective drugs.

ENZYMATIC PROPERTIES OF NAD KINASES

Bacterial NAD Kinase

In the last years several reports have been devoted to the study of NAD kinase from bacteria. In 2000, the first gene coding for the enzyme has been identified in *M. tuberculosis* through homology Searches utilizing partial amino acid sequences obtained from a homogeneous enzyme preparation from *Micrococcus flavus* [24]. Later on, the genes coding for NAD kinases from *E. coli* [25], *M. flavus* [26], *B. subtilis* [27], *Sphingomonas* sp. A1 [28], *Pyrococcus horikoshii* [29] and *Archaeoglobus fulgidus* [30], were identified. Primary structures alignment revealed the presence of highly conserved motifs, including a GGDG motif, a Glycine-rich region, a GXXGF/L motif and a NE/D short motif (Fig. **3**). Their role in substrates binding and catalysis has been confirmed by both mutagenesis experiments and structural studies [30-34], indicating that all NAD kinases contain a highly conserved catalytic region.

All the identified genes have been cloned and expressed in *E. coli* cells. In Table **1** the molecular and enzymatic properties of some of the best characterized recombinant NAD kinases are reported. However, data are also available in the literature on the native enzyme from *E. coli* [35], *B. licheniformis* [36], *B. subtilis* [37], *H. influenzae* [38] and *M. flavus* [24].

Bacterial NAD kinases share a similar subunit molecular weight, of about 30 kDa, and exist in solution as oligomers (Table **1**).

Fig. (3). Archaeal, bacterial and human NAD kinase sequences were aligned using Clustal W. The highly conserved regions are evidenced in bold. Identical and similar amino acid residues are indicated by asterisks and dots, respectively. The species name abbrevations are as follows: *E.co*, *Escherichia coli*; *Sph*, *Sphingomonas sp.*; *M.tb*, *Mycobacterium tuberculosis*; *M.fl*, *Micrococcus flavus*; *P.ho*, *Pyrococcus horikoshii*; *A.fu*, *Archaeoglobus fulgidus*; *B.su*, *Bacillus subtilis.*

The phosphoryl donor ATP can be replaced by other nucleoside triphosphates, including GTP, pyrimidine nucleotides, and their deoxy-derivatives, which are utilized with various efficiencies, as shown in Table **1**. Nucleoside mono- or diphosphates are not used as substrates. Depending on the organism, NAD kinases are either strictly nucleoside triphosphate dependent, like the *E. coli* and *Sphingomonas* sp. enzyme [25,28], or they can also utilize inorganic polyphosphate (poly(P)) as the phosphate donor [24,26,27,29,32]. Poly(P) is a polymer of inorganic ortophosphate residues linked by high-energy phosphoanhydride bonds and it is present in nearly all living organisms, likely representing the primitive energy source for generation of phosphate group potential [39,40]. NAD kinases able to phosphorylate NAD also in the presence of poly(P) have been found in *M. flavus* [24,26], *M. tuberculosis* [24,32], *B. subtilis* [27] and *P. horikoshii* [29], and have recently renamed poly(P)/ATP-NAD kinases, to distinguish them from the strictly ATP-specific NAD kinases, indicated as ATP-NAD kinases. The physiological significance of the ability of poly(P) dependent enzymes to use the polymer is demonstrated by the observation that *M. flavus* NAD kinase is able to use

Table 1. Molecular and Kinetic Properties of Recombinant NAD Kinases from Bacteria and Humans

	M. tuberculosis			*M. flavus*		*B. subtilis*			*E. coli*		*P. horikoshii*	*H. sapiens*	
Subunit MW	33,000			39,000		30,000			30,000		37,000	49,000	
Oligomeric state	tetramer			dimer		dimer			esamer		tetramer	tetramer	
Phosphoryl donor[a]													
ATP	100			100		100			100		100	100	
Poly(P)[b]	300[b]			88[b]		50[b]			0		130[c]	nd	
dATP	96			91		77			42		nd	103	
GTP	47			88		69			56		148	7	
CTP	16			73		nd			60		100	nd	
TTP	33			74		35			40		17	nd	
UTP	88			87		nd			109		125	nd	
dGTP	nd			nd		77			nd		nd	nd	
dCTP	nd			nd		30			nd		nd	nd	
ITP	nd			nd		nd			nd		120	nd	
Metal-ion[d]													
Mg^{+2}	100 (100)			100 (100)		100			100		100 (100)	100	
Mn^{+2}	246 (268)			136 (143)		180			242		62 (100)	290	
Ca^{+2}	39 (34)			61 (65)		104			85		65 (57)	28	
Co^{+2}	29 (55)			28 (51)		15			73		nd (nd)	70	
Cu^{+2}	25 (8)			48 (33)		8			0		158 (100)	0	
Zn^{+2}	51 (30)			51 (30)		89			104		78 (56)	350	
Ni^{+2}	nd			nd		0			nd		36 (80)	0	
Optimum pH[e]	8.0 (6.5)			7.0 (7.0)		9.0 (nd)			7.5		nd (6.8)	7.0-8.0	
Substrates	$S_{0.5}$ (mM)	n_H	Vmax / $S_{0.5}$	Km (mM)	Vmax / Km	$S_{0.5}$ (mM)	n_H	Kcat / $S_{0.5}$	Km (mM)	Kcat / Km	Km (mM)	Km (mM)	Vmax / Km
ATP	2.5	1.5	0.48	0.13	8.39	1.10	2.0	1.24	2.5	22	0.29	3.30	2.0
Poly(P)	1.3	1.4	2.93	1.04	1.52	0.45	2.0	0.14			0.59[f]		
NAD (+ATP)	3.3	1.2	0.36	0.83	1.76	1.00[g]		5.46[g]	2.0	38	0.40	0.54	12.4
NAD (+poly(P))	1.2	1.2	3.16	0.58	2.55	nd		nd			0.30		

[a] values are given as relative activities (%)

[b] hexametaphosphate containing 13 to 18 phosphoryl residues

[c] a mix of poly(P) of different lenght

[d] activity assayed in the presence of ATP (polyP); values are given as relative activities (%)

[e] activity assayed in the presence of ATP (polyP)

[f] $S_{0.5}$ value

[g] Km and Kcat/Km values

nd: not determined

endogeneous inorganic poly(P) isolated from the cells of the microorganism [26]. Other phosphorylated compounds, like glucose-6-phosphate, *p*-nitrophenylphosphate and phospho-enolpyruvate are inert as phosphoryl donors.

Both ATP- and poly(P)/ATP-dependent NAD kinases are specific towards NAD, e.g. adenosine, ADP, AMP and ADP-ribose are not phosphorylated. Very recently the ability of eubacterial NAD kinases to phosphorylate the reduced form of NAD has been revisited, and it has been demons-trated that *M. flavus* and *M. tuberculosis* enzymes are able to phosphorylate NADH, though with lower efficiency [41]. On the contrary, *E. coli* and *Sphingomonas* sp. enzymes possess a strictly NAD specificity [41, 28]. Based on multiple sequence alignment analysis and the crystal structure solution of *M. tuberculosis* NAD kinase in complex with NAD, putative amino acid residues discriminating between NAD and NADH kinases have been identified [41]. In particular, it has been demonstrated that Arg175 in *E. coli* NAD kinase is one of the crucial residues responsible for conferring strict specificity to NAD. Its substitution with Gly or polar amino acid residues (i.e. those residues occupying the position corresponding to Arg175 in NADH kinases) is sufficient to confer the relaxed substrate specificity to the *E. coli* enzyme [41].

NAD kinase activity is strictly dependent on divalent metal-ions. As shown in Table **1**, the most efficient metal

cofactors of the eubacterial enzymes are Mn^{+2} and Mg^{+2}; in *B. subtilis* and *E. coli* NAD kinases Ca^{+2} and Zn^{+2} promote the catalytic activity in a comparable fashion with respect to Mg^{+2} [27,25]. The enzyme from the archaeon *P. horikoshii* displays a different behaviour, in that it is mostly activated by Cu^{+2} ions, which, on the contrary, partly support catalysis in eubacteria [29]. The same metal-ion sensitivity is also maintained when ATP is replaced with poly(P), as the phosphoryl donor.

All eubacterial enzymes exhibit pH optimum values ranging from 7.0 to 9.0 when ATP is used as the substrate, whereas significant lower pH optimum values have been observed when ATP is replaced with poly(P).

The kinetic analysis performed on all bacterial enzymes revealed a different kinetic behaviour depending on the microorganism. In particular, *E. coli* [25,35] and *M. flavus* [24] NAD kinases show linear kinetics towards all substrates used, whereas the *M. tuberculosis* enzyme follows non-linear kinetics, exhibiting a positive cooperativity towards all substrates [32]. Peculiar is the kinetic behaviour of the enzymes from *B. subtilis* and *P. horikoshii*: in particular, *B. subtilis* NAD kinase exhibits a marked positive cooperativity towards both phosphoryl donors [27], whereas the archaeal enzyme follows non-linear kinetics only when ATP is replaced with poly(P) [29]. Both enzymes show linear kinetics towards the phosphoryl acceptor. While, in the presence of poly(P), the activity of *B. subtilis* NAD kinase is only 50% of that measured when ATP is used as the substrate [27], *M. tuberculosis* and archaeal NAD kinases show a marked preference for poly(P) [32,29]. In particular, the mycobacterial enzyme exhibits a 6 times higher catalytic efficiency in the presence of the polymer, because of both an increase in the Vmax of the reaction and higher affinity to poly(P) rather than ATP, and to NAD when ATP is replaced with poly(P) [32].

All the eubacterial NAD kinases so far characterized, with the exception of the *E. coli* enzyme, are inhibited by the product NADP. NADPH and NADH are potent allosteric negative modulators of the *E. coli* enzyme, since their presence results in a pronounced sigmoidal NAD saturation curve [35]. The reduced dinucleotides also inhibit both *M. flavus* and *M. tuberculosis* NAD kinases [24,32]. Interestingly, quinolinate, a central metabolite in NADP biosynthesis has been demonstrated to be a strong allosteric activator of *B. subtilis* NAD kinase [27].

Eubacterial NAD kinases are strongly inhibited by Hg^{+2} and *p*-chloromercuribenzoate, indicating that a SH group of the enzyme is likely involved in catalysis [24,25,27,28]. However, the two compounds exert only a slight inhibition on the archaeal enzyme [29].

In archaeal *Methanocaldococcus* and *Methanococcus* species, the NAD kinase ortholog is fused with a protein endowed with NADP phosphatase activity, forming a novel bifunctional enzyme able to generate intracellular NADP and maintain a suitable NAD/NADP ratio [42]. Like *P. horikoshii* NAD kinase, the bifunctional enzyme exhibits a linear kinetic behaviour and it is capable to utilize also poly(P) as the phosphoryl donor, although with significantly lower efficiency.

Human NAD Kinase

The presence of an activity able to convert NAD to NADP in human sources has been first observed in placenta [43] and subsequently in polymorphonuclear leukocytes [44] and red cells [45]. However the characterization of the human enzyme from a partially purified preparation obtained from neutrophils was carried out more than 20 years later [46]. The enzyme is calmodulin/Ca^{+2} dependent, in that the maximum velocity of the catalyzed reaction was significantly increased by the presence of both effectors [46]. On the other hand, Km values for NAD and ATP (0.3 mM and 0.4 mM, respectively) were not affected by their presence. The optimum pH of the enzyme is in the range 7.5-9.0 and its native molecular mass was 169,000 Da [46].

Four years ago, based on sequence similarity searches by using the bacterial enzyme as the query, the human cDNA for NAD kinase has been identified [47]. The gene is located on chromosome 1p36.21-36.33, and it is expressed in most tissues with the exception of skeletal muscle. The properties of the recombinant human enzyme, purified from *E. coli* cells, are summarized in Table **1** [47]. Human NAD kinase is composed of four identical subunits of about 49 kDa. Its catalytic activity is optimal at 55°C and in the pH range of 7.0-8.0. The enzyme requires a divalent cation such as Zn^{+2}, Mn^{+2} and Mg^{+2} in order of effectiveness. Human NAD kinase is highly selective for the substrates NAD and ATP, showing Km values of 0.54 and 3.3 mM, respectively. The recombinant human enzyme is not calmodulin-dependent and shows molecular and catalytic properties significantly different from those exhibited by the enzyme purified from human neutrophils. This points to the hypothesis that different NAD kinase isoforms might exist in humans. However, further studies will be required to definitively establish the existence of a human calmodulin-dependent NAD kinase, whose presence has been clearly demonstrated both in sea urchin eggs [48,49] and plants [50-58].

STRUCTURAL PROPERTIES OF NAD KINASE

The crystal structure of *M. tuberculosis* NAD kinase has been first solved in its apo-form [34]. The overall quaternary structure is tetrameric, being the minimal functional unit, the asymmetric unit, formed by two protein subunits (Fig. **4A**). Each subunit consists of a α/β N-teminal domain and a C-terminal 12-stranded β sandwich domain, connected by swapped β strands. The molecular architecture is completed by a long C-terminal tail, which anchors the dimerization. The inter-subunit contacts are entirely contributed by residues of the C-terminal domain and the C-terminal extension is located on the external face of the protein. A long crevice housing the enzyme active site on each monomer is present at the domains interface; both the GGDG motif and some residues of the highly conserved Glycine-rich region (spanning residues Asp189-Val210) are located in such a crevice [34]. The GGDG sequence fingerprint is highly conserved in a super-family of enzymes, including diacylglyceride kinase, sphingosine kinase, NAD kinase and 6-phosphofructokinase [31]. Such a motif has been demonstrated to be involved in ADP binding in the 6-phosphofructokinase family [59] and it is proposed to participate in nucleotide-binding in human sphingosine

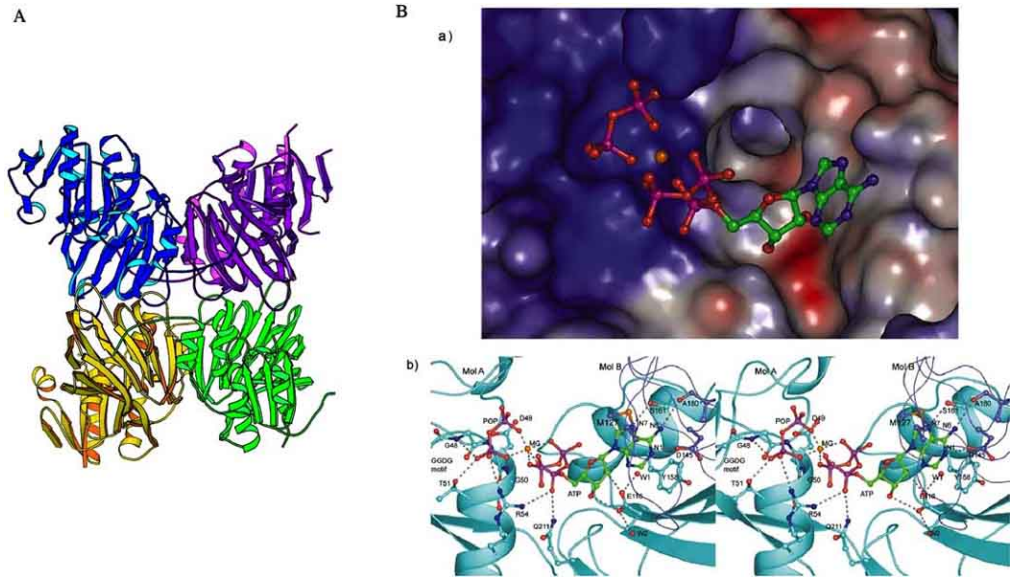

Fig. (4). NAD kinase three-dimensional structure. A, ribbon representation of the *M. tuberculosis* tetramer viewed along one of the dyad axes. Each of the four subunits is colored differently. B, a) electrostatic surface presentation of the ATP-binding site of *A. fulgidus* NAD kinase. ATP is shown as a ball-and-stick model. b) Stereo view of the ATP-binding site in the *A. fulgidus* NAD kinase-ATP structure. The involved molecules are shown in cyan and marine. ATP, pyrophosphate (POP) and residues interacting with NADP are shown as a ball-and-stick model. Magnesium and water are shown as spheres. Hydrogen bonds are shown as dashes in black.
Adapted from [34], and [30] with permission from Elsevier.

kinase [60]. Directed mutagenesis of the GGDG motif of mycobacterial NAD kinase led to complete loss of enzymatic activity [31,33]. The Glycine-rich region is a peculiar feature of all NAD kinases; site-directed mutagenesis of the mycobacterial NAD kinase region demonstrated its essentiality in catalysis and pointed to its involvement in NAD binding [32]. Resolution of the structure of the enzyme complexed with NAD revealed that residues from both subunits of the functional dimer play an important role in NAD binding [33]. Among the residues interacting with the dinucleotide, Asp85 and Gly86 (involved in the diphosphate and adenine ring binding, respectively) belong to the GGDG motif, and Asp189 and Tyr202 (interacting with the pyridine ring) and Thr200 (interacting with the adenine mojety) reside in the Glycine-rich region [33]. In addition, the NE conserved motif (see Fig. **3**) represented by Asn159 and Glu160, is also involved in NAD binding [33].

On superimposition of the tertiary structures of the apo-enzyme and the protein complexed with NAD, a substantial conformational difference in a loop (Gly166-Gly170) involved in the inter-subunit contact was revealed [33]. In particular, Leu169 of such a loop, which interacts with Gly258 of the other subunit in the apo-enzyme, looses the interaction upon NAD binding. Interestingly, the residues corresponding to the loop are not conserved among NAD kinases; this, together with the observation that *M. tuberculosis* NAD kinase is the only exhibiting sigmoidal kinetics towards all substrates, suggest that the loop may be related to the allosteric behaviour [33].

Very recently, the structure of *Archaeoglobus fulgidus* NAD kinase has been solved in complex with ATP, NAD and NADP, revealing detailed information on the binding mode of the phosphoryl donor and on the interaction of the GGDG motif with ATP and NADP [30]. The overall fold of the archaeal enzyme is similar to the structure of mycobacterial NAD kinase. Binding of the different ligands had no significant effect on the overall protein conformation. The substrate NAD and the product NADP share the same binding mode and the interactions of the dinucleotides with the enzyme are similar to those observed in the mycobacterial enzyme complexed with NAD. In the structure of the enzyme complexed with NADP, the 2' sugar phosphate group of NADP forms hydrogen bonds with residues belonging to the GGDG motif. In the structure of the enzyme complexed with ATP, the AMP portion of the ATP molecule was found to bind in the same binding site as the nicotinamide ribose portion of the NAD/NADP molecules, with the adenine ring of ATP positioned in the same orientation as the nicotinamide ring of NAD/NADP. The phosphate tail of ATP protrudes out of the binding pocket and all three phosphate groups are involved in coordination to the magnesium ion (Fig. **4B**). On the other side of the phosphate tail, the magnesium ion is coordinated by a pyrophosphate moiety (proposed to be the β- and γ-phosphate groups of a second bound ATP molecule with the AMP portion disordered) which is involved in interactions with the GGDG motif (Fig. **4B**). Authors propose that the first ATP molecule fortuitously bound to the enzyme in the absence of NAD, and the second ATP molecule was the actual phosphate donor. Therefore the GGDG motif might play a key role in phosphate transfer [30]. Upon these observations and speculations they hypothesized the phosphorylation mechanism for NAD kinase shown in Fig. (**5**). The model implies that the two substrates bind to the active site in an ordered manner, being NAD the first

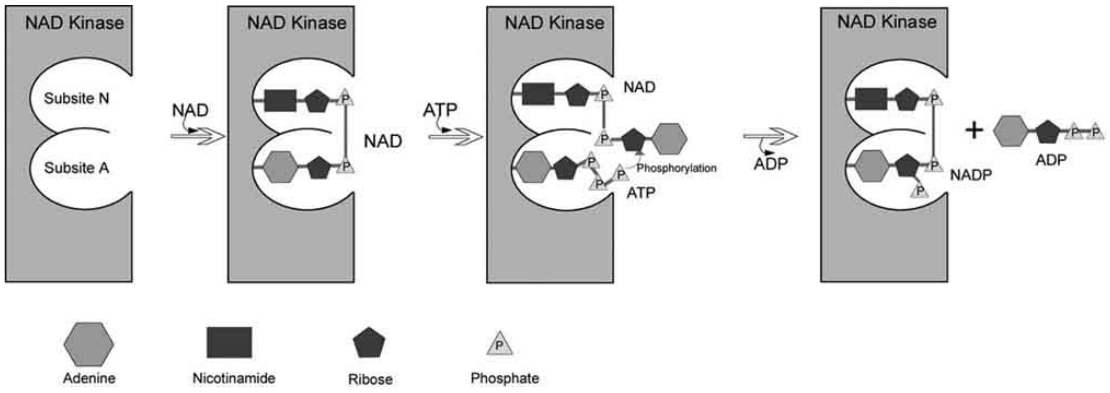

Fig. (5). Proposed mechanism for NAD kinase-catalyzed reaction. Subsite N and subsite A represent the nicotinamide subsite and the AMP subsite, respectively. Reprinted from [30] with permission from Elsevier.

substrate to bind, followed by the phosphoryl donor (ATP or poly(P)) [30]. Such a mechanism needs to be confirmed by further studies, including detailed kinetic analyses.

CONCLUSIONS AND PERSPECTIVES

The major features of human and bacterial NAD kinases have been described, including the most recent advanced knowledge on the kinetic and structural characterization. Of particular importance are the in-depth studies on *M. tuberculosis* NAD kinase, with respect to its structural and functional properties. Unfortunately, the characterization of the human enzyme has not been equally exhaustive. Nevertheless, the availability of the mycobacterial enzyme crystal structure might allow an evaluation of how sequence differences between the bacterial and human enzyme may be exploited for the design of selective inhibitors. The structure of the *M. tuberculosis* enzyme in complex with NAD provides a good starting point for the design process. However, due to its low resolution, some details are diffused and not sharply resolved. This problem could be overcome by the additional information that can be inferred by the structural data reported for the archaeal enzyme.

Of particular interest is the difference existing in the catalytic behaviour between the bacterial and human enzyme. The human enzyme indeed follows linear kinetics, whereas the mycobacterial enzyme exhibits allosteric interactions. This difference, together with the reported strictly ATP-dependency of human NAD kinase, could be instrumental in the designing of new selective antitubercular drugs.

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