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# Nicotinamide Nucleotide Transhydrogenase as a Sensor of Mitochondrial Biology

Salvatore Nesci, Fabiana Trombetti, Alessandra Pagliarani

Department of Veterinary Medical Sciences, University of Bologna

Via Tolara di Sopra, 50 – 40064 Ozzano Emilia, Bologna, Italy.

*Correspondence: salvatore.nesci@unibo.it

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## Abstract

The enzyme nicotinamide nucleotide transhydrogenase (NNT) transfers hydride from NADH to $NADP^+$ coupled to $H^+$ translocation across the inner mitochondrial membrane. In a recent study in *Nature*, Kampjut *et al.* reveal that the bi-functional NNT mechanism rules the $NAD(P)^+$/NAD(P)H interconversion ratio, which in turn regulates antioxidant defence and sirtuin actions.

Pyridine nucleotides are $NAD(P)^+$ coenzymes that can undergo reversible reduction of the nicotinamide ring, which can accept a hydride ion (:$H^-$, a proton plus two electron), yielding the reduced form NAD(P)H. A high $NAD^+$/NADH ratio in cells is required for nutrient oxidation, reflecting its catabolic role, while anabolic reactions feature a low $NADP^+$/NADPH ratio required for biosynthetic reductions. As such, oxidative catabolic reactions and reductive biosynthetic pathways sustained by these pyridine cofactors are segregated in specialized compartments of eukaryotic cells.

Mammalian nicotinamide nucleotide transhydrogenase (NNT), an integral inner mitochondrial membrane (IMM) protein, is a symmetric homodimer formed by two polypeptide chains. In a recent study in *Nature*, Kampjut *et al.* solve the structure of mammalian NNT in various conformations by cryo-electron microscopy (cryo-EM), and reveal insight into the coupling process in NNT [1]. The cryo-EM structure of NNT revealed that each chain bears a matrix-exposed NAD(H)-binding domain (dI) and a NADP(H)-binding domain (dIII), while the hydrophobic transmembrane domain (dII) contains a putative proton channel [1]. The NAD(H)-binding domain is connected to the transmembrane domain by a region named *linker* 1, while the NADP(H)-binding domain is connected to the transmembrane domain by *linker* 2. Therefore, the two hydrophilic domains are indirectly, mutually linked [1] (Fig. 1A). Under physiological conditions, NNT products are driven by Mitchell's proton motive force via protein conformational changes, but the enzyme can also work *in*

*reverse* [2] and act as a NADH/$NADP^+$ fuelled proton pump. Since the detachment and attachment of the nucleotide binding domains, namely the dI–dIII interface, and of the dII–dIII interface, drives the exchange between pyridine nucleotides and $H^+$ translocation, respectively, the bi-functional enzyme catalysis must rely on a coupled reaction mechanism [1]. In this complex reaction cycle, the dIII domain acts as a playmaker to transduce the substrate binding energy [3]. Indeed, when dIII swivels upward, it allows the transfer of the $H^-$ from the dI NAD(H) binding site to the dIII NADP(H) binding site. Accordingly, when dIII swivels down, it allows the translocation of a single $H^+$ from the positive outer side to the negative inner side of the IMM. The dI-dIII association permits $H^-$ transfer and the consequent NADH/$NAD^+$ exchange, while the dII-dIII contact is coupled to $NADP^+$/NADPH exchange and $H^+$ transport [4]. Likely, the NNT mechanism is fully reversible, being able to act in both directions. Accordingly, the difference in bond energies between NADPH and $NADP^+$ adds to the proton motive force to form the thermodynamic force, which is conferred to the dII domain, to decide the direction of the catalytic cycle. Thus, the forward reaction proceeds with high $NADP^+$/NADPH ratio and proton motive force [1] (Fig. 1B). Most likely, NNT can change its "forward" direction to a "reverse" catalysis under pathological conditions [2] so as to maintain the $NAD(P)^+$/NAD(P)H ratio in the mitochondrial matrix. The auto-inhibited double dIII domains face-down conformation in mammalian NNT, which blocks the homeostasis of pyridine cofactors, could represent another relevant mechanism of NNT regulation in mitochondrial biology.

Consistently, NNT regulates mitochondrial metabolism via redox balance of $NAD^+$ and NADPH production. NADH/$NAD^+$ ratio rules Krebs cycle activity, while the NNT "forward" direction, which forms $NAD^+$, increases cell sensitivity to energy demand [5]. Moreover, NADPH other than being required for the biosynthesis of macromolecules, also fuels antioxidant defences. NNT supplies the NADPH reserves that are required to protect mitochondria from superoxide anion, hydrogen peroxide, and related reactive oxygen species (ROS), which may cause oxidative damage. The mitochondrial matrix is exposed to a moderate flux of ROS as normal by-products of mitochondrial metabolism. Indeed, NADPH-oxidase, monoaminoxidase, α-glycerophosphate and dihydrolipoamide dehydrogenase, and other enzymes are mitochondrial production sites of ROS under physiological conditions, while mitochondrial complex I and III are major ROS sources under pathological conditions. Among the predominant chemical changes caused by oxidative stress, post-translational modifications of thiol groups are especially relevant [6]. Thiol sidechain of cysteines can be oxidized to yield various reversible and irreversible redox states of sulfur according to the extent of the change in intracellular redox potential. Therefore, thiol-based redox pairs, such as reduced-glutathione (GSH)/oxidized-glutathione (GSSG) and reduced-thioredoxin (HS-Trx-SH)/oxidized-thioredoxin (Trx-SS), represent sets of low-molecular-weight thiol-containing compounds that act as ROS scavengers in antioxidant systems and contribute to the modulation of ROS-associated signalling events. The redox potential of mitochondrial GSH and Trx systems depends on the NADPH pool provided by the glutathione reductase (GR) and the thioredoxin reductase 2 (TR2), respectively [6]. GR exploits NADPH to synthesize GSH, which in turn, can be used to inactivate peroxides, detoxify electrophiles and, as well as HS-Trx-SH, contribute to the overall thiol content in the mitochondrial matrix (Fig. 1B). Primarily, TR2 uses NADPH to produce HS-Trx-SH, which is the main reductant of peroxidases that degrade inorganic and organic peroxides and reverse methionine sulfoxide to methionine. The NADPH pool is maintained by the combined action of NNT, malic enzyme and isocitrate dehydrogenase 2 (IDH2). Malic enzyme, mainly in brain, muscle and heart, catalyzes malate decarboxylation/dismutation to form pyruvate and generate NADPH from $NADP^+$ in mitochondria. IDH2 isozymes produce NADPH in the Krebs cycle and in general, act in in reductive reactions, while the $NAD^+$-dependent multi-subunit IDH3 is allosterically regulated and is thought to operate in the mitochondrial oxidative decarboxylation of isocitrate to α-keto-glutarate. Conversely, during glutamine oxidation the reductive carboxylation of α-keto-glutarate in the reverse direction of the Krebs cycle are essential metabolic pathways for pluripotent stem cell survival. The IDH2-catalyzed reaction is driven by the NADPH reduction potential, in turn provided by the proton motive force-dependent "forward" NNT reaction [7]. Since the reverse Krebs cycle sustained by the balance of pyridine coenzymes is crucial in proliferative metabolism it

may also sustain proliferation of cancer cells. On these bases, NNT which fuels it, could represent a promising drug target to counteract tumor development.

NNT also produces $NAD^+$, which not only represents the classical coenzyme of dehydrogenase reactions, but also plays a relevant role as a substrate of $NAD^+$-consuming enzymes, including sirtuins. $NAD^+$ formation is the result of $H^-$ transfer mechanism to dI-dIII interface. Mammalian NNT, structurally different from the bacterial counterpart, is characterized by a more rapid enzymatic turnover in forming $NAD^+$ and NADPH. In principle $NAD^+$-sirtuin axis could be supported by NNT, the main mitochondrial $NAD^+$ source, in ruling the metabolic state and constituting a potential tool to mimic calorie restriction and to slow ageing [8]. Mammalian sirtuins (SIRTs) comprise a family of seven proteins (SIRT1–SIRT7) with different subcellular localizations and enzymatic activities [9]. The three mitochondrial sirtuins (SIRT3, SIRT4 and SIRT5) are in the matrix and catalyze $NAD^+$-dependent de-acylase or mono ADP-ribosyltransferase reactions on mitochondrial proteins (Fig. 1B). SIRT3 displays a robust de-acetylase activity in mitochondria, in contrast to SIRTs 4–5, which show little to no activity. SIRT5 primarily demalonylates and desuccinylates mitochondrial proteins. SIRT4 shows ADP-ribosyltransferase, lipoamidase and de-acylase activities, but its role in mitochondrial metabolic regulation remains enigmatic. The $NAD^+$-dependent action of mitochondrial sirtuins regulates energy metabolism and maintains cellular homeostasis in response to physiological stress responses, thus preventing several age-related diseases. Therefore the stimulation of mitochondrial sirtuin activities would be beneficial to human health [10] and positively connected with the NNT forward mode that ensures a high $NAD^+$/NADH ratio. Future studies on mitochondria lacking NNT are required to further test this hypothesis.

Moreover, since the antioxidant system relies on the NADPH bio-availability to provide low-molecular-weight thiol-containing compounds, while sirtuin activities are linked to systemic $NAD^+$ levels, which decline in aging, NNT could represent the metabolic meeting point that translates the changes in $NAD(P)^+$/NAD(P)H pools into adaptive cellular responses to improve lifespan and counteract age-related diseases. Thus, a complete structural and mechanistic understanding of energy transduction regulation in NNT is crucial to define a putative therapeutic role of NNT to counteract mitochondrial dysfunctions arising from ROS damage and protein hyper-acylation.

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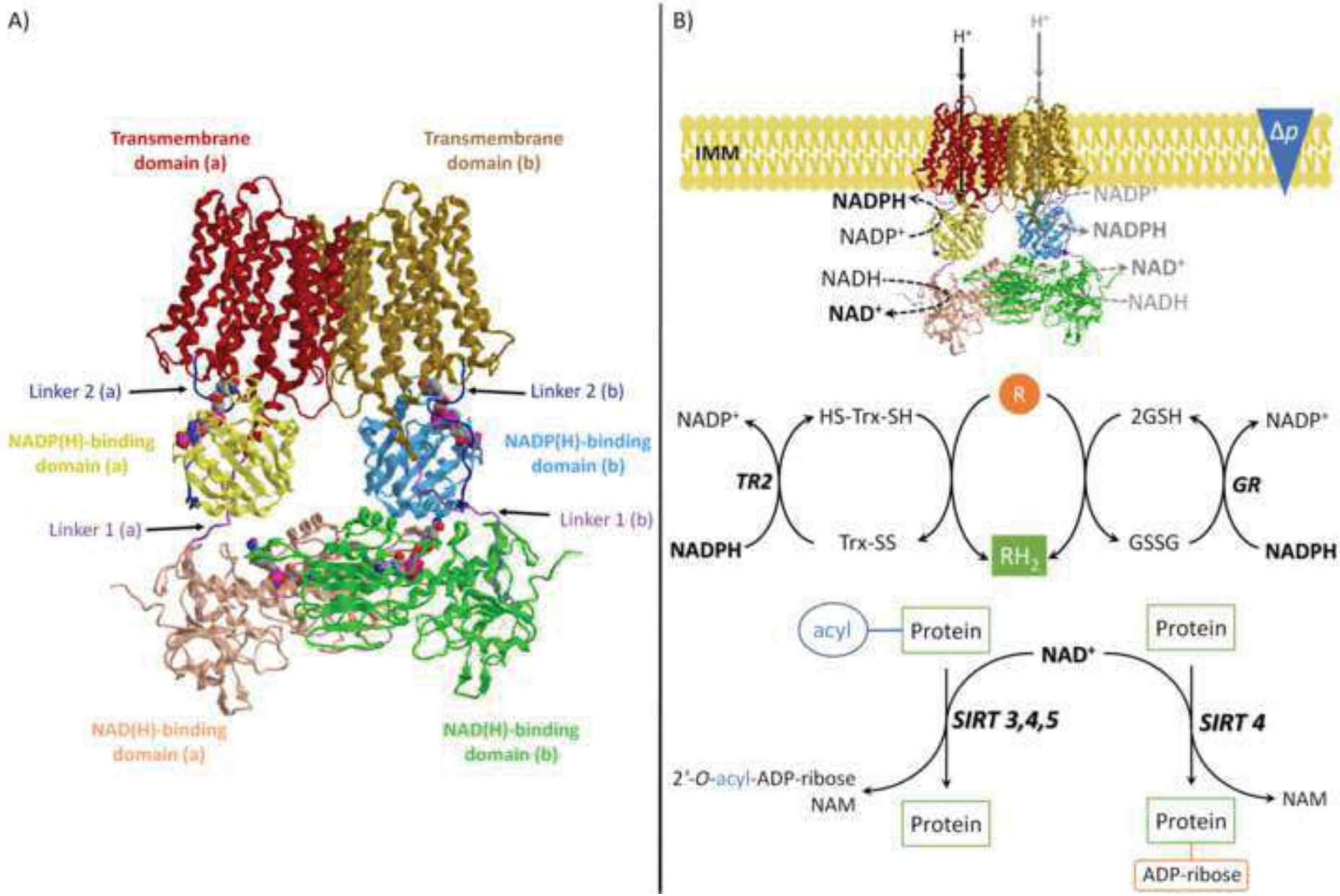
Figure

Figure 1. NNT Structure and Function in Mammalian Mitochondria. A) Domains, linkers and pyridine nucleotide binding sites in the NNT homodimer (modified PDB ID code: 6QTI). NAD(H) and NADP(H), drawn as space filling mode, are bound to their specific binding domains of each monomer. B) NNT catalytic activity and its relationship with the antioxidant system and post-translational modifications of mitochondrial proteins. Upper panel, right side: the "forward" mode of NNT catalysis. Middle panel: two parallel thiol systems driven by NADPH. GSH and HS-Trx-SH pools are involved in redox reactions that participate in the antioxidant system. Lower panel: During catalysis, sirtuins use $NAD^+$ to remove acyl groups (including acetyl, glutaryl, malonyl, succinyl and lipoyl groups) from Lys residues to form 2′-*O*-acyl-ADP-ribose and de-acylate proteins (left side) or for its ADP-ribosyl transferase activity which transfers ADP-ribose from $NAD^+$ to Arg, Cys, Ser or Thr residues of mitochondrial proteins (right side). IMM, inner mitochondrial membrane; Δ*p*, proton motive force; TR2, thioredoxin reductase 2; GR, glutathione reductase; R, oxidized protein; $RH_2$, reduced protein; NAM, nicotinamide.

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