Biochimie 94 (2012) 300–309

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Characterization of human nicotinate phosphoribosyltransferase: Kinetic studies, structure prediction and functional analysis by site-directed mutagenesis

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ARTICLE INFO

Article history:
Received 3 March 2011
Accepted 24 June 2011
Available online 3 July 2011

Keywords:
Human NaPRT
Enzyme kinetics
Homology modeling
Molecular docking
Site-directed mutagenesis

ABSTRACT

Nicotinate phosphoribosyltransferase (NaPRT, EC 2.4.2.11) catalyzes the conversion of nicotinate (Na) to nicotinate mononucleotide, the first reaction of the Preiss-Handler pathway for the biosynthesis of NAD^+. Even though NaPRT activity has been described to be responsible for the ability of Na to increase NAD^+ levels in human cells more effectively than nicotinamide (Nam), so far a limited number of studies on the human NaPRT have appeared.

Here, extensive characterization of a recombinant human NaPRT is reported. We determined its major kinetic parameters and assayed the influence of different compounds on its enzymatic activity. In particular, ATP showed an apparent dual stimulation/inhibition effect at low/high substrates saturation, respectively, consistent with a negative cooperativity model, whereas inorganic phosphate was found to act as an activator. Among other metabolites assayed, including nucleotides, nucleosides, and intermediates of carbohydrates metabolism, some showed inhibitory properties, *i.e.* CoA, several acyl-CoAs, glyceraldehyde 3-phosphate, phosphoenolpyruvate, and fructose 1,6-bisphosphate, whereas dihydroxyacetone phosphate and pyruvate exerted a stimulatory effect. Furthermore, in light of the absence of crystallographic data, we performed homology modeling to predict the protein three-dimensional structure, and molecular docking simulations to identify residues involved in the recognition and stabilization of several ligands. Most of these residues resulted universally conserved among NaPRTs, and, in this study, their importance for enzyme activity was validated through site-directed mutagenesis.

1. Introduction

Besides its well-known function as a redox coenzyme, NAD^+ is also utilized in a number of protein post-translational modification reactions, including histone deacetylation catalyzed by sirtuins [1], and mono- and poly-(ADP-ribosyl)ation reactions [2,3]. Such NAD^+-splitting reactions, involved in fundamental biological processes like cell differentiation, cell survival and apoptosis, call for continuous NAD^+ resynthesis.

Depending on the organism, four different building blocks can be used as a source of the pyridine ring for NAD^+ biosynthesis: Qa [4] in the *de novo* pathway; Na and Nam in the salvage pathways; NamR in the "Preiss-Handler-independent" pathway. Qa, Na and Nam are used by phosphoribosyltransferases, whereas NamR is used by NamR kinase for the production of the mononucleotides NaMN and NMN, in turn converted to their respective dinucleotides $NaAD^+$ and NAD^+ (Fig. 1) [4–6].

Even though Nam levels are much higher than Na levels throughout mammalian living cells, some tissues preferentially use Na for NAD^+ synthesis [7], and indeed Na was found to be more effective than Nam in increasing NAD^+ levels in heart, kidney [8], and red blood cells [9]. Therefore, both precursors appear relevant for NAD^+ biosynthesis, possibly with distinct and complementary roles in different cells.

In mammals, Na is predominantly extracellular and derives directly from the diet or, indirectly, from dietary Nam after deamidation by a gut flora Nam deamidase [4]. In the pyridine

Abbreviations: DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; IPTG, isopropyl β-D-1-thiogalactopyranoside; Na, nicotinic acid; Nam, nicotinamide; NamR, nicotinamide riboside; NaMN, nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide; $NaAD^+$, nicotinic acid adenine dinucleotide; NaPRT, nicotinate phosphoribosyltransferase; NamPRT, nicotinamide phosphoribosyltransferase; PRPP, 5-phosphoribosyl-1-pyrophosphate; Qa, quinolinic acid; RMSD, root mean square deviation.

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doi:10.1016/j.biochi.2011.06.033

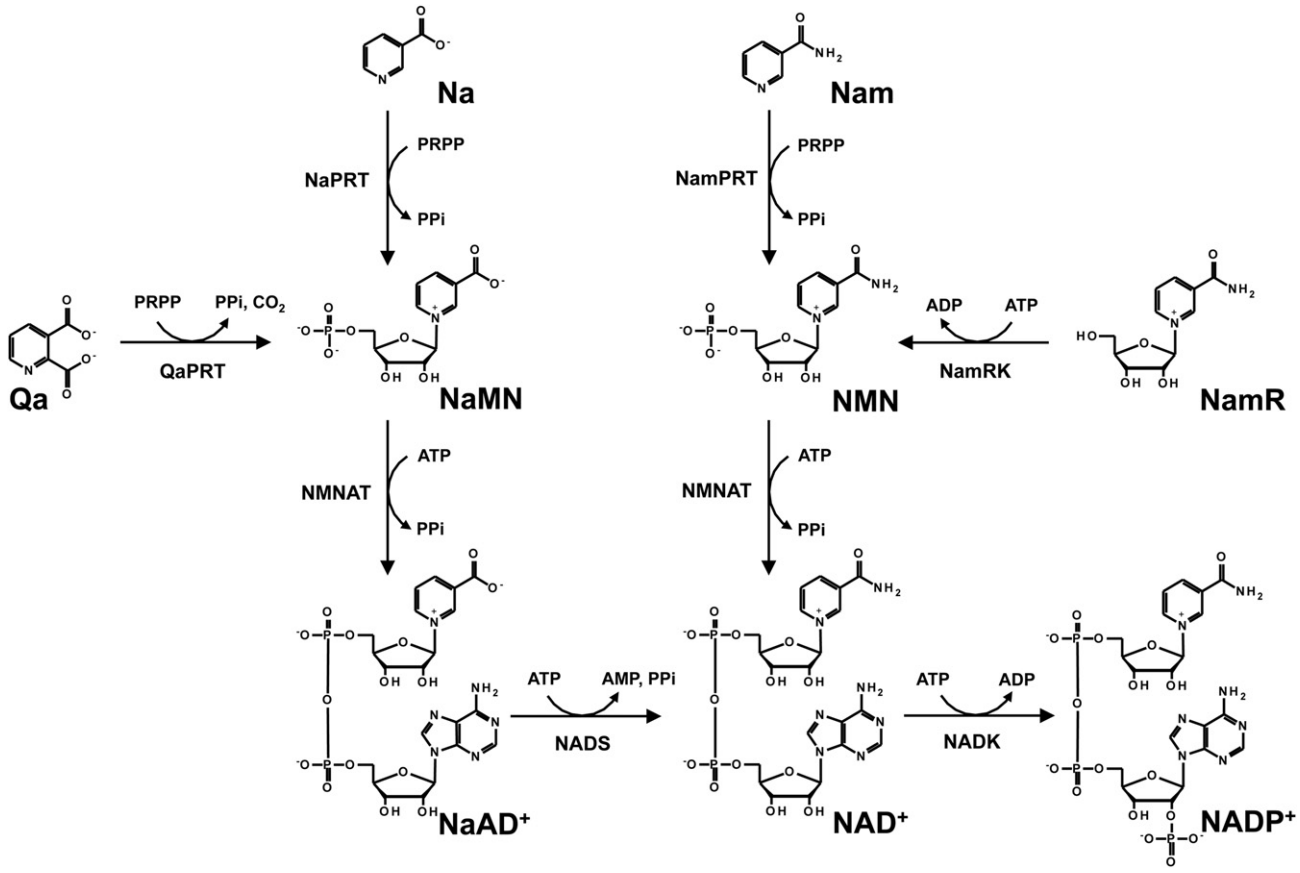

Fig. 1. The NAD^+ biosynthetic pathways. Three pathways (*de novo*, Preiss–Handler, and Preiss–Handler-independent) allow NAD^+ biosynthesis from four distinctive sources of the pyridine ring, *i.e.* Qa, Na, Nam, and NamR (see abbreviations list). NamRK, nicotinamide riboside kinase (EC 2.7.1.22); NMNAT, nicotinamide mononucleotide adenylyltransferase (EC 2.7.7.1); NADS, NAD^+ synthetase (EC 6.3.5.1); NaPRT, nicotinate phosphoribosyltransferase (EC 2.4.2.11); NamPRT, nicotinamide phosphoribosyltransferase (EC 2.4.2.12); QaPRT, quinolinate phosphoribosyltransferase (EC 2.4.2.19).

nucleotide reutilization pathway, Na is the substrate of a nicotinate phosphoribosyltransferase (NaPRT, EC 2.4.2.11) activity, originally named NaMN pyrophosphorylase [10], which catalyzes the synthesis of NaMN from Na and PRPP (Fig. 1). NaPRT is conserved in prokaryotes, archaea, and eukaryotes, indicating the ubiquitous nature of the biosynthetic pathway from Na to NAD^+, the so called "Preiss-Handler pathway" [11].

NaPRT indeed was first identified in humans by Handler together with the different ability of Na and Nam to elevate NAD^+ levels in red blood cells [9,11,12]. The enzyme has also been partially characterized from different sources [5]. Its significance for NAD^+ cellular homeostasis in mammals has been reinforced by the evidence that NaPRT appears to be more expressed in those tissues where Na is the preferential source for NAD^+ biosynthesis, and, in human embryonic kidney cells (HEK293) the addition of Na, but not Nam, markedly elevates NAD^+ levels [13]. This Na effect could be ascribed to the finding that NaPRT, unlike NamPRT, is not subject to feedback inhibition by NAD^+ [13].

The ability of orally administered Na to increase cellular NAD^+ levels *via* NaPRT may also account for some of its vitamin effects [9]. Indeed, this awareness suggested novel applications for the treatment of conditions associated with cellular NAD^+ depletion, such as in photodamaged skin [14]. Furthermore, Na represents one of the oldest lipid-modifying drugs, showing a unique antilipolytic effect [15]. This effect is mediated by its interaction with GPR109A, a Na-receptor on the plasma membranes of adipocytes [16,17]. The lack of reports on the possible modulation of blood Na levels by NaPRT in treated patients warrants special attention on the enzyme characteristics to optimize current pharmacological applications of the vitamin.

In this report, a kinetic characterization of human recombinant NaPRT, including its modulation by several metabolites, is described. In addition, since no three-dimensional structure for mammalian NaPRT is available, we carried out homology-based protein structure modeling using deposited structures of microbial NaPRTs as templates. To identify the residues involved in the recognition and stabilization of enzyme ligands, we also carried out molecular docking simulations and site-directed mutagenesis experiments.

2. Experimental procedures

2.1. Cloning, expression, and purification of human NaPRT

A human NaPRT (UniProt ID: Q6XQN6) ORF was amplified by PCR from the U937 monocyte cell line, using primers carrying appropriate *Eco*RI and *Xho*I restriction overhangs (Naprt-fw and Naprt-rev, Table 1) for directional cloning into the pET32b vector (Novagen). A plasmid fragment encoding thioredoxin, S-tag, and the thrombin and enterokinase recognition sites, was removed by *Eco*RI/*Nde*I digestion and ligation in the presence of linkers A and B (Table 1), which anneal to generate the *Eco*RI and *Nde*I protruding ends. The resulting plasmid was replicated in *Escherichia coli* TOP10, sequence-verified, and transformed into *E. coli* BL21. Bacterial cultures were grown at 25 °C in Luria–Bertani medium supplemented with 100 μg/mL ampicillin. When OD_{600} of 0.6–0.8