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5′-Nucleotidases and their new roles in NAD^+ and phosphate metabolism†

Katrina L. Bogan and Charles Brenner*

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5′-Nucleotidase (EC 3.1.3.5) designates a set of enzymes, which catalyze the hydrolysis of ribonucleoside and deoxyribonucleoside monophosphates into the corresponding nucleosides plus orthophosphate. 5′-Nucleotidases are classified according to subcellular localization, nucleobase specificity and their ability to hydrolyze deoxynucleoside monophosphate substrates. Membrane-bound 5′-nucleotidases are ectoenzymes principally involved in salvage of extracellular nucleosides, and often display a preference toward adenosine monophosphate, thereby modulating signal transduction cascades involving purinergic receptors. Cytosolic 5′-nucleotidases are members of the haloacid dehalogenase superfamily of enzymes, which are two-domain proteins containing a modified Rossman fold as the core and a variable cap structure. Extracellular and intracellular 5′-nucleotidase activities participate in purine and pyrimidine salvage to support balanced synthesis of nucleotides, which is critical for maintaining high fidelity DNA replication. While the production of ribonucleosides from ribonucleotides by 5′-nucleotidases remains the most well studied function, it appears that the physiological functions of these activities are more broad. Indeed, Sdt1, previously termed a pyrimidine-specific 5′-nucleotidase, and Isn1, previously termed an inosine monophosphate (IMP)-specific 5′-nucleotidase, have recently been implicated in catabolic processes in nicotinamide adenine dinucleotide (NAD^+) metabolism, and are regulated by the NAD^+ precursor vitamin nicotinic acid, glucose and phosphate availability in the medium. In addition, Usha, Pho5, Sdt1 and Phm8 are phosphate starvation-induced 5′-nucleotidases with diverse substrate specificities that liberate phosphate under phosphate starvation conditions. Here we review 5′-nucleotidase enzyme structure, catalytic mechanism and substrate specificity and focus on new biological roles for these enzymes in nucleotide, NAD^+ and phosphate metabolism.

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Department of Biochemistry, Carver College of Medicine, The University of Iowa, 51 Newton Rd, 4-403 BSB, Iowa City, IA 52246, USA. E-mail: charles-brenner@uiowa.edu
† This article is part of a themed issue on Biophosphates, and is dedicated to Professor Wojciech J. Stec on the occasion of his 70th birthday.

5′-Nucleotidases are regulators of nucleotide and nucleobase metabolism

5′-Nucleotidase was first reported to dephosphorylate AMP and IMP in heart and skeletal muscle.[1] Since this discovery, similar enzymes have been described in diverse species, tissues

Katrina L. Bogan

Katrina L. Bogan is a 2004 graduate of Providence College and a 2010 PhD from Dartmouth. As a graduate student with Charles Brenner, she demonstrated that nicotinamide riboside and nicotinic acid riboside are normal intracellular metabolites and she identified the 5′-nucleotidases required for their intracellular production. In the spring of 2010, she will begin her postdoctoral fellowship with Michael Welsh at the Howard Hughes Medical Institute and the University of Iowa.

Charles Brenner

Charles Brenner received his PhD from Stanford with Robert Fuller and conducted post-doctoral work at Brandeis with Gregory Petsko. His research, which has been funded by the Leukemia & Lymphoma Society, the March of Dimes, the Beckman Foundation, the Burroughs Wellcome Fund, the Lung Cancer Research Foundation, the National Institutes of Health, and the National Science Foundation, uses interdisciplinary approaches to characterize the normal functions of tumor suppressor genes and to dissect how NAD metabolism is regulated in eukaryotic cells. He was a faculty member at Jefferson (1996–2003) and Dartmouth (2003–2009) before moving to Iowa as head of biochemistry.

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and cell types. Today it is well established that 5′-nucleotidase activities regulate the quantity of nucleotides generated from both *de novo* and salvage pathways (Fig. 1, shown in red). Cellular production of nucleosides from nucleotides not only provides substrates for salvage pathways to regenerate nucleotides but also produces the substrates of nucleoside phosphorylases (in animals, some bacteria and parasitic protozoa) and nucleoside hydrolases (in plants, some fungi and protozoa), which produce nucleobases.[2,3] Maintenance of proper nucleoside metabolizing enzymes, including 5′-nucleotidase and the downstream phosphorylase and hydrolase activities, is critical for human health. Before reviewing the roles of nucleoside metabolism in disease, we review the basics of 5′-nucleotidase activity in nucleotide biosynthesis (Fig. 1).

Nucleotide salvage pathways obtain substrates from two main sources: diet and recycling of macromolecules within the body. DNA, RNA and nucleotides are consumed in the diet and broken down to nucleotides and subsequently nucleosides through the concerted activities of extracellular nucleases, phosphodiesterases and 5′-nucleotidases. Conversion of nucleotides to nucleosides facilitates uptake through nucleoside transporters, after which nucleotides can be regenerated by phosphorylation. Nucleosides can be broken down further to

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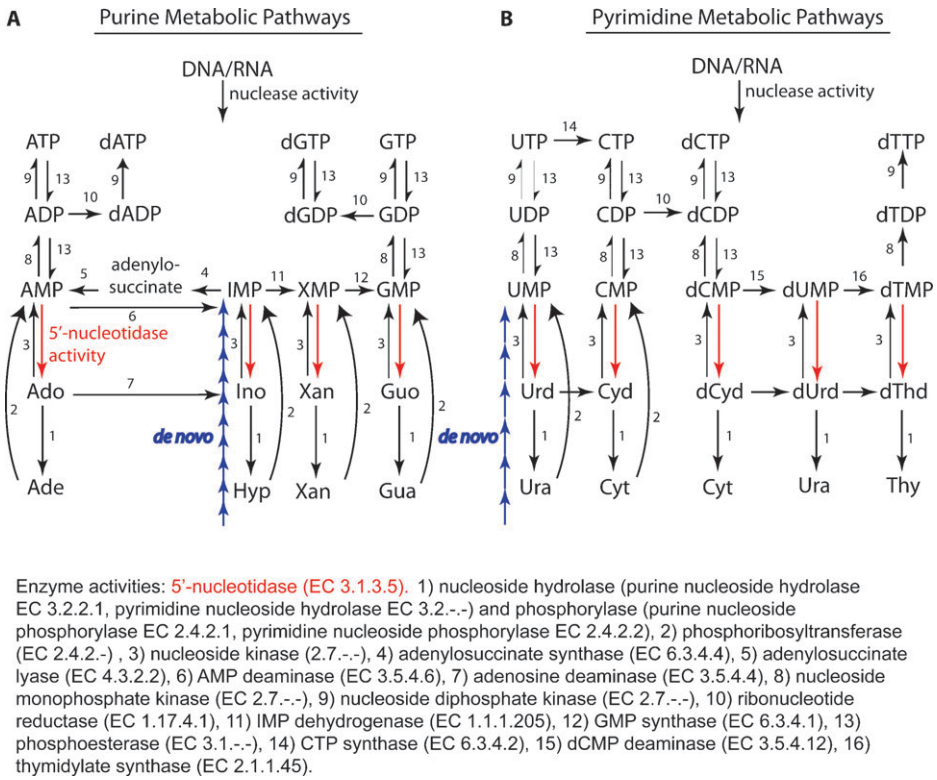

Enzyme activities: 5'-nucleotidase (EC 3.1.3.5). 1) nucleoside hydrolase (purine nucleoside hydrolase EC 3.2.2.1, pyrimidine nucleoside hydrolase EC 3.2.-.-) and phosphorylase (purine nucleoside phosphorylase EC 2.4.2.1, pyrimidine nucleoside phosphorylase EC 2.4.2.2), 2) phosphoribosyltransferase (EC 2.4.2.-) , 3) nucleoside kinase (2.7.-.-), 4) adenylosuccinate synthase (EC 6.3.4.4), 5) adenylosuccinate lyase (EC 4.3.2.2), 6) AMP deaminase (EC 3.5.4.6), 7) adenosine deaminase (EC 3.5.4.4), 8) nucleoside monophosphate kinase (EC 2.7.-.-), 9) nucleoside diphosphate kinase (EC 2.7.-.-), 10) ribonucleotide reductase (EC 1.17.4.1), 11) IMP dehydrogenase (EC 1.1.1.205), 12) GMP synthase (EC 6.3.4.1), 13) phosphoesterase (EC 3.1.-.-), 14) CTP synthase (EC 6.3.4.2), 15) dCMP deaminase (EC 3.5.4.12), 16) thymidylate synthase (EC 2.1.1.45).

Fig. 1 Purine and pyrimidine nucleotide metabolic pathways. In both pathways, red arrows indicate 5′-nucleotidase activity, which act on nucleoside monophosphates generated from the *de novo* pathway (shown in blue) and salvage pathways that originate from RNA, DNA and nucleotide sources. The activity of 5′-nucleotidases produces nucleosides, which are the substrates of nucleoside phosphorylase or hydrolase activities (1) that produce nucleobases. Nucleobases can exit the cell or be converted back to nucleoside monophosphates by phosphoribosyltransferases (2). Similarly, nucleosides can be phosphorylated to form nucleoside monophosphates by nucleoside kinases and/or by nucleoside phosphotransferases (3). (A) The *de novo* pathway for purine biosynthesis includes 11 enzymatic steps represented as blue arrows. The terminal step, which leads to the formation of IMP, is catalyzed by IMP synthase. IMP is then converted to AMP in two steps, catalyzed by adenylosuccinate synthetase (4) and adenylosuccinate lyase (5). These two reactions can be reversed by AMP deaminase (6) activity. Similarly, Ado can be converted to Ino in one step by adenosine deaminase (7). AMP is then a substrate of nucleoside monophosphate kinase (8) and nucleoside diphosphate kinase (9) to form ADP and ATP, respectively. ADP is a substrate of ribonucleotide reductase (10), which produces dADP, which can be phosphorylated to dATP (9). The production of GMP proceeds through XMP as catalyzed by IMP dehydrogenase (11) and GMP synthetase (12). Like AMP, GMP is a substrate of nucleoside monophosphate kinase (8). GDP can be converted to dGDP by ribonucleotide reductase (10), both of which are substrates of nucleoside diphosphokinase (9). Salvage pathways can operate on nucleoside triphosphates or from nucleoside monophosphates from degraded nucleic acids. Free nucleoside tri- and diphosphates are acted upon by various types of phosphodiesterases (13) to produce nucleoside monophosphates, which are potential substrate of the 5′-nucleotidase enzymes (shown in red) and to make nucleosides or nucleobases, which can exit the cell. (B) The *de novo* pyrimidine biosynthetic pathway consists of six steps represented by blue arrows. The final step produces UMP, which gives rise to all other pyrimidine nucleotides. First, UMP is phosphorylated to UDP by nucleoside monophosphate kinase (8), which is phosphorylated again by nucleoside diphosphate kinase (9) to form UTP. UTP is a substrate of CTP synthetase (14), forming CTP. CTP can be dephosphorylated to CDP by a phosphodiesterase activity (13). CDP can be reduced to dCDP by ribonucleotide reductase (10) and dephosphorylated to dCMP by nucleoside phosphoesterase activity (13). dCMP can be converted to dUMP *via* dCMP deaminase (15) and subsequently converted to dTMP by thymidylate synthase (16). dTDP is then formed by a nucleoside monophosphate kinase (8) and dTTP formed by a nucleoside diphosphate kinase (9).

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nucleobases by nucleoside hydrolase and/or phosphorylase activities depending on the organism.

Nucleotide generation from salvaged nucleobases or nucleosides occurs in single enzymatic steps. Nucleobases are converted to nucleotides *via* phosphoribosylation, and nucleosides are phosphorylated by nucleoside kinases (Fig. 1). They are then maintained in cells.[4–6] Nucleoside monophosphates are phosphorylated to nucleoside diphosphates by nucleoside monophosphate kinases.[7] Likewise, nucleoside diphosphates are phosphorylated to triphosphate forms that are used in RNA synthesis and NTP-dependent processes.[8] Deoxyribonucleotides are formed by reduction of ribonucleoside diphosphates by ribonucleotide reductase.[9] Deoxynucleoside diphosphates are subsequently phosphorylated to deoxynucleoside triphosphates, which can be incorporated into DNA. Activity of salvage pathway enzymes is maintained throughout the cell cycle to provide dNTPs for DNA repair and mitochondrial DNA synthesis, whereas enzymes of the *de novo* pathway peak during S-phase to provide substrates for nuclear DNA replication[10] (Fig. 1).

De novo synthesis of purines (Fig. 1A—shown as blue arrows) begins with 5-phosphoribosyl-1-pyrophosphate (PRPP) and proceeds through 11 enzymatic steps to the formation of inosine monophosphate (IMP), the precursor of all purine nucleotides. IMP is then converted to AMP in two steps catalyzed by adenylosuccinate synthetase and adenylosuccinate lyase. The other arm of the purine biosynthetic pathway produces GMP from IMP. This conversion also takes place *via* two enzymatic reactions catalyzed by IMP dehydrogenase and GMP synthetase. In a similar manner, *de novo* synthesis of all pyrimidines proceeds through the formation of uridine monophosphate (UMP) (Fig. 1B). *De novo* synthesis of UMP begins with carbamoyl phosphate and proceeds through five enzymatic steps (shown as blue arrows). Once UMP is formed, it is phosphorylated to UTP by nucleotide kinases. UTP is then converted to CTP by CTP synthetase. CTP is subsequently dephosphorylated to CDP by a phosphodiesterase, which is reduced to dCDP by ribonucleotide reductase. dCMP is then converted to dUMP *via* dCMP deaminase and subsequently to dTMP by thymidylate synthase. dTMP is then phosphorylated by nucleotide kinases. In this way, cells can produce all of the building blocks for DNA and RNA synthesis.

Salvage and *de novo* synthesis converge at the formation of ribonucleoside monophosphates, all of which are potential substrates of 5′-nucleotidases. 5′-Nucleotidases catalyze the reverse reactions of nucleoside kinases, directly opposing the activity of the salvage pathway and nucleotides generated *de novo*. These opposing reactions, termed substrate cycles, exist for almost all ribonucleoside–ribonucleotide pairs, and the relative activity of phosphorylation *versus* dephosphorylation dictates whether nucleoside monophosphates remain in cells and go on to form triphosphates or become dephosphorylated to nucleosides, which can exit the cell.[11] Experiments using radiolabeled nucleosides show that when nucleoside monophosphate levels in cells are high, nucleosides are directed out of the cell, and when nucleoside monophosphate levels are low, nucleosides are brought into cells to serve as substrates for nucleoside kinases,[11] suggesting that 5′-nucleotidase activities are regulated according to the nucleotide and nucleoside content of cells.

Ecto 5′-nucleotidases in adenosine nucleotide and NAD^+ metabolism

The plasma membrane-bound form of 5′-nucleotidase with an outward facing active site is termed ecto-5′-nucleotidase. Human ecto-5′-nucleotidase, encoded by the NT5E gene at 6q14–q21,[12] is referred to by its Cluster of Differentiation (CD) number, CD73, because of its identification as a lymphocyte surface antigen. The enzyme is a 71 kDa polypeptide, which is active on the cell surface as a homodimer.[13] The mature form of the enzyme is processed extensively, including addition of a glycosyl phosphatidylinositol anchor that attaches the polypeptide to the plasma membrane.[13–15] CD73 enzymatic activity is implicated in two major extracellular metabolic pathways with distinct physiological roles, extracellular adenosine nucleotide (Fig. 2A) and NAD^+ metabolism (Fig. 2B).

CD73 belongs to a conserved superfamily of metallophosphodiesterases. Enzymes in this family catalyze hydrolysis of phosphate from diverse substrates including polypeptides, sphingomyelin, RNA, DNA and nucleotides.[16] Structural examination of the related *E. coli* ecto 5′-nucleotidase reveals that the enzyme consists of two domains, an N-terminal domain, which binds two metal ions and contains an aspartic acid–histidine motif that is important for catalysis, and a C-terminal domain, which binds the nucleotide substrate. The active site resides between the two domains. Comparison of structures of the empty enzyme and bound to substrate indicates that catalysis requires the C-terminal domain to rotate around the central axis.[17,18] Structures of enzyme bound to an inhibitor, α,β-methylene-ADP, show strong interaction between two phenylalanine residues and the adenine ring in the substrate binding pocket, likely explaining ecto 5′-nucleotidase substrate preference for adenine nucleotides. Indeed, enzymatic measurements indicate that 5′-AMP is the preferred substrate of CD73, and ribonucleotides are preferred over deoxyribonucleotides.[19–22]

Monoclonal antibodies and quantitative real time-PCR have been used to determine CD73 distribution. It is widely expressed in vertebrate tissues and is prominently expressed on B and T cells and certain types of dendritic cells. It shows variable expression among cell types within other tissues including skeletal muscle, thymus, spleen, kidney and forestomach.[23] Because protein abundance is directly correlated with ecto 5′-nucleotidase transcript levels in the murine system, it is thought to be principally regulated at the mRNA level, mediated by both positive and negative *cis*-regulatory elements.[23,24] CD73 is highly induced by ischemia and hypoxia, and increased expression in cardiac ischemia is mediated by a positive feedback loop involving adenosine.[25,26] Though CD73 is mainly regulated transcriptionally, cross linking of CD38, a cell surface glycoprotein with cyclic ADPribose hydrolase activity, causes upregulation of CD73 *via* export of protein to the cell surface that is independent of new transcription.[27]

The major physiological roles for CD73 in extracellular adenosine metabolism are several-fold. Hydrolysis of AMP

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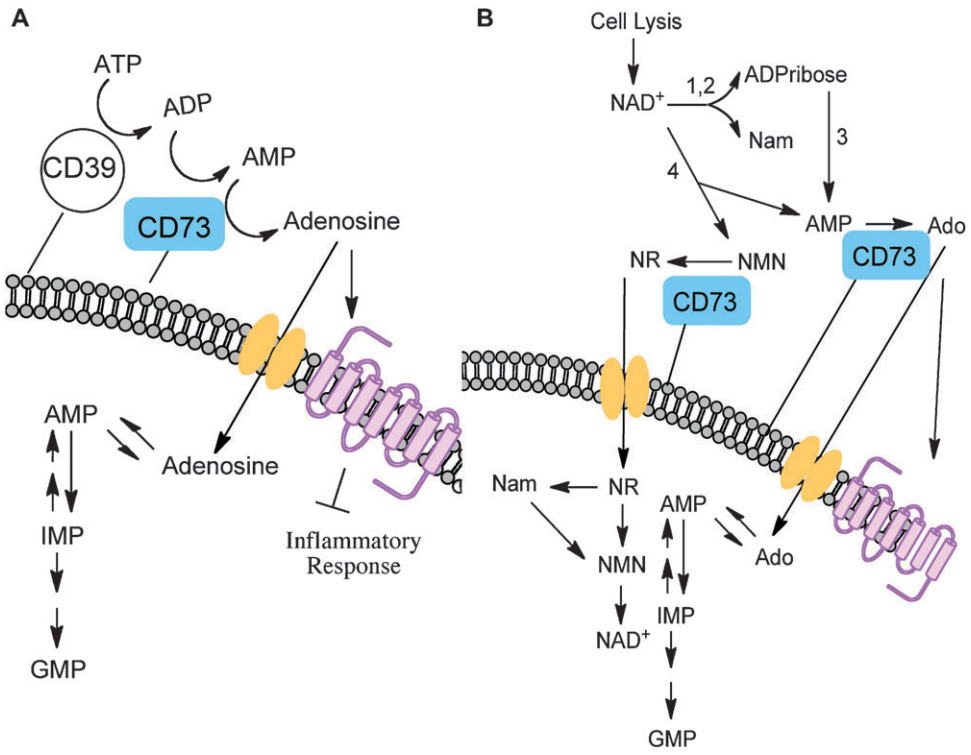

Fig. 2 Extracellular 5′-nucleotidase activities (A) CD73 is a critical mediator of extracellular adenosine metabolism. Extracellular ATP and ADP are substrates of the ecto enzyme CD39, an ATP/ADPase. These reactions produce AMP, which is a substrate of ecto 5′-nucleotidase, CD73. Extracellularly produced adenosine can enter the cell through nucleoside transporters, and enter intracellular adenosine salvage pathways (shown in Fig. 1) or interact with adenosine receptors on the cell surface to dampen the inflammatory response. (B) Extracellular NAD^+, which is generated by cell lysis or other mechanisms, is broken into ADPribose and Nam moieties by NAD^+-glycohydrolases (1) and ADPribosyltransferases (ARTs) (2). ADPribose can be further split to form AMP by ADP-ribose pyrophosphatase (3), which can be subsequently converted to adenosine by ecto-5′-nucleotidase CD73. NAD^+ can also be broken down by NAD^+ diphosphatase (4) to produce NMN and AMP. The AMP goes on to produce adenosine and the NMN can be further broken down to nicotinamide riboside (NR). Both of these reactions are catalyzed by CD73. NR can enter cells through a nucleoside transport system, where it can participate in intracellular NAD^+ biogenesis.

to adenosine forms the extracellular arm of purine salvage such that CD73-produced adenosine can cross the cell membrane *via* nucleoside transporters and be used as a substrate for purine nucleotide production in the cell (Fig. 2A). This activity supplies cells with precursors for energy metabolism and nucleic acid biosynthesis. Extracellular adenosine can also interact with G-protein coupled adenosine receptors, A1, A2a, A2b, and A3, which control diverse physiological responses in the cell including immune function, heart pace, neurotransmission, smooth muscle vasodilatation, platelet aggregation, superoxide anion generation and lipolysis.[28,29] One of the most profound activities of CD73 is the interaction with the P1 purinergic receptor (Fig. 2A) that mitigates the inflammatory response stimulated by stress-induced increased extracellular AMP.[26] Thus, the function of CD73-mediated production of adenosine from AMP is protective against hypoxia and ischemia.[30] Indeed, adenosine produced by CD73 in cardiac tissue leads to coronary vasodilation and correction of the ATP, ADP, AMP, adenosine imbalance that occurs during ischemia and hypoxia.[31,32] Several experimental systems have shown a protective benefit of increased CD73 activity in ischemia.[33] Notably, myocardial infarct due to ischemia is exacerbated when ecto 5′-nucleotidase activity is chemically inhibited or genetically deleted from mouse models. Accordingly, 5′-nucleotidase activators are being considered as therapy for myocardial ischemia.[34] Phenotyping of the cd73-/- mouse in the absence of induced cardiac stress reveals decreased platelet activation and increased leukocyte adhesion to the vascular endothelia.[35]

One of the other major roles of CD73 is in extracellular NAD^+ metabolism (Fig. 2B). Extracellular NAD^+ is degraded by NAD^+-glycohydrolases and ADPribosyltransferases,[36–39] which cleave NAD^+ at the glycosidic ADPribose–nicotinamide linkage to produce free or protein bound ADPribose plus nicotinamide (Nam). Extracellular pyrophosphatases split NAD^+ to AMP and nicotinamide mononucleotide (NMN),[40,41] producing substrates for extracellular 5′-nucleotidase CD73. AMP, cleaved by CD73, produces extracellular adenosine, which either can interact with adenosine receptors or be taken up by cells as shown in Fig. 2A. Earlier, on the basis of sequence similarity between CD73 and the *Haemophilus influenza* NMN 5′-nucleotidase, NadN, we suggested that CD73 also dephosphorylates NMN to nicotinamide riboside for cellular uptake.[42] Both the adenosine and NAD^+-specific activities appear to be important for immune cell function.

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In particular stimulation of human leukocytes is accompanied by increased expression of extracellular NAD^+ metabolic activities, indicating that recycling extracellular NAD^+ equivalents may be used by immune cells to support activation.[43] The extracellular source of NAD^+ and its metabolites remain a subject of interest.

Single celled organisms have also developed mechanisms to recycle extracellular nucleotides. Three gene products from budding yeast have recently been implicated as extracellular nucleotidases (Fig. 4). Npp1 and Npp2 are members of an alkaline phosphatase superfamily that also includes mammalian extracellular 5′-nucleotidases with ATPase and ADPase activity.[44] In addition, Pho5 has recently been implicated in the breakdown of extracellular nicotinamide mononucleotide (NMN) to nicotinamide riboside (NR) for use as an intracellular NAD^+ precursor.[44,45] Extracellular 5′-nucleotidase activity is essential for a number of bacterial species as well. *H. influenza*, a pathogenic bacterium, requires NAD^+ or the precursors NMN or NR to grow in the absence of blood. Due to lack of genes encoding niacin salvage activities, this bacterium cannot utilize either nicotinic acid (NA) or its amide Nam.[46–50] Transport studies indicate that NR is the primary precursor taken up, making the 5′-nucleotidase activity of NadN essential for viability on medium containing NAD^+ or NMN.[51] More recent data indicate that NadN has both NAD^+ pyrophosphatase activity and NMN 5′-nucleotidase activity.[50] However, in a rat infection model, NadN is dispensable, suggesting that NR is a circulating metabolite in vertebrates.[52]

Cytosolic 5′-nucleotidases

Soluble cytosolic 5′-nucleotidases belong to the haloacid dehalogenase (HAD) enzyme superfamily, which includes a diverse group of phosphotransferases. Though HAD reactions can proceed through multiple mechanisms, the active sites in the HAD superfamily generally contain a conserved aspartic acid residue, a lysine, and a nucleophile (usually aspartic acid or serine).[53,54] From the crystal structures of human mitochondrial deoxyribonucleotidase, three conserved sequence motifs were identified that contain critical catalytic residues, which are highly conserved among species. The first Asp in Motif I, DXDX[T/V][L/V/I], forms a phosphorylated enzyme intermediate followed by release of the phosphate to water.[55,56]

Human cytosolic 5′-nucleotidase II (cNII) is a HAD family enzyme encoded by the NT5C2 gene on chromosome 10q24.32. First purified from chicken liver, cNII shows considerable activity on purine substrates dIMP, IMP, dGMP, GMP and XMP.[57–61] Gel filtration experiments and structural analysis indicate that cNII is a tetramer, formed from two homodimers.[58,62–64] Elimination of a stretch of 13 acidic residues abolished these interactions, making the enzyme a monomer with diminished expression level and decreased enzyme activity. Though cNII is ubiquitously expressed among cell types, it is subject to complex allosteric regulation. It is activated by dATP, ATP 2,3-bisphosphoglycerate and diadenosine tetraphosphate.[58,64,65] Structural analysis has revealed possible mechanisms by which these molecules alter enzymatic activity. Modeling diadenosine tetraphosphate at the dimer interface indicates that it may increase binding and dimer formation. 2,3-Bisphosphoglycerate likely works in the same manner, complementing subunit contacts at the dimer interface.[62] Though there are no structures of cNII bound to substrates, comparison with structural data from mitochondrial specific 5′-nucleotidase indicates that residues Arg^{202} and Asn^{158} likely determine the preference for purine nucleotides.[62]

Deficiencies in human pyrimidine 5′-nucleotidase, cNIII, have been identified as one of the major causes of non-spherocytic hemolytic anemia, a condition characterized by low red blood cell counts and hemoglobin content, caused by increased red cell lysis.[66] Fourteen different mutations have been identified in human patients.[67] Such individuals have aberrantly high pyrimidine nucleotide levels in red blood cells.[68] Studies of mutant alleles found in patients indicate that loss of pyrimidine 5′-nucleotidase enzymatic activity leads to disease. Biochemical characterization of the recombinant wild-type cNIII enzyme and disease-associated mutant forms (D87V, L131P, N179S and G230R) showed reduced catalytic activity and thermostability of mutant polypeptides.[69] Together, these data indicate that a loss of 5′-nucleotidase enzyme activity, which controls pyrimidine nucleotide levels in red blood cells, causes increased red blood cell lysis that leads to haemolytic anemia.

Unbalanced nucleotide levels underlie various types of immunodeficiency and vascular disease.[56,70] Patients with adenosine deaminase deficiency have severe immunodeficiency,[71] excessive dATP in lymphocytes and dysregulation of ribonucleotide reductase, which leads to an unbalanced supply of the four deoxyribonucleotides.[72] Mouse models of the same deficiency show abnormal purine deoxynucleoside metabolism and early death *via* liver and intestinal failure.[73,74] Human patients that lack purine nucleoside phosphorylase have T-cell deficiencies, and mouse knockout models show severe immunodeficiency, apparently caused by accumulated dGTP in the mitochondria, which leads to aberrant mitochondrial DNA repair.[71,75] These data underscore the need for tight regulation of 5′-nucleotidase enzymes inside the cell and may help explain the complex allosteric regulation shown by cNII.

Regulation of 5′-nucleotidase activity is of considerable interest for the treatment of patients with nucleoside analog chemotherapeutic prodrugs. Human cytosolic 5′-nucleotidase cN-II has been implicated in drug resistance in patients and has been shown to dephosphorylate 5-fluorodUMP, 3′-azido-3-dTMP, 2′-chloro-2′-deoxyAMP, and related compounds, which represent the monophosphorylated forms of nucleoside prodrugs.[76] Thus, biological and clinical data indicate that 5′-nucleotidase inhibitors may improve clinical outcomes when used in concert with nucleoside analog prodrugs.[76–79]

Fungal-specific 5′-nucleotidase

Though no orthologs of human 5′-nucleotidases are evident, three polypeptides exist in budding yeast that contain conserved catalytic motifs and activities of 5′-nucleotidase. Isn1 and Sdt1 have been characterized biochemically.[80,81] Native Isn1 purified from yeast catalyzes dephosphorylation of IMP with a specific activity of 16 600 μmol min^{-1} mg^{-1}, whereas recombinant Sdt1 hydrolyzes 5′-UMP and 5′-CMP with

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K_M values of 1.2 and 2.3 mM and V_{max} values of 23 and 20 μmol^{-1} min^{-1} mg^{-1}, respectively.[80,81] Biochemical data indicate that Sdt1 is highly specific for 5′-UMP and 5′-CMP, and shows negligible activity on purine nucleoside monophosphates, 3′-monophosphates or nucleoside triphosphates.[81] *In vivo* data indicate that ISN1 is responsible for virtually all IMP 5′-nucleotidase activity in budding yeast cells and is required for cells to release inosine into the culture medium.[82] In addition, overproduction of SDT1 has been shown to protect against the toxicity of pyrimidine base analogs 6-azauracil, 5-fluorouracil and 5-fluorocytosine.[81]

Computer modeling of the Isn1 polypeptide reveals that it is a member of the HAD superfamily with low primary sequence similarity to other members.[83] Analysis of the Sdt1 polypeptide sequence, also a member of the HAD superfamily, indicates ~50% similarity with *Saccharomyces cerevisiae* Phm8, a protein with apparent phosphatase activity for lysophosphatidic acid.[84] In contrast to ISN1, there are SDT1-related genes in *Schizosaccharomyces pombe*, (SPAC24B11), and *Arabidopsis thaliana* (GenBank AC006223).

Recently, we discovered that Sdt1, Phm8 and Isn1 possess NMN 5′-nucleotidase activity *in vitro*. Genetic and metabolomic data indicate that Sdt1 and Isn1 account for dephosphorylation of pyridine nucleotides, nicotinic acid mononucleotide (NaMN) and NMN to nicotinic acid riboside (NAR) and NR, *in vivo*, thereby constituting a novel NAD$^+$ catabolic pathway in yeast[85,86] (Fig. 3). Like other 5′-nucleotidases, these enzymes are highly regulated according to the nutritional state of the cell. In particular, Isn1 protein levels are positively correlated with provision of nicotinic acid and glucose in the growth medium, and overexpression of SDT1 causes toxicity and drives down NAD$^+$ levels.[85]

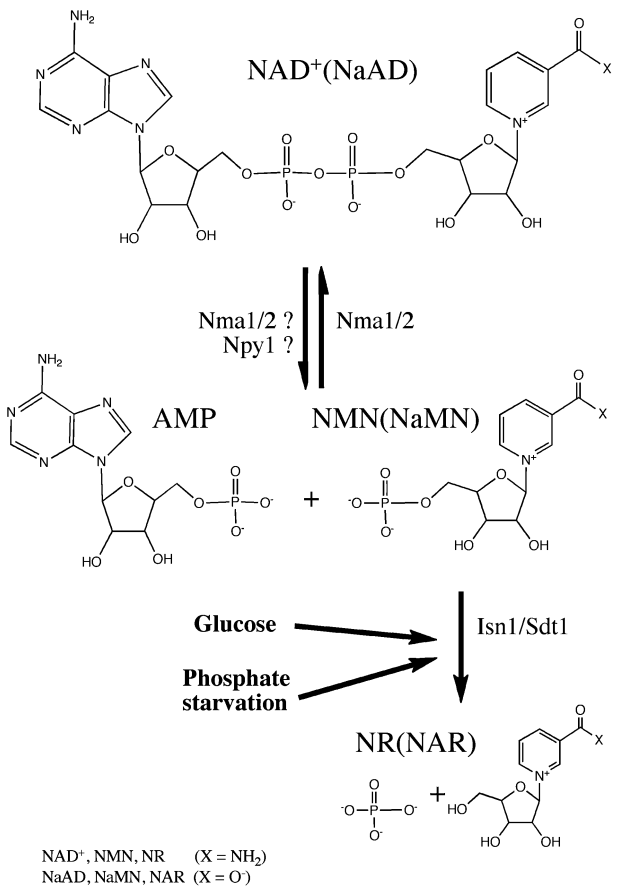

Fig. 3 Fungal 5′-nucleotidase activity in NAD$^+$ metabolism. Though Sdt1 was originally described as a UMP/CMP-specific 5′-nucleotidase and Isn1 was originally described as an IMP-specific 5′-nucleotidase, recent data indicate that Isn1 and Sdt1 are dually responsible for NMN/NaMN conversion to NR/NAR. See the text for a discussion of the possible bases for cellular NaMN and NMN accumulation.

In the course of identifying NaMN and NMN 5′-nucleotidases, we determined that intracellular levels of NaMN and NMN are near 70 and 90 μM, respectively, in standard synthetic yeast growth conditions.[85] Whether there is a specific source of such pyridine mononucleotides is not known (Fig. 3). According to the simplest possibility, NMN and NaMN may accumulate due to the limited forward rates of NaMN and NMN adenylylation. Second, Npy1, a reported NADH/NAD$^+$ pyrophosphatase, might produce NMN and NaMN from NAD$^+$ and NaAD, respectively.[87] Third, if there is sufficient cellular pyrophosphate, NMN and NaMN may be generated by reversible adenylylation reactions from NAD$^+$ and NaAD. Though *in vitro* reversibility is well known,[88] current dogma suggests that abundant pyrophosphatase activities render all pyrophosphate-producing reactions irreversible. However, accumulated pyrophosphate levels in yeast have been reported for more than 60 years,[89] and recent measurements have shown that pyrophosphate concentration can exceed ATP concentration under certain conditions.[90,91] These findings suggest that pyrophosphate is sufficiently available to render NaMN and NMN adenylyltransferase a reversible process.

Inducible 5′-nucleotidases in the phosphate starvation response

The observation that phosphate starvation decreases nucleotide concentration in cells suggests positive regulation of 5′-nucleotidases in low phosphate conditions.[92] Indeed, studies in multiple model organisms indicate that phosphate starvation induces genes encoding phosphate-liberating enzymes. In the cells of the plant *Catharanthus roseus*, both purine and pyrimidine nucleotide levels correlate directly with availability of phosphate in the culture medium, reportedly due to a several fold increase in *de novo* synthesis in medium containing phosphate.[93] Likewise, the purple acid phosphatases of *A. thaliana* are increased transcriptionally by phosphate deprivation,[94] and both tomato plants and suspension culture cells show phosphate starvation-inducible secretion of acid phosphatase.[95] Suspension cell cultures of the mustard plant contain two major inducible acid phosphatase isozymes: vacuolar acid phosphatase and cell wall-nonspecific acid phosphatase, both of which are increased following phosphate deprivation, suggesting a common regulatory mechanism.[96] Microarray studies of several model plants indicate induction of RNAses and phosphatases, which is followed by an increase in hydrolysis of nucleic acids and nucleotides, as a common mechanism in the response to phosphate-starvation.[97–100]

In addition to plants, phosphate-regulated 5′-nucleotidase activity appears to be conserved in some bacteria and yeast. In

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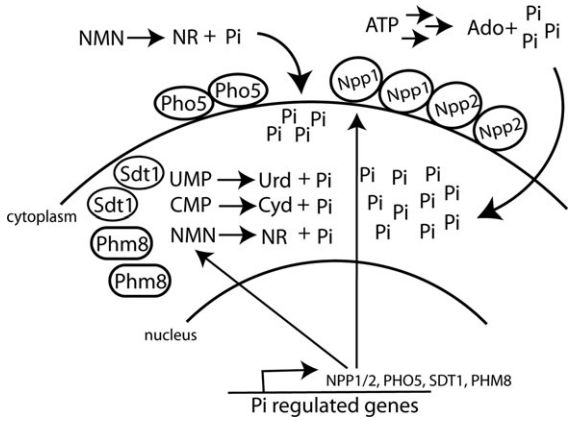

Fig. 4 Phosphate starvation inducible 5′-nucleotidase activities. There is a system, conserved between fungi, plants and fish that activates 5′-nucleotidase activities in response to phosphate starvation. Phosphate starvation induces 5′-nucleotidases such as Npp1, Npp2, Pho5, Sdt1 and Phm8 to mobilized inorganic phosphate for cellular survival.

the bacterium *Corynebacterium glutamicum*, the extracellular 5′-nucleotidase UshA is induced when phosphate is limiting, and is required for growth when nucleotides are provided as the sole source of phosphate, a condition that is likely relevant to bacterial growth in the wild.[101] In budding yeast, nucleotide hydrolysis by Npp1, Npp2 and Pho5 is induced in phosphate limiting conditions[44] (Fig. 4). Moreover, Sdt1 and Phm8 are induced at the transcript level by phosphate starvation (Fig. 4—unpublished data).

Studies using radiolabeled phosphate reveal that although nucleotides are one of the first phosphate sources to be utilized when inorganic sources are depleted, they are also the first to be re-synthesized when inorganic phosphate is made available,[92] indicating a likely role for nucleotide synthesis in recovery from phosphate starvation. Measurements of cellular RNA, protein and nucleotide levels, following phosphate starvation, indicate that each macromolecule is depleted due to phosphate deprivation, suggesting nutrient remobilization as the major cellular response to phosphate starvation. Ribonucleases are considered to play an important role in the remobilization process. Indeed, RNase is highly induced during starvation for phosphate, which is not seen in starvation for the other major macronutrients.[102] Combined with the induced activities of acid phosphatase and 5′- and 3′-nucleotidase activities,[103] this response is expected to deplete intracellular phosphorylated macromolecules in order to provide enough inorganic phosphate to survive an extended period of phosphate starvation. Analysis of phosphate-starved *C. roseus* cells indicates that a conserved mechanism governs biosynthesis of adenosine nucleotides including NAD^+. When cells were resupplied with phosphate after a period of starvation, nicotinate phosphoribosyltransferase and nicotinamidase, the enzymes required for assimilation of NA and Nam into NAD^+, were increased.[92]

Conclusions

5′-Nucleotidases are key enzymes for redistribution of nucleotides and are necessary for reversible transit of nucleosides and nucleobases across cell membranes. Maintaining proper nucleotide ratios is critical for maintaining accuracy in DNA replication and repair. In addition, alterations in 5′-nucleotidase activities have implications in spherocytic hemolytic anemia, immunodeficiency and the efficacy of nucleoside analog chemotherapies.

Arguably, the most novel aspect of 5′-nucleotidase activity is in regulation of NAD^+ metabolism and phosphate acquisition. Regulation of enzyme abundance by vitamins, glucose and phosphate clearly indicates that 5′-nucleotidases are at the crux of multiple regulatory pathways. Because 5′-nucleotidases have relatively broad specificities, metabolic approaches will be necessary to define the sets of compounds from which phosphate is mobilized under starvation and resupply conditions. Future work will define the roles of specific 5′-nucleotidases in specific tissues in the intertwined economies of nucleosides and phosphate in varying conditions.

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