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REVIEW

The GDA1_CD39 superfamily: NTPDases with diverse functions

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Abstract The first comprehensive review of the ubiquitous "ecto-ATPases" by Plesner was published in 1995. A year later, a lymphoid cell activation antigen, CD39, that had been cloned previously, was shown to be an ecto-ATPase. A family of proteins, related to CD39 and a yeast GDPase, all containing the canonical apyrase conserved regions in their polypeptides, soon started to expand. They are now recognized as members of the GDA1_CD39 protein family. Because proteins in this family hydrolyze nucleoside triphosphates and diphosphates, a unifying nomenclature, nucleoside triphosphate diphopshohydrolases (NTPDases), was established in 2000. Membrane-bound NTPDases are either located on the cell surface or membranes of intracellular organelles. Soluble NTPDases exist in the cytosol and may be secreted. In the last 15 years, molecular cloning and functional expression have facilitated biochemical characterization of NTPDases of many organisms, culminating in the recent structural determination of the ecto-domain of a mammalian cell surface NTPDase and a bacterial NTPDase. The first goal of this review is to summarize the biochemical, mutagenesis, and structural studies of the NTPDases. Because of their ability in hydrolyzing extracellular nucleotides, the mammalian cell surface NTPDases (the ecto-NTPDases) which regulate purinergic signaling have received the most attention. Less appreciated are the functions of intracellular NTPDases and NTPDases of other organisms, e.g., bacteria, parasites, *Drosophila,* plants, etc. The second goal of this review is to summarize recent findings which demonstrate the involvement of the NTPDases in multiple and diverse physiological processes: pathogen-host interaction, plant growth, eukaryote cell protein and lipid glycosylation, eye development, and oncogenesis.

Keywords NTPDases · Ecto-ATPases · ATP diphosphohydrolases · Apyrases · Purinergic signaling · Protein glycosylation · Plant growth · Pathogen virulence · Oncogenesis

Abbreviations

NTPDase	Nucleoside triphosphate diphosphohydrolase
ATPDase	ATP diphosphohydrolase
ACR	Apyrase conserved regions
ConA	Concanavalin A
TM	Transmembrane
TMD	Transmembrane domains
ECD	Extracellular domain

Introduction

The GDA1_CD39 superfamily is comprised of nucleoside triphosphate diphosphohydolases (NTPDases) with common motifs in their protein sequences. The family is named after two proteins: the yeast GDPase (GDA1) and a lymphoid cell activation antigen, CD39. The NTPDases that have received the most attention are the ecto-NTPDases, present on the cell surface and involved in purinergic signaling. The importance of extracellular ATP in signaling, proposed by Burnstock in 1972 [1] and validated by numerous studies since, is now well established. At current count, there are close to 4,000 papers and reviews on this subject collected in PubMed. The action of

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extracellular ATP is mediated by the P2X and P2Y purinergic receptors [2, 3]. While hydrolysis of extracellular ATP terminates the action of ATP, it also generates ADP and adenosine, the former activates certain members of the P2Y receptors and the latter is a ligand of the P1 purinergic receptors. Hydrolysis of extracellular ATP to adenosine is catalyzed by several types of ectoenzymes, including the ecto-NTPDases, the ecto-phosphodiesterases/pyrophosphatases, ecto-5′-nucleotidase and alkaline phosphatases. The participation of multiple ecto-nucleotidases in the hydrolysis of extracellular ATP and other nucleotides has been extensively discussed in several excellent reviews [4–8]. Because of their great catalytic activity, the ecto-NTPDases play a more important role in the hydrolysis of extracellular ATP than the other ecto-nucleotidases. A 2006 review focused on the mammalian ecto-NTPDases and provided a concise summary of their molecular properties, tissue distribution as determined by immunolocalization, and studies related to their physiological functions [9].

Ecto-NTPDases in mammalian tissues represent only part of the NTPDase family. There is also a large number of intracellular NTPDases, whose functions are unrelated to purinergic signaling. One of these, an NTPDase5, has been shown to be involved in neoplastic transformation when altered. Plant NTPDases are involved in nodulation and growth. Some NTPDases in parasitic worms and protozoa are necessary for their virulence. The first prokaryotic NTPDase was identified recently and its 3D structure solved in 2010. More exciting discoveries are surely to be made in the coming years. It seems an opportune time to review recent development of this interesting protein family with unexpectedly diverse functions. Findings on NTPDases of both vertebrates and other organisms, with emphasis on their biochemical properties and functions, will be summarized.

Historical perspectives—landmarks

The presence of an ATPase activity on the cell surface of several types of mammalian cells was reported in the 1940s to 1950s [see review in ref. 10]. This activity was named "ecto-ATPase" by Engelhardt in 1957 [11]. A glutaraldehyde-resistant ATPase activity in the plasma membranes of rat liver and hepatomas was subsequently demonstrated by Novikoff et al. [12] in 1958. However, nobody set out to study this enzyme for the next 20 years. My mentor, Efraim Racker, considered the ecto-ATPase to be a "nuisance" as its ATPase activity overwhelmed and masked the Na^+,K^+-ATPase, one of the ion pump ATPases that we were studying in the lab. The unwelcome but persistent presence of the ecto-ATPase activity in membrane preparations explained why many papers on ecto-ATPases published in 1970s to 1980s were contributed by investigators whose main interests were the plasma membrane ion-transporting ATPases. Even Racker was ensnared and published a paper on a viral ecto-ATPase, which originated from the host cell membrane [13]. The seeming lack of a reason for the existence of an enzyme that hydrolyzes extracellular ATP further discouraged a systematic study of this activity. Nevertheless, a substantial literature began to accumulate, most of which described an ATPase activity with characteristics that distinguishes it from the membrane bound F-, P-, and V-type ATPases: (1) hydrolysis of NTPs other than ATP, sometimes also NDPs, in the presence of either Mg^{2+} or Ca^{2+}, and (2) insensitivity to the specific inhibitors of the F-, P-, and V-type ATPases and alkaline phosphatase. However, experimental evidence soon suggested that there must be more than one form of the "ecto-ATPase". Unfortunately, most attempts in purifying the ecto-ATPase(s) met with failure because, unlike the other membrane ATPases, ecto-ATPases are notoriously sensitive to detergent inactivation. The only successful purification of such an enzyme was that of a 67 kDa glycoprotein from rabbit transverse tubule reported by Trueheit and Kirley [14] in 1992. This state of affairs served as the backdrop for the first comprehensive review on ecto-ATPase by Liselotte Plesner [10], who coined "E-type ATPases" as an inclusive term for ATPases with the biochemical characteristics described above, but have been variously called ecto-ATPases [15], ATP-diphosphohydrolases (ATPDases) [16], apyrases [16, 17], and NTPases [18]. The presence of the E-type ATPases in many organisms was recognized in the reviews of both Plesner [10] and Beaudoin et al. [16]. Simultaneously, efforts were made to bring together researchers in the field. The First International Workshop on Ecto-ATPases took place in Mar del Plata, Argentina in 1996; the proceedings published a year later [19]. The small gathering of ecto-ATPase researchers at the meeting was abuzz with excitement because molecular identity of the first ecto-ATPase was revealed in a paper by Handa and Guidotti. These authors purified and cloned a soluble E-type ATPase, the potato apyrase, and observed sequence homology with several other proteins, including a yeast GDPase and human CD39 [20]. CD39, a cell surface antigen on activated B lymphocytes involved in cell adhesion [21] was cloned 2 years earlier by Maliszewski et al. [22], who first noted a sequence homology to the yeast GDPase [23], and speculated that CD39 could also be a nucleotide hydrolase. Handa and Guidotti discerned four conserved regions among these proteins, and named them apyrase conserved regions (ACR1–ACR4). Independently, Vasconcelos et al. [24] also assembled a similar set of proteins based on sequence homology and found a fifth conserved region, ACR5, near the *C* termini of these proteins. Interestingly, the ACR4 sequence corresponded to that of a chymogtryptic peptide of human placental ATPDase purified

and characteriazed by Christoforidis et al. [25] a year earlier. In retrospect, the placental ATPDase was the first mammalian CD39 to be completely purified. At about the same time, the existence of a bovine CD39 was suggested by sequence similarity of several internal peptides of a partially purified ATPDase from bovine aorta [26].

Subsequent functional expression of human CD39 cDNA demonstrated that it is indeed an ecto-ATPDase [27, 28], thus conferring on CD39, together with GDPase, the honor of the paterfamilias of the GDA1_CD39 family. Strong support for the existence of more than one *E*-type ATPase was provided by Chadwick and Frischauff [29] who, using a bioinformatics approach, found four additional human gene sequences related to CD39: human CD39L1-L4. The abundance of the transcripts of these genes in different human tissues was also determined by Northern blot analysis. Full-length cDNA of the human CD39L1 [30, 31], CD39L2 [32, 33], CD39L3 [34], and CD39L4 [35] were subsequently obtained in quick succession. Functional expression demonstrated that these cDNAs all encode *E*-type NTPDases. The CD39 proteins were renamed NTPDase1 (CD39), NTPDase2 (CD39L1), NTPDase3 (CD39L3), NTPDase5 (CD39L4), and NTPDase 6 (CD39L2) when a more unifying nomenclature, nucleoside triphosphate diphosphohydroalse (NTPDase), was agreed upon at the Second International Workshop on Ecto-ATPases and Related Ectonucleotidases [36]. A human Golgi UDPase [37] was designated as NTPDase4. However, a chicken ecto-ATPDase [38], which later was shown to be an NTPDase8, was mistakenly categorized as an NTPDase3 at the time [36, 39].

Characterization of expressed vertebrate NTPDases and functional studies

Cell Surface NTPDases As seen in the annotated sequence alignment of the human NTPDases in Fig. 1, the four cell surface NTPDases, NTPDases 1–3 and 8, have two membrane-spanning domains at their N- and C-termini. The extracellular domains contain the five apyrase conserved regions (ACRs) and ten conserved cysteine residues. In addition, they have four conserved regions (CRs), only two of which are found in the sequences of the intracellular NTPDases 4–7. NTPDases 1–3 and 8 have similar molecular sizes (~500 amino acid) but with variable extent of glycosylation. The glycosylation of these proteins is important for correct protein folding, membrane targeting, and activity [40–43].

NTPDases 1, 3, and 8 have been called ecto-ATPDases because they hydrolyze both ATP and ADP. During the hydrolysis of ATP to AMP by NTPDase1, ADP is not released as an intermediate [44, 45], while the appearance of free ADP could be demonstrated upon hydrolysis of ATP by NTPDases 3 and 8 [46]. NTPDase2 is primarily an NTPase and has been called ecto-ATPase. Several biochemical parameters of the cell surface NTPDases, including ADPase/ATPase and UDPase/UTPase ratios, pH dependence of ATP and ADP hydrolysis, Km values for ATP, ADP, UTP, and UDP, and time course of product formation were determined by Kukulski et al. [46].

Unlike the F-, P-, and V-type ATPases, which are inhibited by specific inhibitors, such as oligomycin, vanadate, ouabain, or thapsigargen, specific inhibitors have not been found for the NTPDases. The mercurials, e.g., *p*-chloromercuriphenylsulfonate, inhibit only NTPDase2 at ~0.1 mM [47–49]. Azide at high concentrations (2–10 mM) inhibits the three ecto-ATPDases, but has no effect on NTPDase2 [25, 34, 50–53]. The extent of azide inhibition of the ecto-ATPDases varies with substrates and divalent ions. Inhibition of ADP hydrolysis by azide is always greater than inhibition of ATP hydrolysis, and inhibition is usually greater with Mg-nucleotides than with Ca-nucleotides [53]. The only other commercially available NTPDase inhibitor is ARL67156 (6-*N, N*-diethyl-D-β-γ-dibromomethlene adenosine triphosphate) [54]. At 100 μM substrate and inhibitor concentrations, ARL67156 inhibited the expressed human NTPDases 1 and 3 more strongly than NTPDases 2 and 8 [55]. It should be pointed out that there are many ongoing efforts at synthesizing isoform specific NTPDase inhibitors that do not affect purinergic receptors. However, this topic is beyond the scope of this review and will not be addressed here.

While the descriptions below focus on the individual NTPDases, a large body of evidence attests to the co-existence of multiple cell surface NTPDases in the same tissue [reviewed in ref. 9]. The coordination of their activities in regulating purinergic signaling of specific physiological process is an important research goal for the future.

NTPDase1 (CD39, ectoapyrase, ecto-ATP diphosphohydrolase, ecto-ADPase) NTPDase1 from human and mouse [22], rat [56, 57], and pig [58], have been cloned and functionally expressed. Northern blot analysis indicated that its transcript is abundant in human placenta, spleen, peripheral blood leukocytes, and lung [28, 29] and rat lung [57]. Human NTPDase1 undergoes palmitoylation of a cysteine residue within its N-terminus and is targeted to the caveolae [59, 60], which should facilitate its interaction with other signaling proteins. Indeed, co-localization of NTPDase1 and the P2Y1 receptor in the caveolae in human placenta was observed [61]. Furthermore, the *N* terminus of human NTPDase1 was shown to associate with RanBPM, a Ran (a small GTPase)-binding protein, that resulted in decrease of ATPase activity [62]. However, Papanikolaou et

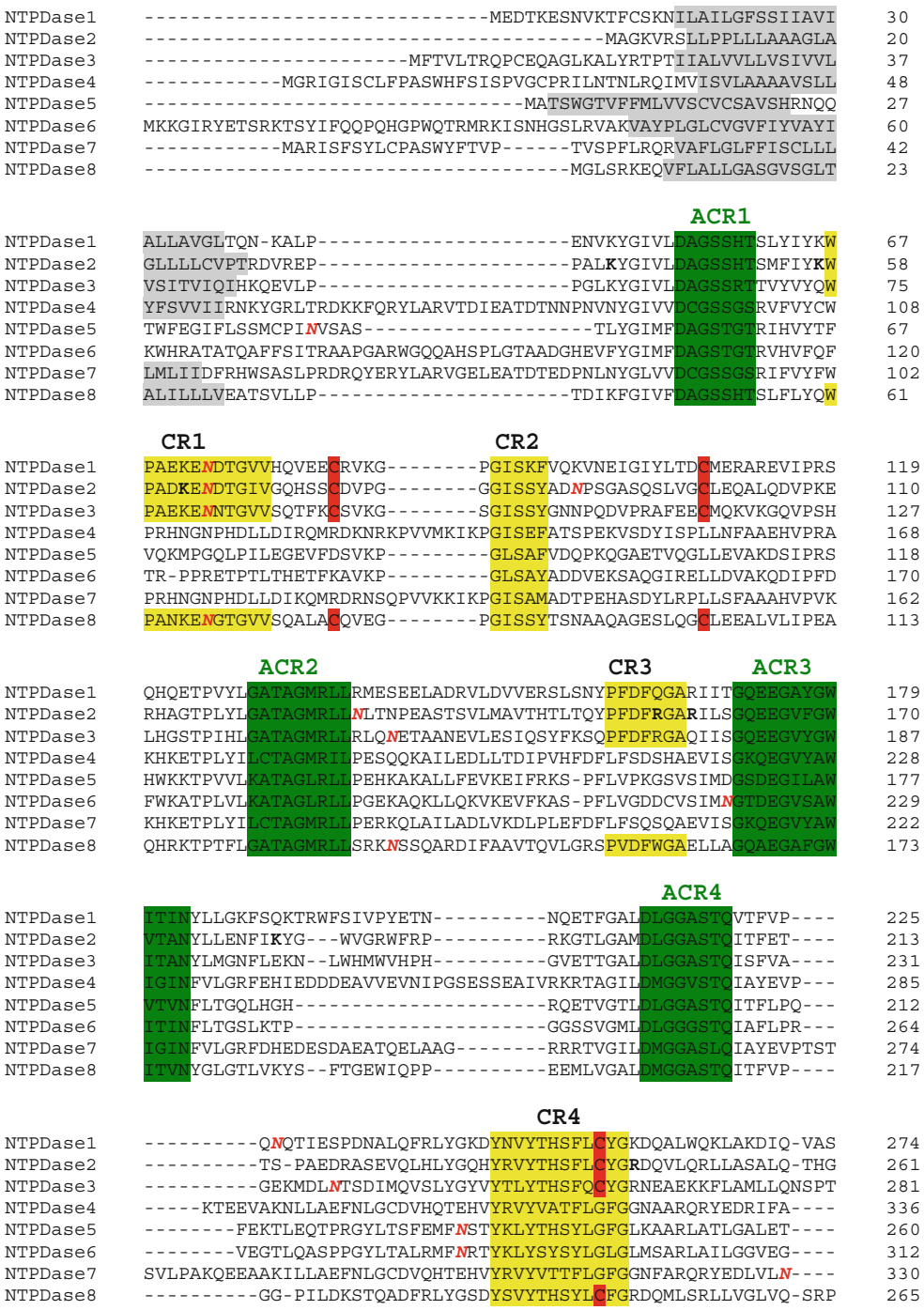
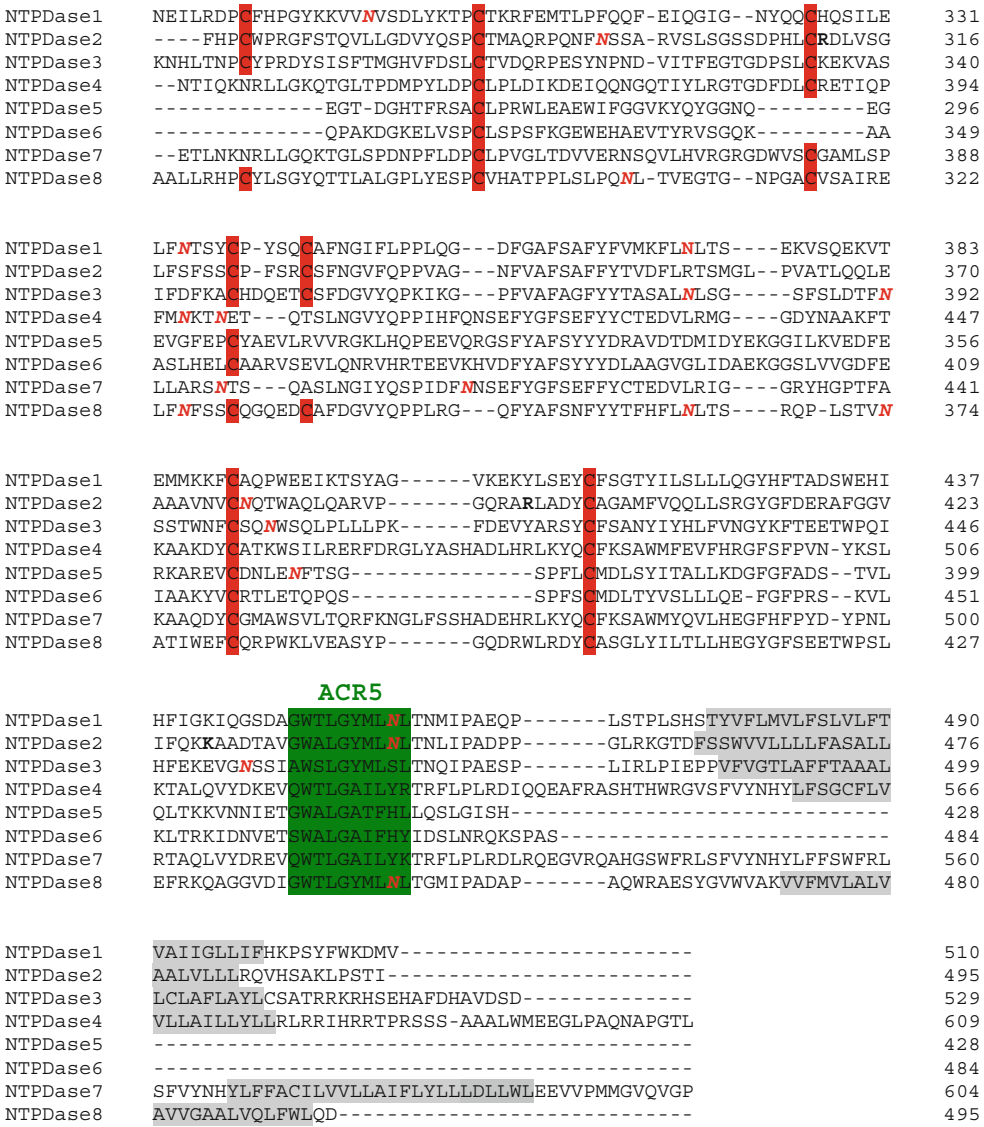


Fig. 1 Alignment of primary sequences of human NTPDases 1–8. Sequence alignment was generated using ClustalW (http://www.ebi.ac.uk/clustalw/). TMDs are shaded in *gray*. The ACRs are shaded in *green*. The conserved regions (CR) are shaded in *yellow*. The conserved cysteine residues are shaded in *red*. The asparagine residues in the putative *N*-glycosylation sites of individual NTPDase are *italicized*, *in bold*, and shown in *red*

al. [63] found that activity and targeting of NTPDase1 were not altered in caveolin-1 knockout mice whereas depletion of cholesterol decreased NTPDase1 activity. These results suggested that NTPDase1 is localized in lipid domains that are enriched in cholesterol, e.g., lipid rafts.

An isoform of human NTPDase1, called placental ecto-ATPDase I, was purified by Makita et al. [64]. It is longer (517 aa) than the NTPDase1 (510 aa) cloned from a lymphoblastoid cell line [22], and the first 11 amino acids of the two proteins are different. A truncated form (306 aa), called placental ecto-ATPDase II, which lacks ACR5 and terminates in a sequence not found in the longer isoform, was later obtained from a human placenta cDNA library [65]. Placental ecto-ATPDase I was active in inhibiting platelet aggregation, but the activity of the truncated form was not determined. While cDNAs of NTPDase1 and both placental ecto-ATPDase I and II were present in the DNAs of human placenta, placental ecto-ATPDase I could not be detected in the DNA of umbilical vein endothelial cells [65].

Fig. 1 (continued)



Because of the location of NTPDase1 on endothelial cells and its ability to hydrolyze ADP, a ligand of the P2Y1 receptor that promotes platelet aggregation, its important role in thromboregulation was immediately recognized. Many in vitro studies followed that showed (1) an inverse correlation of NTPDase1 activity with platelet aggregation [28, 66, 67], (2) a decrease of NTPDase1 activity upon endothelial cell activation [67], and (3) attenuation of ATP signaling in cells expressing an NTPDase1-P2Y1 receptor fusion protein [68]. The last study demonstrated the importance of NTPDase1 in regulating purinergic signaling. The thromboregulatory role of NTPDase1 is also supported by studies using CD39 null mice [69–71]. The *Cd39*$^{-/-}$ mouse was the first NTPDase null organism that became available for studying the physiological functions of NTPDase1 in vivo. While the full-length NTPDase1 was used in the studies cited above, Marcus' group generated a soluble NTPDase1 by removing the two transmembrane domains from the wild-type enzyme and demonstrated that it is also able to regulate platelet aggregation [72, 73].

NTPDase1 was initially found on activated *B*-lymphocytes [22]. In recent years, Robson et al. have shown its wide distribution on macrophages, natural killer cells, and regulatory T cells. The role of NTPDase1 in immune responses is currently under intense investigation [74].

NTPDase2 (CD39L1, ecto-ATPase) NTPDase2 has been cloned from chicken muscle [75], rat brain [57], rat cochlea [76], mouse hepatoma [77], and human tumor cells [30, 31]. The two human NTPDase2 sequences, independently obtained from cDNA libraries of a bladder tumor cell line (ECV-304) [30] and a small cell lung carcinoma cell line (T293) [31], are identical. Although NTPDase2 continues to be referred to as CD39L1 in some publications, the original human CD39L1 sequence [29] has a deletion of a block of 23 amino acids and is not functional. Of the three

splice variants of ecto-ATPase discovered by Mateo et al. [78]: NTPDase2α (495 aa), NTPDase2β (472 aa), and NTPDase2γ (450 aa), the CD39L1 sequence corresponds to that of NTPDase2β. ATPase activity was only obtained with NTPDase2α in transfected CHO cells. The peptides that are missing in NTPDase2β and NTPDase2γ contain a conserved cysteine residue (C399) involved in disulfide bond formation. Mutagenesis of C399 in NTPDase2α produced an inactive protein that had defective glycosylation and was retained in the ER [78], attesting to its importance in proper protein folding of the enzyme.

There are also two splice variants of the rat NTPDase2: NTPDase2α (495 aa) [57, 76] and NTPDase2β (545 aa), which has an extended intracytoplasmic *C* terminus [76, 79]. Transcripts of both forms are present in most rat tissues [76] and both are active, with some differences in biochemical properties, such as substrate preference, Km values for substrates, and sensitivity to suramin and pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid). Rat NTPDase2α was localized in the plasma membranes of the transfected CHO-S cells and its targeting to the location was stimulated by ATP [80]. Rat NTPDase2β was found both at the plasma and intracellular membranes. The longer *C* terminus of rat NTPDase2β contains several phosphorylation sites by protein kinase C (PKC) and casein kinase 2 and its activity was decreased somewhat by inhibitors of PKC, suggesting that its activity may be increased by phosphorylation [79].

Northern blot analysis showed that human NTPDase2 was highly expressed in brain, muscle and testis, but in very low amounts in the liver and lung [29]. Expression pattern of rat NTPDase2α was similar [57, 76]. Thus the cloning of NTPDase2 cDNAs from a mouse hepatoma [77] and a human small cell carcinoma [31], as well as the reports that an NTPDase2 activity was the major ecto-ATPase activity in human hepatoma, Li-7A [81] and small cell lung carcinoma (T293) cells [47] were of interest. These results indicated that gene expression of NTPDase2 is up-regulated in these tumors cells, although its cause is not yet understood. Gene expression of mouse NTPDase2 in the mouse Hepa1c1c7 hepatoma cells was further induced by dioxin treatment [77], but NTPDase2 induction by dioxin is not universal and varies with cell type [39]. Induction of NTPDase2 in the Sertoli cells has been shown in hypophysectomized rats treated by testosterone and in follicle stimulating hormone treated Sertoli cells [82]. However, its role in purinergic signaling of Sertoli cells [83] and their proliferation requires further investigation.

The most illuminating study on the physiological function of NTPDase2 concerns its role in the eye development of *Xenopus laevis*. Massé et al. showed that overexpression of NTPDase2 caused ectopic eye-like structures and increased expression of the eye field transcription factors (EFTFs), Pax6, Rx1 and Six3. Their results suggested that (1) NTPDase2 acts upstream of the EFTFs and (2) increased transcription of the EFTFs is a consequence of *Xenopus* P2Y1 receptor activation by ADP, the product of ATP hydrolysis catalyzed by NTPDase2. The involvement of P2Y1 receptor and NTPDase2 in this process was proven when simultaneous knock down of the two genes prevented expression of Pax6 and Rx1and eye formation. The authors suggested that this is a widespread mechanism in initiating eye development [84].

NTPDase3 (CD39L3, HB6) NTPDase3 has been cloned from human [34] and rat [85] brains and mouse spleen [86]. The expressed human NTPDase3 is the subject of a series of probing studies by Kirley et al. aimed at identifying amino acid residues important for NTPDase3 expression, enzyme activity, membrane targeting, and regulation [87–89, and see "Mutagenesis Studies of Cell Surface NTPDases" section below]. These investigators also detected an alternatively spliced form, NTPDase3β, in the human lung cDNA library. NTPDase3β is shorter (459 aa) than NTPDase3α (529 aa) and lacks the C-terminus that includes ACR5. NTPDase3β was expressed and targeted to the membrane; however, it had no measurable activity. When COS cells were co-transfected with the cDNAs of NTPDase3α and 3β, the amount of the active NTPDase3α delivered to the plasma membrane was reduced [90]. These results suggested that NTPDase3β may play a role in regulating NTPDase3α activity and purinergic signaling in tissues that express both proteins.

Immunoreactive NTPDase3 was detected in the neurons of adult brain. The immunopositive hypothalamic cells also expressed hypocretin-1/orexin-A. It was suggested that the neuronal NTPDase3 may modulate feeding, sleep–wake, and other behaviors [91]. In zebrafish, NTPDase3 expression was observed in sensory neurons and in a subset of hypocretin-producing cells. More importantly, NTPDase3 expression overlapped with that of several P2X receptors [92], further confirming the important role of ATP mediated signaling in the regulation of sleep and the processing of sensory inputs. The distribution of NTPDase2, the other NTPDase that is highly expressed in the brain, has been studied by Zimmermann and coworders. Braun et al. [93] reported that NTPDase2 was located in the subventricular zone and the rostral migratory stream of the adult rat brain and suggested that the enzyme has an important role in neural development and differentiation. Thus NTPDase2 and NTPDase3 appear to have different functions in the brain. Future studies that simultaneously examine the localization of both NTPDases in the same specimens will be useful in shedding light on their specific functions in the brain.

Besides brain, NTPDase3 is also highly expressed in the pancreas [29]. Recently, Loavoie et al. [94] reported the detection of NTPDase3 in Langerhans islet cells in mouse, rat, and human pancreas sections. More interestingly, they showed that inhibition of NTPDase3 activity by two compounds, BG0136 (1-naphthol-2,3-bisulfonic acid disodium salt) and NF279, increased insulin release from rat β-cell line INS-1 (832/13) cells under conditions of low glycemia, most likely by affecting P2 receptor activation.

NTPDase8 (ecto-ATP-diposphohydrolase, ecto-ATPDase, ecto-apyrase) The first reported NTPDase8 sequence (AF041355), assembled from two partial sequences, was that of the chicken oviduct ecto-ATPDase [38]. An enzyme with identical biochemical properties to the oviduct ecto-ATPDase [95, 96] was also present in the chicken liver [96, 97]. The full-length chicken liver ecto-ATPDase cDNA (AF426405) was subsequently cloned and expressed [97]. The chicken ecto-ATPDase sequence has 12 potential *N*-glycosylation sites, which accounts for the higher molecular mass of the mature protein. The slightly different sizes of the purified chicken oviduct and liver ecto-ATPDase [97] most likely resulted from different extent of N-glycosylation of the polypeptide in the two respective tissues. In the chicken liver, the ecto-ATPDase is localized at the bile canaliculi [97]. A similar ecto-ATPDase was detected in the chicken stomach and partially purified. Its N-terminal amino acid sequence is nearly identical to that of the chicken liver enzyme [98]. Subsequently, NTPDases that are homologous to the chicken ecto-ATPDase and designated as NTPDase8, were cloned from mouse and rat livers [99, 100]. The murine NTPDase8 is highly expressed in the liver and is also localized to the bile canaliculi [100]. Surprisingly, in contrast to other ecto-ATPDases, the expressed recombinant rat NTPDase8 was not inhibited by azide [100]. Because of their location, NTPDase8 may be involved in secretory functions as suggested for some ecto-ATPDases [101]. However, its physiological function is unknown at this time.

Human NTPDase8 was independently cloned in the laboratories of Knowles [52] and Sévigny [100] from liver. In addition to the 1.5 kb coding cDNA, a 1.7 kb cDNA, which contains two introns, was detected in human liver RNA by RT-PCR using primers corresponding to the 5′- and 3′-ends of the coding region of human NTPDase8 [52]. HEK293 cells transfected by the 1.7 kb cDNA had no measurable ATPase activity. These results indicated that the two introns were not spliced out in either human liver or in the transfected HEK293 cells. Surprisingly, the amount of the 1.7 kb transcript exceeded that of the 1.5 kb transcript in human liver [52].

The human NTPDase8 has Km for MgADP that is 30–40 times lower than the Km of chicken NTPDase8, although affinities for MgATP are similar [52, 97]. The MgADPase activities of both NTPDase8 are strongly inhibited by 10 mM azide. The two enzymes show different pH–activity curves with respect to ATP and ADP hydrolysis in the presence of Mg^{2+} and Ca^{2+}. The most striking difference is their different sensitivity to detergents. The chicken NTPDase8 is not inactivated by most of the commonly used detergents in membrane solubilization [52, 96], whereas the human NTPDase8 is inactivated 50–90% by alkyl-glucosides, Triton X-100 and NP-40 [52]. Both rat and porcine liver NTPDase8 are also inactivated by Triton X-100 [100, 102].

Intracellular NTPDases The intracellular NTPDases, NTPDases 4–7, are also integral membrane proteins. NTPDases 4 and 7 are anchored to the membrane by two transmembrane domains at the N- and C-termini whereas NTPDases 5 and 6 have only one transmembrane domain at their N-termini. In contrast to the cell surface NTPDases, the intracellular NTPDases have only four conserved cysteine residues in their extracellular domains, two of the four CRs, and 2–3 potential N-glycosylation sites compared to six or more in the cell surface NTPDases (Fig. 1). Glycosylation of the intracellular NTPDases appears to be unnecessary for their processing or activity. Notably, NTPDases 5 and 6 are primarily GDPases or UDPases and do not hydrolyze NTPs. The intracellular NTPDases have different functions than the cell surface NTPDases.

NTPDase 4 (Golgi UDPase, LALP70v) NTPDase4 was cloned from a human brain cDNA library by Wang and Guidotti [37] and was initially named human Golgi UDPase. The expressed protein hydrolyzed GDP, CDP, and TDP at comparable rates. UDP was the best substrate, but its ADPase activity was negligible. Rates of NTP hydrolysis were ~60% of that of NDP hydrolysis. UDPase activity with Mg^{2+} was less than 20% of that with Ca^{2+}. Unlike the human cell surface NTPDases, human NTPDase4 activity was not inactivated by Triton-X100. The localization of the enzyme in the Golgi was demonstrated by immunofluorescence in transfected COS cells and its disappearance after the cells were treated with brefeldin, which disrupts the Golgi assembly [37]. The Golgi UDPase is identical to LALP70v [103] and is a splice variant of LALP70, both cloned by Biederbeck et al. [104]. LALP70 has an additional eight amino acids, VSFASSQQ, downstream of ACR4. In contrast to the Golgi localization of LALP70v/Golgi UDPase, LALP70 was shown to be localized in lysosomes and autophagic vacuoles in transfected PaTu 8902 cells [104]. The two variants differed in their substrate preference and dependence on divalent ions. The nucleotidase activity (substrate not specified) of LALP70 was lower with Mg^{2+} than with Ca^{2+} whereas

the activity of LALP70v was similar with either Mg^{2+} or Ca^{2+} [103], the latter contradicted the results of Wang and Guidotti [37].

NTPDase 5 (CD39L4, ER UDPase) Human NTPDase5 was first cloned from a macrophage cDNA library. Mulero et al. [35] reported that both membrane-bound and secreted forms of the enzyme were obtained from transfected COS7 cells. The secreted form was generated as a result of cleavage of the signal peptide (amino acid 1–20), which coincides with the N-terminal transmembrane domain. The amount of the soluble form decreased when protein secretion was inhibited by brefeldin [35]. In their biochemical characterization of NTPDase5, Mulero et al. [105] focused on the ADPase activity of the protein, even though the Km for ADP was extraordinarily high (12.7 mM) and its ADPase activity was one fourth of its UDPase activity [35].

A soluble form of NTPDase5, named ER UDPase, was purified from bovine liver by Trombetta and Helenius. The enzyme hydrolyzed UDP, GDP, and CDP at comparable rates but had negligible ADP activity. The same investigators also cloned the mouse homologue and generated antibody for immunolocalization. The subcellular distribution of NTPDase5 was similar to the ER marker, calnexin, in several types of transfected cells [106].

While the ER location and its UDPase activity suggested that NTPDase5 promotes reglucosylation involved in glycoprotein folding in the ER [106], a totally unexpected role of NTPDase5 in oncogenesis was revealed in a series of studies by Notario et al. These investigators showed that an oncogene, *cph* (later renamed *mt-PCPH*), which was activated during chemical carcinogenesis of Syrian hamsters, caused transformation of NIH3T3 cells [107]. The related protooncogene, *PCPH*, encodes a polypeptide of 469 aa, while the *mt-PCPH* has a point mutation and encodes a truncated protein (246 aa) that lacks ACR5 and has a different *C* terminus [108]. PCPH was later shown to be identical to the hamster NTPDase5 [109]. Both PCPH and mt-PCPH displayed similar GDPase activity when expressed in *Escherichia coli*; however, only mt-PCH could act synergistically with H-Ras in transforming NIH3T3 cells [110]. Other experimental evidence to support the role of NTPDase5 in oncogenesis and tumor development has been presented. Only a few of the studies will be cited here: (1) loss of PCPH during progression of human laryngeal neoplasia [111], (2) alteration of PCPH in human breast cancers [112], and (3) increased expression of PCPH in human prostate cancer cells which enhanced their invasiveness [113] as well as conferring cisplatin resistance [114]. These studies have now been complemented by evidence presented in a most exciting recent report. Read et al. [115] generated *Entpd5*$^{-/-}$ mice and found that there was greatly increased proliferation of hepatocytes in both young and aged mice as well as development of hepatocellular neoplasia with age. These results provided further evidence correlating aberration of NTPDase5 with tumorigenesis.

NTPDase 6 (CD39L2) Three groups reported on the cloning and expression of human and rat NTPDase6 in the same year [32, 33, 116]. Human NTPDase6 is highly expressed in the heart [32]. Yeung et al. showed that both soluble and membrane-bound forms of the enzyme were produced in COS cells transfected with human NTPDase6 cDNA. Unfortunately, these authors used ADP as the substrate in their biochemical characterization, which was shown by Hicks-Berger et al. [33] to be a very poor substrate. The soluble enzyme had the highest hydrolysis activity with GDP and IDP. UDP and ADP hydrolysis rates were, respectively, ~30% and ~3% that of GDP [33]. Membrane-bound and soluble proteins were also obtained in CHO cells transfected by rat NTPDase6 cDNA [116]. The GDPase activity of the soluble rat NTPDase6 also exceeded its UDPase and ADPase activity. Although some cell surface localization by immunofluorescence analysis was observed, the majority of the NTPDase6 was associated with the Golgi [116]. The function of NTPDase6 was most clearly demonstrated with the yeast enzyme as will be discussed later.

NTPDase 7 (LALP1) NTPDase7 has been cloned from both human and mouse brain, but it is expressed in most tissues [117]. In spite of sequence differences, NTPDase7 is similar to NTPDase4 in that both have two transmembrane domains and hydrolyze some NTPs as well as NDPs. In fact, the UTPase activity of human NTPDase7 was greater than its UDPase activity. Fluorescence microscopy of HEK293 cells transfected with a LALP1–GFP fusion protein indicated it was distributed in intracellular vesicles [117]. It was noted that the NTPDase7 sequence in Genbank (AF269255; Fig. 1) differs from that in the published paper in the C-terminus.

Gene expression in Xenopus and zebrafish All eight NTPDase genes of both *Xenopus laevis* and *Xenopus tropicalis* have been isolated and their sequences characterized [118]. Some of the *Xenopus* NTPDases may regulate purinergic signaling in these animals, as ATP and adenosine regulate fatigue or run down of swimming in tadpoles. Massé et al. also determined expression of NTPDases 1–8 in the different tissues of the adult frog as well as temporal and spatial expression of NTPDases 1–7 during embryogenesis. Each of the NTPDases had a distinct expression pattern during embryogenesis suggest-

ing that they have different functions in this process [118]. Rosemberg et al. [119] reported that the NTPDase7 gene is absent in zebrafish, while there are three NTPase2 and two NTPDase5 paralogs. Gene expression of the seven NTPDases in zebrafish brain, liver, and heart was analyzed by RT-PCR. Differences in the expression levels of these genes in the three tissues were less distinct than that for the mammalian NTPDases [29, 57, 99]. Hydrolysis of CaATP and CaADP was determined using the post-nuclear fraction of the tissue homogenates of zebrafish brain, liver, and heart, which would represent the sum of the activities of multiple nucleotide hydrolases. Thus, the contribution by the NTPDases could not be quantified. No effort was made to determine the protein expression of the seven NTPDases in the three zebrafish tissues [119].

Characterization of expressed NTPDases of other organisms and functional studies

Drosophila melanogaster

A CD39-like gene in *D. melanogaster* was first reported by Chadwick and Frischauf [29]. A search of the *Drosophila* genome sequence revealed that there is only one NTPase gene which generates four transcripts, NTPase-RA, RB, RC, and RD. NTPase RC and RD encode an identical protein of 271 amino acids, that lacks ACR1 and ACR2. NTPase-RA and RB encode proteins of 461 and 464 amino acids, differing only in the first few amino acids at the N-termini. The protein encoded by NTPase-RA was cloned, expressed in S2 insect cells and characterized [120]. Its primary sequence indicated that it is a homolog of mammalian NTPDase6. The expressed *Drosophila* NTPDas6 is also an NDPase, the highest activity being obtained with GDP, IDP, and UDP as the substrates. Its higher UDPase activity distinguishes it from the mammalian NTPDase6, whose UDPase activity is only ~30% of its GDPase activity [33, 116]. The UDPase activity of *Drosophila* NTPDase6 was inhibited by azide, glutaraldehyde, and oxidative cross-linking [120]. Fluorescence microscopy showed that the protein was located primarily in the ER of the transfected S2 cells. Unlike the mammalian NTPDase6, no soluble form of the *Drosophila* NTPDase6 was detected in the culture media. The ER location of the enzyme indicates that its major role is participation in protein glycosylation. The finding that the only *Drosophila* NTPase gene encodes an intracellular NTPDase and not a cell surface NTPDase suggests that purinergic signaling pathways may not exist in this organism. This conclusion is compatible with the absence of genes encoding the P2 receptors in the *Drosophila* genome [121].

Plants

Apyrases that contain the five ACRs have been cloned from pea (*Pisum sativum*) [122], potato (*Solanum tuberosa*) [20, 123], the legume, *Dolichos biflorus* [124], Arabidopsis (*Arabidopsis thaliana*) [125], soybean (*Glycine soja*) [126], and cotton (*Gossypium hirsultum*) [127].

Without a doubt, the potato apyrase enjoys a place of honor in the history of NTPDase research as the determination of its sequence was instrumental in gathering seemingly unrelated proteins under the same umbrella [20]. Actually, the existence of the potato apyrase has been known for decades before its cloning, and the first effort in isolating the protein was reported more than 60 years ago [128]. This was followed by many published studies from the laboratory of Traverso-Cori on the potato apyrase isoforms and their biochemical properties [17, 129, 130]. It is marketed by Sigma and is widely used in experiments when rapid elimination of ATP is needed. So what is the function of this abundant protein? Earlier speculation that potato apyrase is involved in starch synthesis has yet to be proven. On the other hand, strong evidence that the potato apyrase affects growth has been presented by Riewe et al. [123]. In this massive study using a combination of experimental approaches, the investigators found two other apyrase cDNAs (Stapy2 and Stapy3) in addition to that previously cloned, Stapy1 [20]. Stapy3 (455 aa) is similar in size to Stapy1 (454 aa) whereas Stapy2 encodes a truncated protein (284 aa) that lacks ACR5. Silencing of the apyrase genes with RNAi led to markedly decreased apyrase activity as well as general retardation in growth and changes in tuber morphology. The apyrases are abundant in the tuber making it a good source for enzyme purification. Their location in the apoplast makes them accessible to extracellular ATP. The authors suggested that loss of the potato apyrases perturbs the relay of signals from extracellular ATP, resulting in stunted plant development [123].

Before the cloning of the potato apyrase, the sequence of a purified garden pea apyrase had already been obtained. The pea apyrase was associated with chromatin and its activity was stimulated by calmodulin [131]. Both potato and pea apyrases are soluble proteins even though sequences deduced from their cDNAs contain hydrophobic regions in their N-termini. Roux et al. [125] later cloned two apyrases, Atapy1 (471 aa) and Atapy2 (472 aa), from *A. thaliana* and expressed them in *E. coli*. Both *Arabidopsis* apyrases hydrolyzed ATP and ADP with an ADPase/ATPase ratio of ~0.25, but only Atapy1 binds calmodulin. Knockout of both Arabidopsis apyrases completely blocked pollen germination [132] and plant growth [133] due to reduced cell elongation. In vitro studies showed that addition of apyrase antibodies to germination media

inhibited growth rate of pollen tubes, but increased the concentration of extracellular ATP [133]. Roux and Steinebrunner proposed that an optimal concentration of extracellular ATP is required for maximal growth. Excess extracellular ATP inhibits growth but can be remedied by its hydrolysis by apyrases [134]. Two cotton apyrases with similar functions, GhAPY1 (472 aa) and GhAPY2 (469 aa), have also been cloned. Cotton fiber elongation was inhibited by addition of apyrase antibodies or apyrase inhibitors [127].

Besides their effect on plant growth, some apyrases are involved in nodulation of legumes by rhizobia. A carbohydrate binding protein, lectin-nucleotide phosphohydrolase (LNP), was isolated from the roots of the legume, *D. biflorus* [124]. LNP binds the Nod factors produced by rhizobia, which is likely the first step in initiating nodulation. LNP (462 aa) hydrolyzed ATP and ADP and its nucleotidase activity was increased upon binding to chitooligosaccharides (Nod factors) [124]. Two apyrases, GS50 and GS52, were found in another legume, soybean. The two apyrases have different tissue and subcellular distribution, with GS52 being localized in the plasma membrane [126]. Definitive evidence for the involvement of GS52 in soybean nodulation was presented by Govindarajulu et al. who showed that: (1) expression of GS52 was induced early in response to inoculation of the symbiotic bacterium and (2) silencing of GS52 in soybean roots by RNAi resulted in reduced number of mature nodules [135]. It is yet to be determined if the catalytic activity of the nodulins is important for the nodulation process, however, extracellular ATP is likely to be involved in signaling of events crucial for nodulation [136].

The reports cited above demonstrated that plant NTPDases are involved in growth and symbiosis between plants and microbes by regulating the concentration of extracellular ATP [134, 136]. However, purinergic receptors in plants have not been identified.

Pathogenic organisms

NTPDases of several parasites that are the causative agents of important human and animal diseases have been cloned. The best characterized are those from *Toxoplasma gondii* [18, 137–139], *Schistosoma mansoni* [140, 141], and *Trypanosoma cruzi* [142]. The first prokaryote NTPDase, from *Legionella pneumophila*, was discovered recently by Hartland's group [143], who also solved its 3D structure [144]. The readers are referred to an excellent and comprehensive review by Sansom et al. [145] which summarized published investigations on the role of NTPDases of pathogenic organisms in their interaction with host cells. This section will focus mainly on the molecular and biochemical aspects of these NTPDases and recently published results on functions.

Toxoplasma gondii

Two NTPases (628 aa) were cloned from *T. gondii* and characterized independently by Asai et al. [18, 137] and Joiner's group [138, 139] in the early 1990s. The two isoforms (NTPaseI and NTPaseII) have 97% sequence homology but differ in their utilization of NDP. NTPaseI is an NTPase whereas NTPaseII is an NTPDase [18]. Substrate preference is dictated by a block of 12 amino acids outside the ACRs [139]. Unlike most other NTPDases, the *Toxoplasma* NTPases require dithiols for full activity. The concentration of dithiothreitol or dithioerythritol needed, >2 mM, is sufficient to reduce disulfide bonds in the enzyme [18]. The effect of dithiothreitol on the *Toxoplasma* NTPases contrasts with that on some mammalian cell surface NTPDases, which are inactivated after prolonged incubation with dithiothreitol [146–148]. This is understandable since the cell surface NTPDases have five pairs of disulfide bonds in their extracellular domains that are important in maintaining structural integrity and activity [148]. NTPaseII is expressed in avirulent strains whereas both NTPases are expressed in virulent strains [139]. The *Toxoplasma* NTPases have become a target in drug development. Knock down of NTPaseII inhibited parasite proliferation, suggesting that NTPases play an important role in parasite replication [149]. This conclusion was supported by a recent study which showed that two monoclonal antibodies against NTPDaseII inhibited NTPase activity and increased the survival time of mice challenged with the parasite [150].

Schistosoma mansoni

Vasconcelos et al. [151] first reported the presence of an ATPDase activity on the external surface of the tegument of the worm, *S. manosoni*. Two proteins with ATPDase activity were detected in a partially purified preparation [24]. It was proposed that the ecto-ATPDases help the organism escape host hemostasis by preventing ADP-induced platelet aggregation. Two ATPDases, SmATPDase1 (544 aa) and SmATPDase2 (564 aa), that differ significantly in sequences were subsequently cloned [140, 141]. SmATPDase1, which is similar to the vertebrate NTPDase1, encodes a protein with two transmembrane domains and is located on the external surface at all stages of the parasite life cycle except egg [140]. SmATPDase2 shares only 27% amino acid identity with SmATPDase1. It has a single transmembrane domain and could be secreted [141], and is therefore more similar to NTPDases 5 and 6. However, its substrate specificity has not been determined. SmATPDase2 is present in the tegument

syncytium in addition to the apical and basal membranes. It was suggested that SmATPDaseI and II perform complementary functions [141].

Trypanosoma cruzi

Fietto et al. reported the presence of an *E*-type ATPase activity in live intact *T. cruzi* epimastigotes. A protein of 58 kDa could be immunoprecipitated by the antiserum to *T. gondii* NTPase. Immunolocalization with the antibody detected the protein on the external surface of all forms of the parasite [142]. An NTPDase gene, named *T. cruzi* NTPDase-1, was cloned and expressed [152]. Its protein sequence (636 aa) indicated that it is related to NTPDase5. Santos et al. evaluated the importance of the *T. curzi* NTPDase-1 in the infective process and showed that ARL 67156, gadolinium and suramin, and an NTPDase-1 antibody markedly reduced trypomastigotes infectivity. Mice infected with the NTPDase-1-inhibited trypomastigotes produced lower levels of parasites and survived longer [152].

Legionella pneumophila

L. pneumophila is the causative agent of Legionnaire's disease. Its genome contains two genes, *lpg0971* and *lpg1905*, that encode proteins containing the five ACRs. Inactivation of *lpg 0971* had no effect on bacterial replication within macrophages, alveolar epithelial cells, or an amoeba, *Hartmannella vermiformis*, but inactivation of *lpg1905* had a negative impact [143]. The sequence of Lpg1905 is most closely related to vertebrate NTPDase1, but it has only a signal sequence at its N-terminus and the protein is secreted. Lpg1905 expressed in *E. coli* hydrolyzed ATP, GTP, ADP, and GDP and its activity was inhibited by ARL67156 [143, 153]. Hartland's group generated several Lpg1905 mutants that were either inactive or had impaired ATPase activity. When introduced into a replication-defective *L. pneumophila* mutant, the Lpg1905 mutants that had no enzyme activity were not able to confer the ability to replicate in the macrophage THP-1 cells. On the other hand, an Lpg1905 mutant that suffered only partial loss of enzyme activity could restore replication of the same bacteria in the THP-1 cells [143]. These results support the conclusion that catalytic activity of the Lpg1905 NTPDase is critical for *L. pneumophila* infection and its virulence.

Tetrahymena

A soluble NTPase of *Tetrahymena thermophila* was purified by Smith et al. [154]. The 66 kDa protein hydrolyzed all NTPs, although GTPase activity was only ~24% of the ATPase activity. ADPase activity was negligible. The ATPase activity was inhibited by ConA. While the enzyme was purified from the growth media, both activity and cross-reactivity with the chicken NTPDase2 antibody were detected in the body and ciliary membranes, suggesting that the enzyme exists as an ectoenzyme. The *Tetrahymena* ecto-ATPase is probably involved in regulation of signaling by extracellular ATP and GTP, which are depolarizing chemorepellents in both *Tetrahymena* and *Paramecium* and cause avoiding reactions in these organisms. *Tetrahymena* binds extracellular ATP and GTP with high affinity, although their receptors have not been identified [see review in ref. 155].

The cDNA encoding the purified *Tetrahymena* NTPase was not identified. At present, sequences of two putative *T. thermophila* NTPase cDNAs, XP_001025149.1 (454 aa) and XP_00106399.1 (481 aa), can be retrieved from the database. They share only 34% identity and 52% similarity in their amino acid sequences. Interestingly, both sequences show homology to the human NTPDase1 with also ~30% identity. XP_001025149.1 has a hydrophobic region at its N-terminus and may be the candidate cDNA for the purified ecto-ATPase [154].

Yeast

The GDPase of *Sacchromyces cerivisiae* (Gda1p), extensively studied by Hirschberg's group, was the very first NTPDase to be cloned [23]. Gda1p (518 aa) is an NTPDase6 that is located in the Golgi. It is highly specific for GDP with little UDPase activity. Its major function is the hydrolysis of GDP to GMP, whose transport out of the Golgi is obligatorily coupled to uptake of nucleotide sugars, the substrates for glycosyltransferase reactions that add carbohydrate moieties to proteins and lipids [156]. A similar NTPDase6 of *Schizosaccharomyces pombe*, Spgda1p (556 aa), differs somewhat from the *S. cerevisia* Gda1p in that it hydrolyzes both UDP and GDP [157].

In addition to the Gda1p/NTPDase6 protein, Gao et al. [158] found a homologous protein, YND1 (630 aa), that is required for Golgi N- and O-glycosylation in *S. cerevisia*. YND1, which is identical to Apy1p characterized by Zhong and Guidotti [159], has broad substrate specificity with respect to NDPs and NTPs. A homolog of *S. cerevisiae* YND1/Apy1p, Spynd1, is present in *S. pombe*. Yeast Gda1p and YND/Apy1 may have partially redundant functions. Individual disruption of either gene did not affect cell viability but disruption of both genes was lethal [158].

YND1/Apy1 has the five ACRs typical of all NTPDases and has a single transmembrane domain. However, unlike NTPDases 5 and 6, the transmembrane domain of YND1/Apy1 is near the C-terminus. A vertebrate homolog of YND/Apy1 has not yet been reported. Nevertheless, the different topology of these proteins may merit adding an NTPDase9 to the NTPDase family.

Purified NTPDases

NTPDases purified from natural sources In the early literature on "ecto-ATPases" of mammalian tissues, most investigators noted that this activity decreased markedly in the presence of most detergents [see review in ref. 10], except for digitonin, an expensive and often impure detergent. While this sensitivity to detergents distinguished the "ecto-ATPases" from the F-, P-, and V-type ATPases, it was a real headache for anyone who attempted their purification, the first step toward molecular identification. Therefore, purification of any ecto-ATPase from natural sources not only represented valiant efforts but also great achievement. Table 1 is a summary of the NTPDases that have been purified to homogeneity, as determined by SDS-PAGE analysis, from tissues or native organisms.

It is now clear that detergent inactivation is limited only to the mammalian cell surface NTPDases, since NTPDases 5 and 6 are not so affected. There is also a species difference with respect to NTPDase8. NP-40 and Triton X-100 inactivate both human and rat NTPDase8 [52, 102], but not chicken NTPDase8 [52, 96]. Interestingly, this species difference does not extend to NTPDase2, as both chicken and human NTPDase2 are inhibited by Triton X-100 [48, 146]. Detergent inactivation also accounts for the disparity of reported specific activities of purified NTPDases. Most purified NTPDases shown in Table 1 have specific activities of ~1,000–10,000 μmol min^{-1} mg^{-1}. Their extraordinarily high activity is another hallmark that distinguishes them from the F-, P-, and V-ATPases. The low specific activity of the purified human plancenta NTPDase1, ~20 μmol min^{-1} mg^{-1}, seen in Table 1 could be attributed to the use of Triton X-100 in solubilizing the placenta membranes [25]. In spite of this low activity, the enzyme was completely purified as mentioned earlier. On the other hand, while the rat liver ecto-ATPase purified by Lin also appeared to be homogeneous [160], oligonucleotide probe prepared according to a tryptic peptide sequence of the purified protein led to the cloning of biliary glycoprotein I (BGP-1) [161], a cell adhesion molecule unrelated to NTPDases. This error suggested that the single band on the SDS gel was a mixture of rat NTPDase8 and BGP-1. The rat liver NTPDase8 with high specific activity has now been purified by Fausther et al. [100].

Purification of recombinant NTPDases expressed in bacteria The elucidation of the 3D structure of a protein is necessary in the complete understanding of its biochemical mechanism. Unfortunately, this is not an easily achievable goal for the NTPDases because of their low abundance and their association with the membrane. However, the unique topology of membrane-bound NTPDases with a large exoplasmic domain that contains all the catalytic groups offers a second choice. As summarized in Table 2, the ecto-domains of rat NTPDases 1, 2, and 3 [162] and the soluble forms of human NTPDases 5 and

Table 1 NTPDases purified from natural sources

NTPDase	Tissue	Substrate	Specific activity (μmol min^{-1} mg^{-1})	References
NTPDase1	Human placenta	MgADP	20.8	Christoforidis et al. [25]
	Human placent a	CaATP	15–20	Makita et al. [64]
	Porcine brain cortex synaptosomes	CaATP	69.4	Kukulski & Komoczyński [201]
NTPDase2	Rabbit skeletal muscle *T*-tubules	MgATP	6,600	Treuheit et al. [14]
	Chicken gizzard	MgATP	88.6 1,681 (+ConA)	Stout and Kirley [174]
	Porcine brain cortex synaptosomes	CaATP	79.6	Kukulski & Komoczyński [201]
NTPDase5	Bovine liver	CaUDP	5,460	Trombetta & Helenius [106]
NTPDase6	Yeast	CaGDP	3,080	Yanagisawa et al. [202]
NTPDase8	Rat liver	CaATP	19.1	Lin [160]
	Rat liver	Ca(Mg) ATP	454	Fausther et al. [100]
	Chicken oviduct	MgATP	806	Strobel et al. [95]
	Chicken liver	MgATP	1242	Knowles et al. [97]
Pea nuclei NTPase		MgATP	428.6	Chen et al. [131]
Potato apyrase		CaATP	10,000	Handa & Guidotti [20]
Toxoplasma NTPase1		MgATP	2,540	Asai et al. [18]
Toxoplasma NTPase2		MgATP	446	Asai et al. [18]
Tetrahymena thermophila		MgATP	2,011	Smith et al. [154]

Table 2 Bacterially expressed NTPDases that have been purified

NTPDases	Substrate	Specific activity (μmol $min^{-1}mg^{-1}$)	References
Rat NTPDase1 ectodomain	CaATP	294.5	Zebisch and Sträter [162]
Rat NTPDase2 ectodomain	CaATP	40.6	Zebisch and Sträter [162]
Rat NTPDase3 ectodomain	CaATP	128.7	Zebisch and Sträter [162]
Human NTPDase5 (sol)	CaGDP	566.7	Murphy-Piedmonte et al. [163]
Human NTPDase6 (sol)	CaGDP	750	Ivanenkov et al. [164]
Arabidopsis apyrase 1	Mg,CaATP	59.9	Steinebrunner et al. [125]
Arabidopsis apyrase 2	Mg,CaATP	58.9	Steinebrunner et al. [125]
Legionella pneumophila	MgATP	1,182	Sansom et al. [153]

6 [163, 164] have been expressed in bacteria and purified. These recombinant proteins were produced in inclusion bodies, and refolding after denaturation in guanidine hydrochloride and disulfide bond reduction was necessary. In general, the soluble, bacterially expressed NTPDases retained most of the characteristics of the full-length enzymes expressed in eukaryotic cells, but some properties were altered. As mentioned earlier, glycosylation of NTPDases 5 and 6 is not required for their activity whereas the activity of full-length cell surface NTPDases is adversely affected by inadequate glycosylation. The lower activity of the ecto-domains of rat NTPDases 1–3 may result from a lack of glycosylation since this post-translational modification pathway does not exist in *E. coli*. On the other hand, it is also possible that the lower activity is the consequence of the removal of the transmembrane domains and that glycosylation per se is not important for ATPase activity of the ecto-domains. This question cannot be resolved until the effect of glycosylation on the activity of the ecto-domains of NTPDases becomes known.

The effect of glycosylation on plant apyrases has not been investigated. The two full-length apyrases of *A. thaliana*, that contain a hydrophobic region at their N-termini, were expressed in *E. coli*. The bacterially expressed proteins were insoluble and required purification under denaturing conditions before renaturation. The proteins were ~70% pure [125] and the activity seemed low. The putative signal peptide sequence (the first 34 amino acids) was removed from *L. pneumophila* NTPDase [143] before being expressed in *E. coli*. Solubility of the recombinant protein was apparently not an issue.

Regulation of cell surface NTPDase activity

The activity of cell surface NTPDases is regulated by several highly unusual mechanisms, the most interesting being the unexpected role of transmembrane domain interaction in modulating enzyme activity. The cell surface NTPDases have two transmembrane domains (hereon abbreviated as TMDs): one at the N-terminus (TMD1) and one at the C-terminus (TMD2). This type of membrane topology is seen only with the P2X receptors, the epithelial Na^+ channel, and the acid-sensing ion channel [165–167]. All three ion channels are trimeric and their TMDs form the pore for the ions. Channel opening is regulated by ligand binding in the extracellular domains (ECD). As will be described below, ATP binding to the ECD of cell surface NTPDases also affects TMD mobility. More importantly, membrane perturbation, which affects the TMDs, is conveyed to the catalytic sites in the ECD and alters the activity of the cell surface NTPDases.

Substrate inactivation and regulation

In two papers published in the mid-1980s, Beeler et al. described some unusual properties of the microsomal E-type ATPases of rat tissues [168, 169]. They noted that: (1) ATPase activity decreased with time, which they called ATP-dependent inactivation, (2) ATP (substrate) inactivation was exacerbated in the presence of detergents and at higher temperatures, and (3) substrate inactivation was prevented if the membranes were first incubated with the tetravalent lectin, ConA, or a chemical cross-linking reagent, glutaraldehyde. Similar results were obtained by other investigators using membrane preparations of rat tissues. In her review, Plesner [10] commented on these findings and suggested that it may be specific only for the rat E-type ATPases.

The chicken transverse tubule E-type ATPase appeared not to undergo substrate inactivation, but the enzyme did not obey Michaelis–Menten kinetics. Two Km values were obtained indicating high affinity (μM) and low affinity (mM) sites for ATP [170, 171]. Moreover, its activity decreased at higher temperature, which could be prevented by preincubation with ConA or glutaraldehyde. ConA was

ineffective if added after ATP, UTP or AMPPNP. Megías et al. proposed a model in which (1) ATP binds to both an active site and a regulatory site in the enzyme and (2) the enzyme has a high and low activity state, the latter characterized by negative substrate cooperativity. Membrane perturbation by temperature and detergents promotes formation of the low activity state whereas dimer formation, induced by lectin and glutaraldehyde, promotes formation of the high activity state. Dimer formation is prevented when the regulatory site is occupied by ATP [171]. This model could account for the results of Beeler et al. [168, 169] and those obtained with the chicken gizzard ecto-ATPase [172, 173]. A marked ConA stimulation, 19-fold, persisted in the purified chicken gizzard ecto-ATPase, an NTPDase2 [174], but the regulatory effect of ATP was not investigated. Substrate inactivation, decrease of activity at higher temperature, and the effect of ConA and glutarladehyde on preventing substrate and temperature inactivation were recapitulated in the recombinant human NTPDase2 expressed in HEK293 cells, but it displayed normal Michaelis-Menten kinetics with or without ConA [31]. Recombinant NTPDase3 expressed in COS-1 cells displayed a linear time course of ATP hydrolysis but its activity was stimulated by ConA [41]. Recombinant human NTPDase8 did not undergo substrate inactivation [52], nor stimulated by ConA. The effects of substrate, temperature, and lectin on human NTPDase1 and the four rat cell surface NTPDases have not been investigated. Nevertheless, it is obvious that different NTPDases do not respond to these parameters in the same manner.

It should be emphasized that while substrate inhibition is a common regulatory mechanism for many enzymes, substrate inactivation is rare. It is also clear that the effects of detergents, high temperature and substrate inactivation are not correlated and need to be determined for the individual NTPDase.

Regulation of cell surface NTPDases by their transmembrane domains

The inactivation of membrane *E*-type ATPases by detergents at concentration lower than their critical micelle concentrations was initially baffling. When the predicted membrane topology of the cell surface NTPDases became known, investigators began to focus their attention on the two TMDs, the structural elements impacted by the lipid soluble detergents. There is now firm evidence that the interaction of TMDs and their mobility in the lipid bilayer regulate catalysis occurring in the ECD. Some of the salient points on the interplay of TMDs and catalytic site of the rat NTPDase1/CD39 were elaborated in a review by Grinthal and Guidotti in 2006 [175]. Other relevant and more recent findings on this topic are summarized below.

1) *Decrease of activity and alteration of enzymatic properties after removal of the two transmembrane domains*. Engineered soluble human NTPDase1 [72], NTPDase2 [176, 177] and NTPDase8 [52], rat NTPDase1 [178], and chicken NTPDase8 [179], comprised of only the ECD of these NTPDases, have been expressed in mammalian cells, purified, and characterized. As expected, the activity of the soluble NTPDases was not affected by detergents. Wang et al. showed that larger amounts of rat NTPDase1 ECD protein was required to obtain the same activity as the full-length enzyme [178], evidence for the lower specific activity of the ECD. Truncated mutants containing only one TMD were also less active [178]. In the study by Wang et al., activity of the full-length NTPDase1 was determined using membrane preparations. We were able to make a more direct comparison and showed that the purified chicken NTPDase8 ECD had, at most, one tenth the activity of the purified full-length enzyme [179]. Assuming that most cell surface NTPDases, when purified, will have specific activity in the range of ~1000 μmol min^{-1} mg^{-1} (see Table 1), the generally lower activity of the purified NTPDase ECDs (20–150 μmol min^{-1} mg^{-1}) [52, 72, 162, 177–179] indicates that the presence of both TMDs is necessary for the cell surface NTPDases to achieve maximal activity.

Removal of the TMDs from cell surface NTPDases also resulted in alteration of enzymatic properties. Several characteristics: activation by Ca^{2+}, ADPase/ATPase ratios, Km values for substrates, pH–activity curves, and CaATPase/MgATPase ratios, differ between the ECDs and the full-length enzymes as reported in studies carried out with rat NTPDase1 [178], human NTPDase2 [176, 177], and human and chicken NTPDase8 [52, 179]. More interestingly, in contrast to the full-length enzyme, the human NTPDase2 ECD remained active at higher temperatures and was no longer susceptible to substrate inactivation [177]. Thus, the TMDs of human NTPDase2 are also involved in substrate inactivation of the enzyme in addition to influencing catalytic mechanism,

Opposite and entirely unexpected results were obtained with the chicken NTPDase8 ECD. Chicken NTPDase8 is unique in its resistance to detergent inactivation. It shows a linear time course of ATP hydrolysis and its activity increases with temperature [180]. These properties were expected to be retained by the ECD, but the soluble chicken NTPDase8 displayed a temperature dependent loss of activity in the presence of ATP, ADP, and Pi [179], i.e., the ECD became susceptible to inactivation by the substrates and product. Clearly, removal of the TMDs of the chicken NTPDase8 converted it from a stable to an "unstable" enzyme. Substrate and Pi inactivation persisted in truncated mutants that contain only TMD1 or TMD2, but disappeared in the native enzyme or a chimera in which the chicken NTPDase8 ECD is

anchored to membrane by the two TMDs from human NTPDase2 [179]. These results highlight the requirement of two TMDs for the maintenance of a stable chicken NTPDase8.

2) *Rotational mobility of the transmembrane domains is required for maximal activity of NTPDases 1, 2, and 3.* Grinthal and Guidotti used oxidative cross-linking of strategically placed cysteine residues in the TMDs of rat NTPDase1 to examine the interaction of TMDs [181]. They found, surprisingly: (1) the TM helices do not have specific orientation, (2) cross-linking occurred more rapidly between cysteine residues introduced into the TM helices near the extracytoplasmic surface, and (3) locking the TM helices by intra- or inter-molecular disulfide bonds resulted in marked loss of activity. The authors concluded that movement of the two TM helices within an NTPDase1 molecule is critical for enzymatic activity [181]. Oxidative cross-linking of the TMD was reduced when the reaction was carried out in the presence of ATP and ADP, but not AMP or Pi, suggesting that TM mobility becomes restricted upon substrate binding. These results were taken to be good indications of communication between TMDs and the ATP binding site.

Rotational mobility of the TMDs also appears to be important for the activity of human NTPDases 2 and 3. Human NTPDase2 contains a single cysteine residue (C26) in TMD1, three residues away from the cell surface. Oxidative cross-linking of the wild-type human NTPDase2 resulted in dimer formation and ~70% loss of activity. Similar results were obtained with mutants that contain a single cysteine residue in TMD2 at approximately the same depth as C26 of the wild-type enzyme. The amount of dimer formed upon oxidative cross-linking was dependent on the position of the cysteine residue, and correlated with loss of activity. Intramolecular cross-linking obtained with mutants containing C26 in TMD1 and an introduced cysteine residue in TMD2 also resulted in significant loss of activity due to intramolecular cross-linking, although some of the effect could be attributed to intermolecular cross-linking. Interestingly, the residual activity of the cross-linked NTPDase2 mutants became resistant to inactivation by detergents and high temperature [49]. Inhibition of human NTPDase3 activity, following oxidative cross-linking of mutants with cysteine residues introduced into the TMDs, was recently reported by Gaddie and Kirley [182].

Rotational mobility of the TM helices appeared to be not important for chicken NTPDase8 activity. Although the protein contains a free cysteine residue in its TMD1, it is located in the middle of the helix and inaccessible for oxidative cross-linking. We then constructed chimeras in which either TMD1 or both TMDs of chicken NTPDase8 are substituted by that of human NTPDase2. Because of the presence of C26 in TMD1 of these chimeras, dimers could be obtained upon oxidative cross-linking. The activity of these cross-linked chimeras was actually increased (unpublished results).

3) *The effect of detergents on the mobility of transmembrane domains*. The data in Table 3 shows the effect of commonly used detergents on the activity of human NTPDase2 and human and chicken NTPDase8. The human NTPDase2 was inhibited by most detergents except digitonin. The chicken NTPDase8 activity was not affected by any of the detergents, while human NTPDase8 was only strongly inhibited by Triton X-100, NP-40 and dodecyl β-D-maltoside. Triton X-100 also caused a loss of 80% activity of the full-length rat NTPDase1 [25, 178]. The effect of detergents was initially attributed to an increase in membrane fluidity, shifting the equilibrium between the less active monomers and the more active oligomers toward the monomers [171, 173, 178]. However, the question of oligomeric NTPDases has not been completely resolved. In a recent paper, the inhibitory effects of Triton X-100, some lysophopholipids, and unsaturated fatty acids on rat NTPDase1 activity were attributed to increased membrane elasticity [183]. These compounds, as well as the removal of cholesterol, increased the rates of oxidative cross-linking of the TMDs while abolishing the protective effect of ATP on dimer formation. These results were interpreted to mean that an optimal flexibility of both the ECD and TMDs must be

Table 3 Effect of various detergents on the ATPase activity of human NTPDase2 and human and chicken NTPDase8

Detergents	Human NTPDase2	Human NTPDase8	Chicken NTPDase 8
None	100	100	100
NP-40	3.2±1.6	9.14±0.06	118.6±4.6
Triton X-100	3.0±1.0	17.9±5.8	126.6±0.8
Deoxycholate	73.9±6.9	95.1±8.3	101.2±3.3
CHAPS	41.3±1.1	80.5±8.4	99.8±3.8
Octylglucoside	8.6±1.1	46.4±1.3	102.8±1.5
Dodecyl β-D-maltoside	40.1±3.5	21.0±2.1	125.0±7.3
Digitonin	238.6±1.1	102.4±3.0	120.2±5.2

maintained for the communication between the active site and the TMDs. A unifying hypothesis invoking two functional states of the monomeric NTPDase1 was proposed. In the fully functional state of NTPDase1, there is optimal rotational mobility of the TMDs and coupling between TMD motions and substrate binding. When these are disturbed by removal of either or both TMDs or the presence of agents that affect the mechanical properties of the membrane resulting in increased mobility of the TM helices, the enzyme is shifted into the second functional state of low activity. This model provides a satisfactory explanation of the experimental findings of rat NTPDase1. A complete model would also need to account for the effect of lectins and glutaraldehyde on NTPDase activity.

In a study aimed at identifying amino acid residues in human NTPDase3 that mediate the communication between the active site and the TMDs, Gaddie and Kirley presented evidence that two conserved proline residues in NTPDase3, in the linker regions between TMD1 and ACR1 and TMD2 and ACR5, may be responsible. Mutation of these proline residues resulted in (1) reduced enzyme activity and (2) reduced protective effect of ATP in diminishing dimer formation [182], suggesting that they are important in the coupling of ATP binding and rearrangement of the TM helices. It will be interesting to determine if such residues exist in other NTPDases.

4) *Inhibition of NTPDases 2 and 3 by modification of intramembrane cysteine by sulfhydryl reagents*. Sulfhydryl reagents, such as mercurials and *N*-ethymalemides, react with free cysteine residues and are inhibitors of enzymes in which cysteine residues are involved in activity. Human and chicken NTPDase2 are inhibited by *p*-chloromercuri-phenylsulfonate (pCMPS) [31, 47, 173], although none of the ACRs contains a free cysteine residue. The only free cysteine in these enzymes is in TMD1. When C26 in human NTPDase2 was mutated to serine, pCMPS was no longer inhibitory, proving that C26 is the target of pCMPS modification. Since reaction with pCMPS adds a bulky group to the S atom in C26, its inhibitory effect might result from reducing the mobility of TMD1. However, mutants in which a single cysteine residue was introduced into the TMD2 near the cell surface were inhibited only 15%–40% by pCMPS compared to ~80% inhibition of the wild-type enzyme, even though reaction with pCMPS did occur [177]. This result suggested that inhibition of NTPDase2 by pCMPS may be the combined effect of reduced helical mobility of TMD1, interference with TMD1–TMD2 interaction, and alteration of the conformation of ACR1 which is a short distance away from C26.

In human NTPDase3, a free cysteine residue (C501) in TMD2 was the target of pCMPS, and partial loss of activity was obtained [184]. C501 is located in the middle of TMD2 and should not have been accessible to pCMPS. Murphy et al. suggested that an aqueous pore in the region allows pCMPS to access C501.

5) *The importance of the strength of transmembrane domain interaction in preserving NTPDase activity in the presence of detergents*. In an effort to determine if the TMDs are responsible for the different sensitivity of human NTPDase2 and chicken NTPDase8 to detergent inactivation, we generated chimeras in which one or both of the chicken NTPDase8 TMDs were grafted onto the human NTPDase2 ECD (human NTPDase2 chimeras). This was also done with the chicken NTPDase8 ECD and human NTPDase2 TMDs (chicken NTPDase8 chimeras). Human NTPDase2 did not tolerate the exchange of TMD well and expression and activity of the chimeras were reduced. Nevertheless, the human NTPDase2 chimera which contains the two chicken NTPDase8 TMDs and intervening peptides up to ACR1 and ACR5 became resistant to NP-40 inhibition and no longer underwent substrate inactivation [177]. In contrast, expression of all chicken NTPDase8 chimeras was similar to wild-type enzyme with retention of activity. Activity exceeding that of the wild-type enzyme was actually obtained when both TMDs were replaced by those of human NTPDase2. All chimeras were inhibited more than 90% by 0.1% NP-40 [180, and unpublished data]. Taken together, these results indicated that resistance to detergent inhibition requires the presence of both TMDs of the chicken NTPDase8 regardless of the ECD attached to them. A comparison of the amino acid sequences of the TMDs of the human NTPDase2 and chicken NTPDase8 showed that the latter contain a larger number of polar and small amino acids that generally promote a stronger interaction of membrane helices [185], as well as motifs that favor helical interaction [186]. Further exploration of the relationship between amino acid sequences and strength of membrane helix interaction will be a most worthwhile pursuit in our general understanding of membrane protein structure.

Oligomerization and NTPDase activity

As described above, the activity of NTPDase2 and NTPDase3, whether in native membranes or in the recombinant form, was increased if the membranes were preincubated with ConA, or the lysine-specific cross-linking agents, glutaraldehyde and disuccinimidyl suberate [31, 41, 147, 169, 171, 173, 174, 187]. These results led to the proposal that the NTPDase oligomer is more active than the monomer [147, 168, 171, 173]. Wang et al. showed that the digitonin-solubilized rat NTPDase1 had a greater density than the Triton X-100-solubilized enzyme using sucrose gradient centrifugation. Estimation of the size of the more active species obtained in

digitonin suggested a tetramer [178]. Even though this view was later revised by the same authors because of lack of clear evidence of a stable NTPDase1 tetramer [175], the notion that all cell-surface NTPDases exist as tetramers somehow persists in the literature. While association of NTPDase monomers undoubtedly occurs in the membrane as demonstrated by blue native PAGE analysis of several NTPDases, the oligomers contained variable number of monomers [188, 189]. This behavior is unlike other membrane proteins, e.g., the P2X receptors, which require a defined quaternary structure to function. In the author's opinion, the "stimulating" effect of lectins and chemical cross-linking agents has less to do with promoting a defined oligomeric structure of NTPDase, but comes about as a result of alteration of the ECD (in the oligomer) in a way which prevents substrate inactivation and confers resistance to inhibitors and detergents [31, 171, 173].

While evidence for a stable tetrameric NTPDase is generally lacking, Gaddie and Kirley [89] recently provided strong evidence for the existence of a stable dimer for the full-length human NTPDase3. NTPDase3 of many species has a conserved glutamine (Q44 in human NTPDase3) that is located in TMD1 and near the extracellular surface. These authors showed that (1) both Q44A and Q44C mutants had only 30% of the activity of the wild-type enzyme, (2) inhibition of the activity of these mutants by Triton X-100 was attenuated when compared to that of the wild-type enzyme, (3) disulfide bond formation occurred spontaneously in Q44C mutant, and (4) ~50% of the Q44C protein existed as dimer which resisted reduction by dithiothreitol. The authors concluded that the conserved glutamine is responsible for NTPDase3 dimer formation via hydrogen bonding. Dimer formation cannot occur when Q44 is mutated resulting in loss of activity to a similar level as seen with the wild-type enzyme in the presence of Triton X-100. In the same study, the authors obtained the surprising result that NTPDase3 dimer persisted in the presence of Tween-20 and the activity was stimulated 2.5-fold [89]. This effect is unique to NTPDase3 since Tween-20 inhibited both NTPDases 1 and 2. Separation of the monomeric and dimeric NTPDase3 and determination of their activity would resolve the issue if an oligomeric NTPDase is indeed more active than the monomer.

Mutagenesis studies of cell surface NTPDases

Early mutagenesis studies focused on determining the importance of amino acids in the ACRs for enzyme activity. To facilitate discussion in this and the next sections, the sequences of the five ACRs are shown below. The numbering used is that of rat NTPDase2, whose ECD structure was elucidated by Zebisch and Sträter [190] in 2008. The strictly conserved amino acid residues are in bold. The DXG sequences in ACR1 and 4 are present in members of the actin/hexokinase/hsp70 family and are known as the phosphate1 and phosphate 2 binding motifs [191].

ACR1	**D**A**G**SSHT	51
ACR2	GA**TAG**M**RLL**	128
ACR3	**G**QD**E**G	166
ACR4	**DLGG**AS**TQ**	198
ACR5	**W**AL**G**	436

An impressive number of mutants have been generated for human NTPDase3 by Kirley et al. including those in which the strictly conserved amino acid residues were altered. The readers are referred to a review by Kirley et al. [87] that summarized their studies on NTPDase3 as well as those on other NTPDases prior to 2006. These studies showed that aspartate and glycine in ACR1 and 4, glutamate in ACR3, and serine and glutamine in ACR4 are essential for enzyme activity. Arginine in ACR2 can be substituted by lysine but not alanine. Mutation of tryptophan in ACR5 resulted in lower protein expression but, surprisingly, its ATPase activity was increased.

Two studies demonstrated that mutation of the same residue has different effects on the activity of full-length and soluble NTPDases. The histidine residue in ACR1 is conserved in NTPDases 1, 2, and 8 but not in the other NTPDases (Fig. 1). H59 in NTPDase1 was shown to be the target of diethylpyrocarbonate [192], a histidine-specific modifying agent that inhibits many NTPDases [48, 193, 194]. When H59 in the full-length rat NTPDase1 was mutated to glycine or serine, the enzyme lost 90% of its ATPase activity, but less than 50% of its ADPase activity. In contrast, mutation of H59 in the soluble NTPDase1 did not alter ATPase activity while increasing ADPase activity [195]. Similarly, mutagenesis of the histidine residue in ACR1 of human NTPDase2, H50, decreased the activity of the full-length enzyme but not that of the soluble enzyme [176]. Musi et al. [189] reported that mutation of aspartate and serine residues in ACR1 and aspartate in ACR4 to alanine decreased the CaADPase activity of the full-length human NTPDase1 to less than 50% of the control, whereas the same mutation resulted in a ~twofold increase of CaADPase activity in the soluble enzyme [196, 197]. The authors attributed the difference to a more limited movement of protein domains that contain these residues in the membrane-bound NTPDase1 [189]. However, in view of the numerous examples where a single amino acid mutation affects protein expression, the effect of mutation on protein folding and formation of the optimal active site must also be taken into consideration together with the critical role they play in catalysis.

Because lysine is involved in binding the negatively charged phosphates of ATP of many ATPases, the effect of

mutagenesis of several conserved lysine and arginine residues in human NTPDase2 was determined. Only mutation of K62, in the conserved region 1 (CR1) which immediately follows ACR1 (see Fig. 1), had a significant effect. Mutation of K62 to alanine resulted in loss of protein expression. Substitution of K62 by arginine restored expression and some activity, which was stimulated by ConA [43]. The corresponding lysine in human NTPDase3 is K79 and similar results were obtained with the K79A and K79R mutants. Basu et al. suggested that a positively charged amino acid in the vicinity of the conserved glycosylation site (N81) of human NTPDase3 is necessary for ConA binding [198]. The effect of mutating this conserved asparagine was studied in three enzymes: rat NTPDase1 [42], human NTPDase2 [43], and human NTPDase3 [40]. Mutation of N73 in rat NTPDase1 to serine resulted in loss of only 50% activity. Mutation of N81 in NTPDase3 to aspartate or glutamate resulted in loss of activity and the activity could not be stimulated by ConA, suggesting that the oligosaccharide attached to N81 in the wild-type NTPDase3 provides the ConA binding site [41]. The N64Q mutant of human NTPDase2 lost 95% of activity, but almost ~60% wild-type enzyme activity was obtained after the membrane was preincubated with ConA [43], indicating that N64 is not the exclusive ConA binding site in human NTPDase2. We postulated that lack of glycosylation at N64 perturbed the protein structure and the active site of NTPDase2, which is corrected upon binding ConA. The different results obtained with mutating the same conserved asparagine in the three NTPDases may be related to the different amino acids that the asparagine is mutated to or the different expression systems used in the these studies. It is also likely that they have different functions in the different NTPDases. This last point needs emphasizing since one often finds statements in literature which imply that an experimental observation with one cell surface NTPDase can be applied to others. There are now many examples which prove to the contrary.

Structural determination

All cell surface NTPDases contain ten conserved cysteine residues in their ECD. The disulfide structures were first investigated with human NTPDase3 using single and double mutants of the ten cysteines [148]. The results suggested pairing of specifc cysteines in disulfide bond formation, which may also occur in other NTPDases. The knowledge of the positions of the disulfide bonds was necessary in the generation of a theoretical 3D model of NTPDase3 using homology modeling [148]. Computational analysis showed extensive fold similarity between NTPDase3 and a bacterial exopolyphosphatase that had been crystallized [199], and the latter molecule was used as a template for modeling. Albeit some gap in the middle of the protein, Ivanenkov et al. developed a model for the NTPDase3 ECD that turns out to be quite similar to the recently published NTPDase crystal structures [144, 190]. Kozakiewicz et al. [200] also used the same exopolyphosphatase structure in homology modeling of potato apyrase and defined the putative Ca^{2+} and ATP binding sites of the enzyme.

While information obtained from modeling is useful, the uncertainty associated with such exercises can only be expelled by elucidation of the crystal structure of a protein. Such a feat was achieved for the field of NTPDases when Zebisch and Sträter published the first crystal structure of an NTPDase in 2008 [190]. The protein crystallized was the bacterially expressed rat NTPDase2 ECD, which is not glycosylated. Its activity was lower than that expected of the full-length enzyme but its ADPase/ATPase ratio was increased (~0.7 vs 0.1) [162]. Four different structures at resolutions between 1.7 and 2.1 Å were obtained: (1) the apoenzyme; (2) enzyme complexed with AMPPNP and Ca^{2+}; (3) enzyme with bound AMP, phosphate, and Ca^{2+}; and (4) enzyme with bound AMP. The structures showed the same two lobes as in the theoretical model, each with an extended RNase H fold, seen in structures of members of the actin/hexokinase/hsp70 family. The pairing of the ten cysteine residues agrees with that proposed for NTPDase3 [148] and it has the closest structural homology to exopolyphosphatase. Ca^{2+} and the substrate analog, AMPPNP, bind at the cleft between the two lobes. The arrangement of amino acid residues in the active site confirmed the importance of the strictly conserved residues in the ACRs: (1) Ca^{2+} is bound by oxygen atoms of β-, γ-phosphates and also to D in ACR1 and 4, T in ACR2, E in ACR3, W in ACR5 and α-phosphate via four water molecules; (2) the H and two S in ACR1, T in ACR2 and GAS in ACR4 are involved in binding O atoms in the β-, and γ-phosphates; (3) R in ACR2 and S and Q in ACR4 position E in ACR3 and a water molecule for a nucleophilic attack of the γ-phosphate; (4) hydroxyl groups of ribose are hydrogen bonded to two R residues and a D residue in the *C* terminal half of the protein; and (5) the adenine ring is in the vicinity of the same R residues as well as a Y and an A residue that interact with adenine, or other purine and pyrimidine bases, via π stacking interactions. The structure obtained with Ca^{2+}-AMP-Pi, which mimics Ca^{2+}-ADP, shows that Pi occupies the same position as the γ-phosphate of AMPPNP, suggesting that the same hydrolytic site is used for both ADP and ATP hydrolysis.

A second NTPDase structure, that of the *L. pneumophila* Lpg1905 NTPDase (Lp1NTPDase), was published in 2010 [144]. Three structures, the apoenzyme, enzyme complexed

with the inhibitor ARL67156, and enzyme complexed with AMPPNP, were obtained. In spite of low sequence identity (26%) and different numbers of disulfide bonds, the bacterial NTPDase and the rat NTPDase2 ECD structures are nearly superimposable. Like the rat NTPDase2 ECD, residues in ACR1 and ACR4 of Lp1NTPDase are also located in the ATP binding site. However, the contacts of phosphates of AMPPNP with Lp1NTPDase are mostly water mediated and exhibit more flexibility. There are also differences in the residues that bind the β- and γ-phosphates. In Lp1NTPDase, the ACR1 residues mediate binding with the γ-phosphate whereas ACR4 residues mediate binding with the α- and β-phosphate. In rat NTPDase1 ECD, ACR1 residues mediate binding of the β-phosphate while ACR4 residues bind the γ-phosphate. In contrast to the rat enzyme, no water molecule is seen near the catalytic glutamate residue in Lp1NTPDase. The authors suggested that the γ-phosphate needs to be repositioned for hydrolysis. Lp1NTPDase is strongly inhibited by ARL67156, an ATP analog [145]. The structure with bound ARL67156 is very similar to that with bound AMPPNP, but ARL67156 makes more direct contact with the protein. The β- and γ-phosphates in ARL67156 have slightly different positions than the same phosphate groups in AMPPNP, which allows the inhibitor to have more hydrogen bonding with the ACR1 region. In the future, it will be interesting to determine the structural basis for the greater sensitivity of NTPDases 1 and 3 than NTPDases 2 and 8 to inhibition by this compound [55].

Concluding remarks

Research on NTPDases has progressed at an accelerated pace since molecular identification of CD39 in 1996. With the discovery of an NTPDase in bacteria, there is little doubt that NTPDases occur throughout the evolutionary tree. As described in some detail in this review, the NTPDases of higher eukaryotes have different biochemical properties, splice variants, subcellular distribution, and tissue distribution. The physiological functions of NTPDase1 in thromoboregulation, inflammation, and immune suppression are being elucidated both in in vitro and in vivo studies, the latter with the *Cd39* null mice. Researchers are also making inroads in understanding the physiological role of NTPDase3 in brain functions and insulin secretion. One looks forward to exciting development concerning NTPDase5 in oncogenesis, now that *Ntpdase5* null mice have been generated. The importance of the NTPDases of plants and pathogenic organisms are just beginning to be appreciated. Future research will provide a better understanding of the mechanism by which they stimulate plant growth and impart virulence to pathogens.

Even though we finally have our first glimpse of an NTPDase molecule, many unresolved questions require further studies with respect to the structure-function relationship of NTPDases.

Catalytic mechanism The basis for the preference of NTP and NDP of the different NTPDases is unknown. The crystal structures of the two NTPDases indicated that both ATP and ADP are bound at the active site. What are the reasons for the inability of NTPDase2 to hydrolyze NDP and the inability of NTPDases 5 and 6 to hydrolyze NTP? Why do some NTPDases prefer a particular purine or pyrimidine nucleotide as the substrate? Why does removal of the two TMDs from cell surface NTPDases usually result in a higher ADPase/ATPase ratio, i.e., an increased ability to hydrolyze NDP? Why is ADP not released from the full-length NTPDase1 during ATP hydrolysis, but is released by the soluble NTPDase1? Why is this characteristic not displayed by NTPDases 1, 3, and 8?

Structural determination The rat NTPDase2 ECD used for structural determination was obtained from *E. coli*, therefore not glycosylated. It needs to be determined whether glycosylation of the ECD changes its activity and structure. The different biochemical properties of the full-length cell surface NTPDases and their soluble ECDs indicate that removal of TMDs alters substrate binding, substrate preference, and catalytic mechanism. Therefore, obtaining the structure of a full-length cell surface NTPDase with its two TMDs still remains the ultimate goal. Since glycosylation of NTPDases 4–7 appears to be not essential, and the soluble forms of human NTPDases 5 and 6 have already been expressed in bacteria [163, 164], a comparison of these ECDs with their full-length enzymes, which are bound to the membrane by a single TMD, may be a goal that can be attained more easily.

Regulation of catalysis of cell surface NTPDases by transmembrane domains This most unique regulatory mechanism, not reported for other membrane proteins, needs to be further explored. More mutagenesis studies of the full-length cell surface NTPDases with focus on the TMDs are needed to understand how the interaction of the TMDs and their mobility affect catalysis at the active site in the ECD. Efforts should also be made in the search for residues that are involved in the coupling of the TMD and the active site.

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