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The Stability of Chicken Nucleoside Triphosphate Diphosphohydrolase 8 Requires Both of Its Transmembrane Domains[†]

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ABSTRACT: Chicken nucleoside triphosphate diphosphohydrolase 8 (NTPDase8) is a cell surface ectonucleotidase with a large extracellular domain (ECD) containing the active site and is anchored to the membrane by two transmembrane domains (TMDs) at the N- and C-termini. Unlike other cell surface NTPDases that have been characterized, the chicken NTPDase8 is not susceptible to substrate inactivation or agents that cause membrane perturbation. To determine if the stability of the enzyme is inherent in its ECD, the cDNA construct of the soluble chicken NTPDase8 was expressed and the protein purified. The ATPase activity of the purified soluble chicken NTPDase8 was less than 15% of that of the purified full-length enzyme. Strikingly, in contrast to the membrane-bound enzyme, the activity of the soluble chicken NTPDase8 decreased with time in a temperature-dependent manner as a result of inactivation by ATP, ADP, and P_i. Truncated mutants in which the ECD is anchored by a single TMD at either the N- or the C-terminus by the native chicken NTPDase TMDs or a TMD from a different NTPDase, human NTPDase2, also displayed a nonlinear time course of ATP hydrolysis. While removal of the N- or C-terminal TMD affected protein expression differently, the truncated mutants were generally similar to the soluble chicken NTPDase8 with respect to ATP, ADP, and P_i inactivation. Other biochemical characteristics, e.g., ATPase/ADPase ratios, inhibition by azide, and affinity for ATP, were also altered when one or both of the TMDs were removed from the chicken NTPDase8. These results indicate that (1) both TMDs of the chicken NTPDase8 are required to maintain stability of the enzyme and maximal catalytic activity and (2) the conformations of the ectodomain in the soluble enzyme and the truncated mutants differ from that of the full-length chicken NTPDase8.

Nucleoside triphosphate diphosphohydrolases (NTPDases)[1] are integral membrane proteins capable of hydrolyzing nucleoside triphosphates (NTPs) and/or nucleoside diphosphates (NDPs). NTPDases 1, 2, 3, and 8 are present on the cell surfaces while NTPDases 4−7 are found in intracellular organelles (*1*). The cell surface NTPDases are anchored to the cell membranes with two transmembrane domains (TMDs) at their N- and C-termini. Because the extracellular domains (ECDs) of these proteins constitute the bulk (~90%) of the polypeptide as well as containing the conserved regions important for enzyme activity, engineered proteins comprising only the ECDs of these NTPDases, which are water-soluble, retain catalytic activity (*2*−*7*). Recently, the structure of the rat NTPDase2 ECD obtained by expression in bacteria was elucidated (*8*).

While the ECDs of the cell surface NTPDases manifest catalytic activity, all published reports indicate that they have reduced activity compared to the membrane-bound forms (*2*−*7*). They also differ from the full-length enzymes in their enzymatic properties; among these are altered preference for divalent ions (*5*−*7*) and nucleotide substrates (*4*, *5*, *7*). The intermediate reaction product of ATP hydrolysis, ADP, is released by the soluble rat CD39/NTPDase1 but not the membrane-bound enzyme (*9*). Mutation of a conserved histidine residue decreased the activity of the full-length rat CD39/NTPDase1 but not that of its ECD (*4*). Another demonstration of the markedly different characteristics of the membrane-bound and soluble forms was obtained with the human NTPDase2. We showed that the full-length human NTPDase2 was susceptible to substrate (NTP) inactivation resulting in a nonlinear time course of ATP hydrolysis as well as decrease of activity at temperatures higher than 37 °C (*7*, *10*). In contrast, the activity of the human NTPDase2 ECD was not affected by these parameters. The soluble human NTPDase2 displayed a linear time course of ATP hydrolysis, and its activity increased with temperature (*7*). Thus, the human NTPDase2 ECD freed of the two TMDs becomes stable, suggesting that the structure of the free ECD of human NTPDase2 is different than when it is anchored to the membrane.

Inactivation by most detergents is a common characteristic of most cell surface NTPDases, indicating that catalysis occurring in the ECDs is modulated by interaction of the two TMDs. However, there is no correlation between detergent sensitivity and substrate inactivation. This was clearly illustrated by the expressed human NTPDase8 cloned from human liver. Like the human NTPDase2, the membrane-bound human NTPDase8 is inhibited by a variety of detergents. However, unlike the human NTPDase2, its activity does not decline with time or at high temperatures (*5*). These characteristics are still maintained in the human NTPDase8 ECD while it becomes resistant to detergent inactivation (*5*).

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[1]Abbreviations: NTPDase, nucleoside triphosphate diphosphohydrolase; ECD, extracellular domain; TMD, transmembrane domain; TMD1, N-terminal transmembrane domain; TMD2, C-terminal transmembrane domain; AMPPCP, β,γ-methyleneadenosine 5′-triphosphate; AMPCP, α,β-methyleneadenosine 5′-diphosphate; NP-40, Nonidet P-40; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; TBS, Tris-buffered saline; PNGase F, peptide:*N*-glycosidase F; Endo H, endoglycosidase H.

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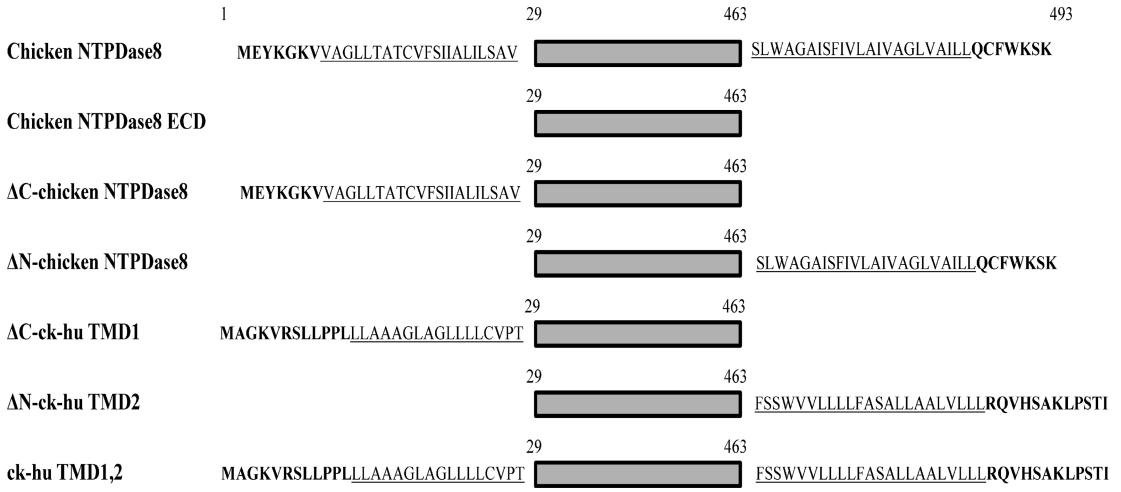

FIGURE 1: Schematic representation of the wild-type chicken NTPDase8, the chicken NTPDase8 ECD, the ΔC-chicken NTPDase8, the ΔN-chicken NTPDase8, the ΔC-ck-hu TMD1, the ΔN-ck-hu TMD2, and the ck-hu TMD1,2. The wild-type chicken NTPDase8 consists of 493 amino acid residues. The shaded region (aa 29−463) represents its extracellular domain (ECD). Amino acid sequences in bold represent the short intracytoplasmic domains in the wild-type chicken NTPDase8 and human NTPDase2. Underlined amino acid sequences represent the transmembrane domains (TMDs) in these proteins.

The chicken NTPDase8 is unique among the cell surface NTPDases in that it is stable in the presence of detergents. The enzyme has been purified from its native tissues, liver and oviduct, after initial solubilization with 5% NP-40 (*11*, *12*). Protein obtained from mammalian cells transfected with the chicken NTPDase8 cDNA was also resistant to detergent inactivation (*12*, *13*). Both the native and expressed chicken NTPDase8 maintain a linear time course of ATP hydrolysis (*12*, *13*). Thus, we expected the same stability to be maintained in the soluble chicken NTPDase8. To our surprise, we found that removal of the two TMDs of the chicken NTPDase8 resulted in an "unstable" enzyme in that the ECD became susceptible to substrate as well as product inactivation in a temperature-dependent manner. We further demonstrated that anchorage of the ECD by a single TMD at either the N- or the C-terminus did not restore stability to the chicken NTPDase8 ECD. However, stability of the enzyme was maintained when both TMDs of the chicken NTPDase8 were replaced by TMDs of a different NTPDase, human NTPDase2. Thus, anchorage of the ECD by both TMDs is required to generate a stable chicken NTPDase8.

MATERIALS AND METHODS

Materials. Oligonucleotides used for PCR and DNA sequencing were purchased from IDT (San Diego, CA). TOP10 *Escherichia coli* cells, DH5α *E. coli* cells, *Taq* DNA polymerase, pcDNA3.1/V5-His-TOPO cloning kit, pSecTag2B vector, Lipofectamine, Dulbecco's modified Eagle's media (DMEM), Opti-MEM, trypsin−EDTA, and antibiotics were purchased from Invitrogen. *Pfu* Turbo DNA polymerase was from Strategene. The DNA miniprep kit and QIA gel extraction kit were from Qiagen. Fetal calf serum and newborn calf serum were from Gemini-Bio Products (Calabasas, CA). Restriction enzymes, peptide:*N*-glycosidase F (PNGase F) and endoglycosidase H (Endo H), were from New England Biolabs. SDS−PAGE reagents, Affi-Gel Blue gel, Bio-Rad Dc protein assay kit, and dye reagent concentrate for protein assays were from Bio-Rad Laboratories. Prestained protein markers were from Invitrogen. Goat anti-mouse antibody conjugated to horseradish peroxidase was from GE Healthcare. Goat anti-mouse IgG conjugated to alkaline phosphatase was from Promega. His·bind kit was from Novagen. PVDF membrane, [γ-^{32}P] ATP, and Kodak Blue XB-1 film were from Perkin-Elmer Life Sciences Inc. Alkaline phosphatase substrate tablets (NBT/BCIP) were from Roche. Adenine nucleotides, β,γ-methyleneadenosine 5′-triphosphate (AMPPCP), α,β-methyleneadenosine 5′-diphosphate (AMPCP), detergents, and all other biochemical reagents were from Sigma Chemical Co. DNA sequencing was provided by the San Diego State University Microchemical Core Facility. The mouse anti-chicken NTPDase8 monoclonal antibody, MC-18, was the generous gift of Dr. Randy Strobel.

Generation of Soluble Chicken NTPDase8 cDNA. The full-length chicken NTPDase8 consists of 493 amino acid residues. Amino acids 1−28 (containing TMD1) and 464−493 (containing TMD2) were removed to produce the chicken NTPDase8 ECD (Figure 1). In the generation of the cDNA encoding the ECD by PCR, chicken NTPDase8 cDNA (accession number AF426405) inserted in the expression vector pcDNA3 was used as the template. The forward primer (5′-GCATACTGCAAGCTT-*GATGTGAAGGATG*-3′) contained a *Hin*dIII site (underlined) and nucleotides that annealed to nt 85−97 of the chicken NTPDase8 cDNA (italic). The reverse primer (5′-GCTCGAAT-TCGCGGCCGC*GGGCTGCTGCC*-3′) contained a *Not*I site (underlined) and nucleotides that annealed to nt 1378−1389 of the chicken NTPDase8 cDNA (italic). Conditions for PCR with *pfu* DNA polymerase were similar to that described previously (*5*, *7*). The ~1.3 kb PCR product was digested with *Hin*dIII and *Not*I and ligated with the expression vector, pSecTag2B, which was digested by the same endonucleases. The PCR product was inserted downstream of the Ig *k*-chain leader sequence and upstream of sequences encoding the myc epitope and His tag in the pSecTag2B vector.

Generation of ΔC-Chicken NTPDase8 cDNA. The ΔC-chicken NTPDase8 polypeptide consists of amino acids 1−463 of the chicken NTPDase8 (Figure 1). Two oligonucleotides that annealed to nt 1−24 (forward: 5′-ATGGAGTATAAGGG-GAAGGTTGTT-3′) and nt 1369−1389 (reverse: 5′-TTAGGG-CTGCTGCCCCTTGAC-3′) of the chicken NTPDase8 cDNA were used as primers in PCR. Conditions for PCR with *Taq* DNA polymerase were similar to that described previously (*5*, *7*). An ~1.4 kb (1389 bp) DNA fragment was obtained and ligated to

pcDNA3.1/V5-His-TOPO vector by TA cloning. After transformation in TOP10 *E. coli* cells, plasmid DNA was isolated from several colonies. Orientation of the ~1.4 kb DNA insert in pcDNA3.1/V5-His-TOPO was determined by restriction enzyme mapping using *Pst*I. Colonies containing recombinant plasmids with ΔC-chicken NTPDase8 cDNA of the correct orientation were propagated and plasmid DNA isolated.

Generation of ΔN-Chicken NTPDase8 cDNA. The ΔN-chicken NTPDase8 polypeptide consists of amino acids 29−493 of the chicken NTPDase8 (Figure 1). The cDNA was generated by PCR using the chicken NTPDase8 cDNA as the template. The forward primer was the same as that used for generating the cDNA of the chicken NTPDase8 ECD. The reverse primer, 5′-GCTCGAATTCGCGGCCGC*TTATTTG-GATTTCC*-3′, contained a *Not*I site (underlined) and nucleotides annealing to the stop codon and nt 1469−1479 of the chicken NTPDase8 cDNA (italic). The PCR product obtained was digested with *Hin*dIII and *Not*I and ligated to pSecTag2B which had been digested with the same restriction enzymes as described above. Plasmid DNA containing the ΔN-chicken NTPDase8 cDNA was isolated from transformed TOP10 *E. coli* cells.

Generation of ck-hu TMD1,2, ΔC-ck-hu TMD1, and ΔN-ck-hu TMD2 cDNAs. Three chimeric cDNAs, ck-hu TMD1, ck-hu TMD2, and ck-hu TMD1,2, inserted in pcDNA3 were first generated by joining two DNA fragments using two-step PCR based on the strategy of overlap extension as previously described (*7*, *13*). Ck-hu TMD1 encodes a protein in which the N-terminus (aa 1−28) of the chicken NTPDase8 is substituted with the corresponding region (aa 1−29) of the human NTPDase2, which includes its TMD1. Ck-hu TMD2 encodes a protein in which the C-terminus (aa 465−493) of the chicken NTPDase8 is substituted with the corresponding region (aa 461−495) of the human NTPDase2, which includes its TMD2. Ck-hu TMD1,2 encodes a protein in which the TMD1 and TMD2 of the chicken NTPDase8 are replaced by those of the human NTPDase2 (Figure 1). Ck-hu TMD1 and ck-hu TMD2 cDNAs in pcDNA3 were used as the templates for PCR in the generation of ΔC-ck-hu TMD1 and ΔN-ck-hu TMD2. The ΔC-ck-hu TMD1 protein contains the N-terminus of the human NTPDase2 and lacks the C-terminus of the chicken NTPDase8 (Figure 1). The strategy in cloning this construct was identical to that for generating ΔC-chicken NTPDase8, except ck-hu TMD1 cDNA was used as the template and the forward primer, 5′-AATGGCCGGGAAGGTGCGGTC-3′, annealed to nt 1−21 of the human NTPDase2 sequence in ck-hu TMD1. The ΔN-ck-hu TMD2 protein contains the C-terminus of the human NTPDase2 and lacks the N-terminus of the chicken NTPDase8 (Figure 1). The strategy in cloning this construct was identical to that in generating the ΔN-chicken NTPDase8 cDNA, except ck-hu TMD2 cDNA was used as the template and the reverse primer, 5′-GCTATTCGCGGCCGC*TTAAATGGTGCTTG*-3′, contained a *Not*I site (underlined) and nucleotides annealing to the stop codon and nt 1399−1410 in the cDNA encoding ck-hu TMD2 (italic).

Transient and Stable Transfection in HEK293 Cells. HEK293 cells were maintained in DMEM containing 5% fetal calf serum, 5% newborn calf serum, and 1% penicillin/streptomycin at 37 °C in an atmosphere of 5% CO_2/95% air. Transient transfection in six-well plates with 1 μg of recombinant plasmid DNA using Lipofectamine was as described previously (*5*, *7*). To obtain stably transfected cells, HEK293 cells were first transfected with plasmid DNA in six-well plates. Two days after transfection, the cells harvested from one well were placed in a T25 flask with 5 mL of media and allowed to attach overnight. Geneticin (for cells transfected with recombinant pcDNA3 and pcDNA3.1-V5-His-TOPO plasmids) or zeocin (for cells transfected with recombinant pSecTag2B plasmids) was then added in 100 μg/mL increments until concentration reached 300 μg/mL geneticin or 200 μg/mL zeocin, respectively. The established geneticin- or zeocin-resistant HEK293 cells were then propagated and used for membrane preparation.

Preparation of Plasma Membranes from Stably Transfected HEK293 Cells. Plasma membranes were prepared from five to ten 10 cm plates of HEK293 cells stably transfected with recombinant plasmids containing the cDNAs of the wild-type and chimeric chicken NTPDase8 and their truncated mutants. Cells were harvested by trypsinization and washed once with isotonic buffer (25 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.01 M KCl) to remove residual serum and trypsin. Membranes were prepared from the homogenized cells by ultracentrifugation and sucrose gradient centrifugation as previously described (*5*, *7*, *10*, *13*). The membranes could be stored at −20 °C for several months without loss of activity. The protein concentrations of the membranes were in the range of 1.3−3.3 mg/mL.

Purification of Chicken NTPDase8 ECD. HEK293 cells stably transfected with recombinant pSecTag2B containing cDNA encoding the chicken NTPDase8 ECD were established as described above and maintained in media containing zeocin. Approximately 300 mL of cell culture media was collected for the purification of the secreted chicken NTPDase8 ECD. In the first step, proteins precipitating between 40% and 70% ammonium sulfate saturation were collected by centrifugation. The pellet was dissolved in a minimal volume of 50 mM Tris-HCl, pH 8, and applied to a nickel (His·bind) column with a bed volume of 2.5 mL. After washing with 25 mL of binding buffer (5 mM imidazole, 20 mM Tris-HCl, pH 7.9, and 0.5 M NaCl), the column was washed with 5 mL each of 20, 40, and 60 mM imidazole in 20 mM Tris-HCl, pH 7.9, and 0.5 M NaCl. The His-tagged chicken NTPDase8 ECD protein was eluted with 500 mM imidazole in 20 mM Tris-HCl, pH 7.9, and 0.5 M NaCl and ten 1 mL fractions were collected. Fractions containing ATPase activity were combined and used for biochemical characterization of the chicken NTPDase8 ECD.

ATPase and ADPase Assays. ATPase activity of transiently transfected cells was determined in a 0.5 mL reaction mixture containing 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM KCl, 0.1 mM NaN_3, 5 mM $MgCl_2$, 5 mM ATP, and 20−50 μg of cell proteins. ATPase reactions were initiated with the addition of ATP and allowed to proceed at 37 °C for 10 min. After termination of the reaction by the addition of 0.1 mL of 10% trichloroacetic acid, denatured proteins were removed by centrifugation. An aliquot (0.1−0.3 mL) of the supernatant was used for P_i determination by adding 2 mL of a colorimetric reagent consisting of 1 volume of 10 mM ammonium molybdate, 1 volume of 5 N H_2SO_4, and 2 volumes of acetone as described previously (*11*). Absorbance was measured at 355 nm. ADPase activity of membranes and purified ECD was also determined using colorimetric assay of P_i.

For the biochemical characterization of membranes and purified ECD, ATPase activity was determined using radioactive ATP as the substrate. Reactions were carried out in a 0.25 mL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 1 mM [γ-^{32}P]ATP (~150−400 cpm/nmol) with

10−50 μg of membrane proteins or ∼0.5−1 μg of chicken NTPDase8 ECD. After termination of the reaction by adding 0.05 mL of 1.8 N $HClO_4$, [^{32}P]P_i released was separated from radioactive ATP by isobutyl alcohol−benzene extraction after complexing with ammonium molybdate (*5*, *7*).

All enzyme assays were carried out in duplicate. Results presented were the average of at least three separate experiments ± standard deviation.

Protein concentrations of cell lysates and membranes were determined using Bio-Rad Dc reagents A and B with bovine serum albumin as the standard. The absorbance was measured at 750 nm. Protein concentration of the chicken NTPDase8 ECD was determined using Bio-Rad dye reagent, with bovine serum albumin as the standard. The absorbance was measured at 595 nm.

Deglycosylation by Endo H and PNGase F and Glutaraldehyde Treatment. Membranes containing the wild-type chicken NTPDase8, ck-hu TMD1,2, and the truncated mutants (10 μg of protein) and chicken NTPDase8 ECD (1 μg) were treated with 500 units of Endo H or PNGase F according to the manufacturer's instructions, except dithiothreitol was left out of the denaturation solution since reduction of the disulfide bonds of chicken NTPDase8 markedly decreased its binding to the specific monoclonal antibody, MC18 (*11*, *12*). After incubation at 37 °C (24 h with Endo H and 3 h with PNGase F), the reactions were terminated by the addition of 2× SDS gel sample buffer and heating.

To determine the extent of oligomerization, membranes containing the wild-type chicken NTPDase8, ck-hu TMD1,2, and the truncated mutants (12.5 μg) or chicken NTPDase8 ECD (1.8 μg) were incubated in a 50 μL solution containing 20 mM Mops, pH 7.2, and 5 mM $MgCl_2$ without and with 10 mM glutaraldehyde for 30 min at 22 °C. The reactions were terminated by the addition of 2.5 μL of 0.25 M lysine.

SDS−PAGE, Silver Staining, and Western Blot Analysis. Samples to be analyzed were mixed with 2× SDS gel sample buffer without reducing agents and heated at 100 °C for 3 min. SDS−PAGE was carried out in 7.5% polyacrylamide gel according to the method of Laemmli (*14*). Silver staining of the gel was performed as described previously (*11*).

For Western blot analysis, MC-18 (500-fold dilution in TBS−2% BSA) was used as the primary antibody and goat anti-mouse IgG conjugated to alkaline phosphatase as the secondary antibody (5000-fold dilution in TBS−2% BSA). Immunoreactive protein bands were detected after incubating the blot with solution containing the alkaline phosphatase substrates, NBT/BCIP, according to the manufacturer's instructions.

To quantify protein expression of the ck-hu TMD1,2 and the truncated chicken NTPDase8 mutants in transient transfection experiments, PVDF membrane was blocked in a solution of TBST (TBS containing 0.2% Tween 20 and 0.02% azide) containing 5% nonfat milk for 45 min. The blot was then incubated in a solution containing MC-18 (500-fold dilution in TBST−5% nonfat milk) for 45 min, followed by washing and incubation with goat anti-mouse antibody conjugated to horseradish peroxidase (2000−3000-fold dilution in TBST) for 30 min. After washing, ECL reagent (10 mL of luminol with 3 μL of 30% H_2O_2) was applied to the PVDF membrane for 1 min, and the membrane was exposed to Kodak Blue XB-1 film in the dark room for 30 s to 10 min. Band intensities were quantified using Image J analysis.

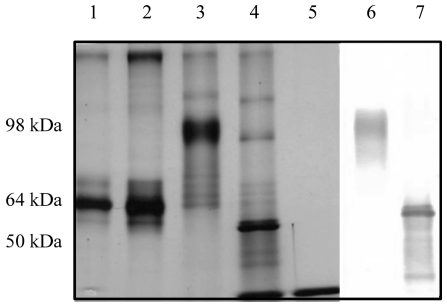

FIGURE 2: SDS−PAGE and Western blot analysis of the purification of the chicken NTPDase8 ECD. The chicken NTPDase8 ECD was secreted by HEK cells stably transfected with the recombinant pSecTag2B containing cDNA encoding the chicken NTPDase8 ECD. The soluble protein was purified from the culture media as described in Materials and Methods. Protein samples (0.25 μg of protein for silver-stained gel and 0.125 μg of protein for Western blot analysis) were treated with SDS gel sample buffer without reducing agent and subjected to SDS−PAGE on 7.5% acrylamide gel. After electrophoresis, the gel containing lanes 1−5 was silver stained. The gel containing lanes 6 and 7 was used for Western blot analysis with the chicken NTPDase8 specific monoclonal antibody, MC18. Key: lane 1, culture media containing secreted chicken NTPDase8 ECD; lane 2, proteins precipitated by 40−70% ammonium sulfate saturation; lane 3, proteins eluted from the His·bind column with solution containing 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 500 mM imidazole; lane 4, the same protein as in lane 3 treated with PNGase F; lane 5, PNGase F; lanes 6 and 7, Western blot analysis of the same samples as in lanes 4 and 5.

RESULTS

Expression and Purification of Soluble Chicken NTPDase8. The chicken NTPDase8 ECD was secreted into the culture media in which HEK293 cells stably transfected with its cDNA inserted in pSecTag2B were maintained. The media were collected for purification of the soluble chicken NTPDase8 using ammonium sulfate fractionation and affinity column chromatography as described in Materials and Methods. Nearly all of the ATPase activity and ∼20% of total proteins were recovered in the fraction that was precipitated between 40% and 70% ammonium sulfate saturation. Removal of the majority of the contaminating proteins with little loss of activity was accomplished using a His·bind column. The specific activity of the partially purified chicken NTPDase8 ECD was 23.7 μmol min^{-1} mg^{-1} in the experiment shown in Figure 2 and represented ∼1400-fold purification.

Purification of the chicken NTPDase8 ECD was demonstrated by SDS−PAGE and Western blot analysis (Figure 2). Silver staining of the SDS gel showed that a major protein band of ∼60 kDa was present in both the culture media and the salted out proteins (Figure 2, lanes 1 and 2) but was removed during affinity chromatography (Figure 2, lane 3). The purified chicken NTPDase8 ECD eluted from the His·bind column contained a prominent protein band of 98 kDa (Figure 2, lane 3), which accounted for 56% of the eluted proteins according to Image J analysis. The diffuse 98 kDa protein disappeared almost completely upon treatment of the chicken NTPDase8 ECD sample by PNGase F with the simultaneous appearance of a sharp ∼50 kDa protein that corresponds to the deglycosylated chicken NTPDase8 ECD (Figure 2, lane 4). This result indicates that there was negligible contamination of the purified chicken NTPDase ECD by other proteins of similar size. The additional band with a molecular mass of 36 kDa seen in the sample of deglycosylated chicken NTPDase8 ECD (Figure 2, lane 4)

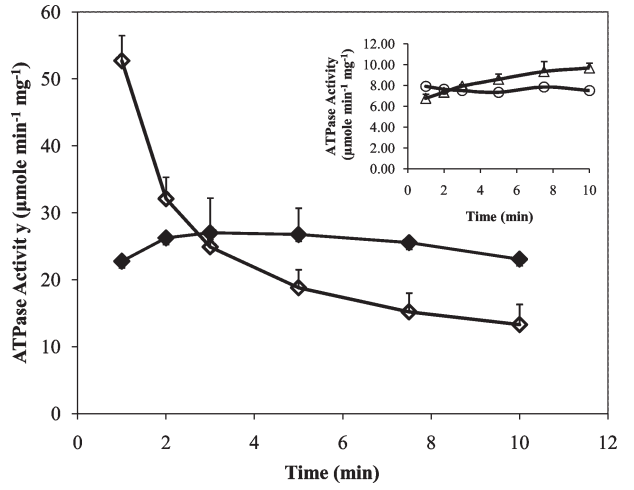

FIGURE 3: Time course of ATP hydrolysis by the chicken NTPDase8 ECD without and with prior glutaraldehyde cross-linking. Purified chicken NTPDase8 ECD (3 μg) was treated in a 100 μL mixture containing 20 mM Mops, pH 7.2, and 5 mM $MgCl_2$ without (◇) and with (◆) 10 mM glutaraldehyde for 30 min at 22 °C. After termination of the cross-linking reaction with 10 μL of 0.25 M lysine, the enzyme mixtures were placed on ice. Aliquots (0.3−1.0 μg) were used for ATPase determination for the indicated times at 37 °C (n = 3). The inset shows the time course of ATP hydrolysis of the full-length chicken NTPDase8 (○) and the ck-hu TMD1,2 (△) at 37 °C using membranes containing the wild-type chicken NTPDase8 and the ck-hu TMD1,2 chimera.

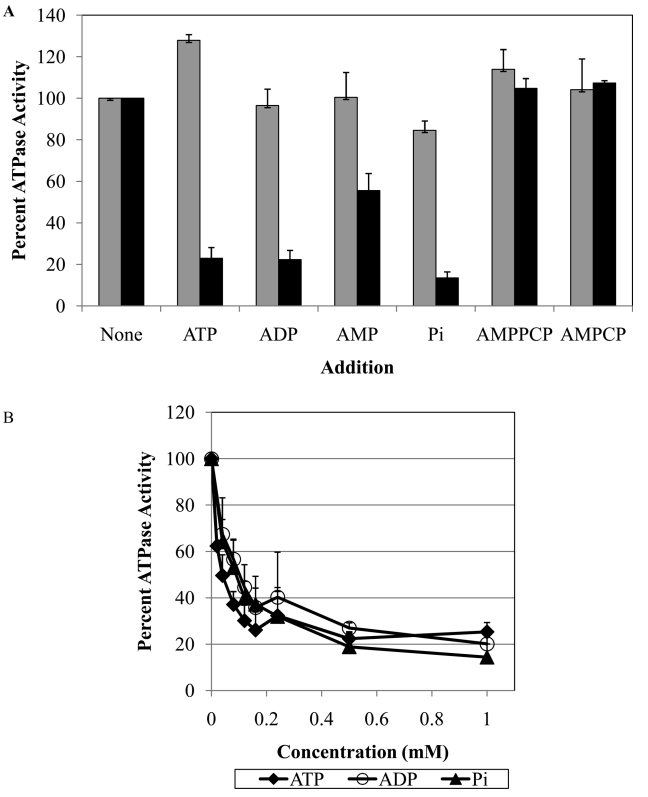

FIGURE 4: Effect of preincubation with nucleotides and P_i on the ATPase activities of full-length chicken NTPDase8 and its ECD. (A) Full-length chicken NTPDase8 (gray bars) or soluble chicken NTPDase8 (black bars) was incubated in a 0.25 mL reaction mixture containing 50 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ in the absence or presence of 1 mM ATP, ADP, AMP, P_i, AMPPCP, and AMPCP for 5 min at 37 °C. Reactions were initiated by the addition of 0.50 μmol of [γ-^{32}P]ATP to tubes containing ADP, AMP, P_i, AMPPCP, and AMPCP and 0.25 μmol of [γ-^{32}P]ATP to tubes containing ATP. Reaction time was 1 min at 37 °C. The amounts of protein used in the assays were 6.95 μg of membrane containing chicken NTPDase8 and 1 μg of chicken NTPDase8 ECD (n = 3). (B) The chicken NTPDase8 ECD was preincubated with the indicated concentrations of ATP (◆), ADP (○), or P_i (▲) for 5 min at 37 °C and assayed for ATPase activity as described in the legend of panel A .

corresponded to PNGase F (Figure 2, lane 5). The identification of the 98 and 50 kDa proteins with the glycosylated and deglycosylated chicken NTPDase8 ECD was confirmed by Western blot analysis using a chicken NTPDase8 specific monoclonal antibody, MC18 (Figure 2, lanes 6 and 7). Since the predicted molecular mass of the ECD polypeptide (48.1 kDa) with the myc epitope and His tag (~1.8 kDa) and extra amino acid residues is ~51 kDa, a molecular mass of 98 kDa of the expressed protein indicated that the secreted protein was highly glycosylated, in agreement with the presence of 12 potential N-glycosylation sites in the extracellular domain of the chicken NTPDase8 (*12*).

To determine if there is any misfolded protein in the purified chicken NTPDase8 ECD, an aliquot was incubated with Affi-Gel Blue gel, which only binds nucleotidases with native tertiary structures (*7*). Under conditions where all of the ATPase activity of the chicken NTPDase8 ECD was bound, there was no detectable immunoreactive protein in the supernatant after centrifugation. This result indicated that all of the purified chicken NTPDase8 is properly folded.

The Chicken NTPDase8 ECD Undergoes Inactivation by ATP, ADP, and P_i. The ATPase activity of the full-length chicken NTPDase8, whether in membrane fractions or purified, increased linearly with time up to 10 min at 37 °C, so that a straight line was obtained when plotting specific activity with time (Figure 3 inset, line with open circles). Unexpectedly, the ATPase activity of the chicken NTPDase8 ECD decreased rapidly with time so that the specific ATPase activity obtained in a 10 min reaction was ~25% of that obtained in a 1 min reaction (Figure 3, curve with open symbols). This was not due to denaturation of the enzyme. Incubation of the chicken NTPDase8 ECD at 37 °C for 10 min in the assay mixture without ATP resulted in ~25% loss of activity, far less than the 75% reduction of activity that was routinely observed in a 10 min ATPase assay.

To determine the cause of the loss of activity with reaction time, we examined the effect of preincubation of the chicken NTPDase8 ECD with nucleotides and P_i on its activity. The ECD was first preincubated in a solution containing buffer and Mg^{2+}, without or with 1 mM ATP, ADP, AMP, and P_i for 5 min at 37 °C before initiation of the ATPase reaction by the addition of 2 mM [γ-^{32}P]ATP. Only 1 mM [γ-^{32}P]ATP was added to the assay tubes in which the chicken NTPDase8 ECD was preincubated with 1 mM ATP. The specific radioactivity of [γ-^{32}P]ATP was corrected for the nonradioactive ATP that was already present in the solution in the calculation of ATPase activity in these assay tubes. After addition of [γ-^{32}P]ATP, ATP hydrolysis was allowed to proceed for 1 min to minimize the loss of activity. Preincubation of the chicken NTPDase8 ECD with ATP, ADP, AMP, and P_i for 5 min reduced the ATPase activity to 23%, 22%, 56%, and 13% of the control in which no nucleotides or P_i was present during the preincubation (Figure 4A, black bars). In contrast, preincubation of the full-length chicken NTPDase8 with nucleotides and P_i did not affect its ATPase activity (Figure 4A, gray bars). The latter result was expected from the linear time course obtained for the wild-type enzyme.

Inactivation of the chicken NTPDase8 ECD by P_i is interesting in that this effect has not been reported for other NTPDases. The human NTPDase2, which is primarily an NTPase, is susceptible to inactivation by NTP but not P_i (data not shown). The effect of P_i on the activity of the chicken NTPDase8 ECD is not due to product inhibition. The addition of up to 5 mM P_i in the reaction mixture did not diminish the ATPase activity.

Since P_i is produced from hydrolysis of ATP and ADP during preincubation of the chicken NTPDase8 ECD with the nucleotides, the subsequent loss of activity could be due solely to the inactivation of the enzyme by P_i. This possibility appeared to be supported by the data obtained with the nonhydrolyzable adenine nucleotide analogues. No loss of activity was obtained when the chicken NTPDase8 ECD was preincubated with AMPPCP and AMPCP (Figure 4A). However, we found that AMPPCP also had no effect on the human NTPDase2 which is susceptible to inactivation by NTP substrates (data not shown). These results are interpreted to mean that NTP inactivation of the human NTPDase2 occurs only upon substrate turnover. Therefore, the lack of inactivation by AMPPCP and AMPCP does not constitute evidence against substrate inactivation of the chicken NTPDase8 ECD. We conclude that loss of ATPase activity of the chicken NTPDase8 ECD with time is attributed to inactivation of the enzyme by ATP, ADP, and P_i. The concentrations of ATP, ADP, and P_i in the preincubation mixture that resulted in 50% reduction of activity were respectively 0.04, 0.11, and 0.09 mM (Figure 4B).

The chicken NTPDase8 ECD also differed from the full-length enzyme with respect to the effect of temperature on its ATPase activity. The activity of the full-length enzyme increases with temperature up to 60 °C (inset of Figure 5A), whereas the ATPase activity of the chicken NTPDase8 ECD decreased at temperatures higher than 25 °C even when assayed for 1 min (Figure 5A). This unusual temperature effect was related to the greater rate of substrate and product inactivation of the chicken NTPDase8 ECD at higher temperatures. The first-order rate constants of loss of activity were 0.12, 0.21, 0.55, 0.80, and 1.69 min^{-1}, respectively, at 14, 21.5, 29, 37, and 50 °C for the experiment shown in Figure 5B.

Previously, we reported that substrate inactivation of the full-length human NTPDase2 was abolished when membranes containing the enzyme were treated with glutaraldehyde (*10*). Glutaraldehyde treatment of the chicken NTPDase8 ECD had dual effects: (1) it was inhibitory as the activity of the glutaraldehyde-treated chicken NTPDase8 ECD was ~40% of that of the untreated protein in a 1-min assay, and (2) it abolished the loss of activity with time and the glutaraldehyde-treated enzyme now displayed a linear time course (Figure 3, curve with closed symbols). The latter result suggested that the glutaraldehyde-treated chicken NTPDase8 ECD is not susceptible to substrate and product inactivation, which was confirmed in an experiment where the glutaraldehyde-treated chicken NTPDase8 ECD was preincubated with nucleotides and P_i before the addition of [γ-^{32} P]ATP. Instead of the 70−80% reduction of activity seen with the untreated ECD preincubated with ATP, ADP, and P_i (Figure 4, black bars), the ATPase activities of glutaraldehyde-treated chicken NTPDase8 ECD preincubated with ATP, ADP, and P_i were respectively 95%, 80%, and 89% of the control (not shown). Western blot analysis revealed that, in contrast to the wild-type chicken NTPDase8, which formed dimers upon treatment by glutaraldehyde (compare lanes 1 and 2 of Figure 6), the glutaraldehyde-treated chicken NTPDase8 ECD remained as monomers (compare lanes 3 and 4 of Figure 6), indicating that glutaraldehyde treatment caused internal cross-linking of the protein rather than intermolecular cross-linking. It is concluded that intramolecular cross-linking of the chicken NTPDase8 ECD by glutaraldehyde alters the conformation of the protein so that it becomes resistant to substrate and product inactivation in spite of some loss of activity.

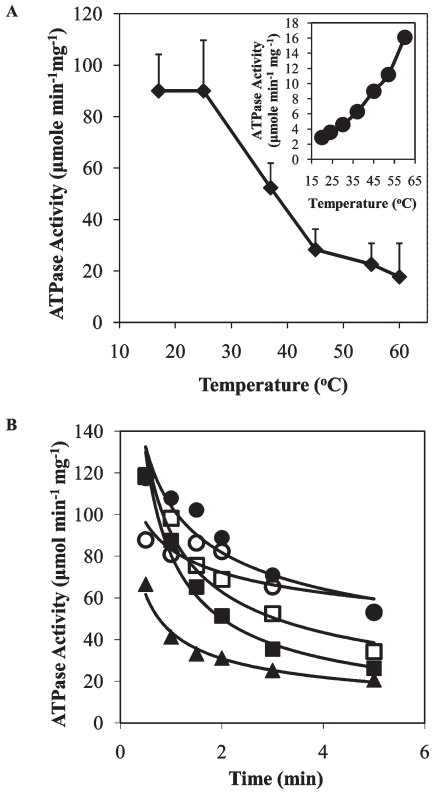

FIGURE 5: Effect of temperature on the ATPase activity of the chicken NTPDase8 ECD. (A) ATPase reactions were carried out in a 0.25 mL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM [γ-^{32}P]ATP, and 1 μg of purified chicken NTPDase8 ECD for 1 min at the indicated temperatures (n = 3). The inset shows the effect of temperature on the ATPase activity of the full-length chicken NTPDase8 using 6.95 μg of membranes. (B) ATPase assays of the chicken NTPDase8 ECD in a 0.25 mL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, and 0.3 μg of purified chicken NTPDase8 ECD at the indicated times at 14 °C (○), 21.5 °C (●) 29 °C (□), 37 °C (■), and 50 °C (▲) (n = 3). Standard deviations were not shown.

Other Differences between the Biochemical Properties of Soluble and Full-Length Chicken NTPDase8. Published reports showed that full-length and soluble cell surface NTPDases have different divalent ion preferences. While similar ATPase activity was obtained for full-length human NTPDase2 and NTPDase8 with either Mg^{2+} or Ca^{2+}, the CaATPase activities of the human NTPDase 2 and 8 ECDs were 4−5 times greater than their MgATPase activities (*5*, *7*). This was also observed with the bacterially expressed ectodomain of rat NTPDase2 (*6*). Even more remarkably, the ZnATPase activity of the soluble rat NTPDase2 was 30-fold higher than its MgATPase activity and 7-fold higher than its CaATPase activity (*6*). Figure 7A shows that (i) maximal MgATPase activity of the full-length chicken NTPDase8 was approximately 2-fold greater than its maximal CaATPase activity and (ii) while maximal ZnATPase activity was comparable to the maximal MgATPase activity, the ZnATPase activity declined markedly at higher Zn^{2+} concentrations and was ~10% of that obtained with

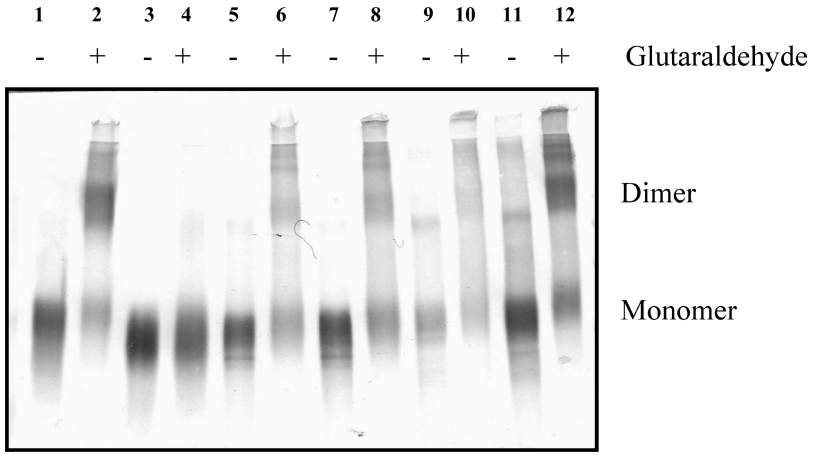

FIGURE 6: Effect of glutaraldehyde treatment on the chicken NTPDase8 and mutants. Membranes (10 μg of protein) containing wild-type chicken NTPDase8, ΔN-chicken NTPDase8, ΔN-ck-hu TMD2, ΔC-ck-hu TMD1, ck-hu TMD1,2, and chicken NTPDase8 ECD (1 μg protein) were treated without (lanes 1, 3, 5, 7, 9, and 11) and with 10 mM glutaraldehyde (lanes 2, 4, 6, 8, 10, 12) as described in Materials and Methods for 30 min at 22 °C. After addition of of lysine to terminate the reactions, aliquots were used for Western blot analysis with MC18. Key: lanes 1 and 2, wild-type chicken NTPDase8 (3 μg); lanes 3 and 4, chicken NTPDase8 ECD (0.07 μg); lanes 5 and 6, ΔN-chicken NTPDase8 (1.5 μg); lanes 7 and 8, ΔN-ck-hu TMD2 (1.5 μg); lanes 9 and 10, ΔC-ck-hu TMD1 (3.7 μg); lanes 11 and 12, ck-hu TMD1,2 (1.5 μg).

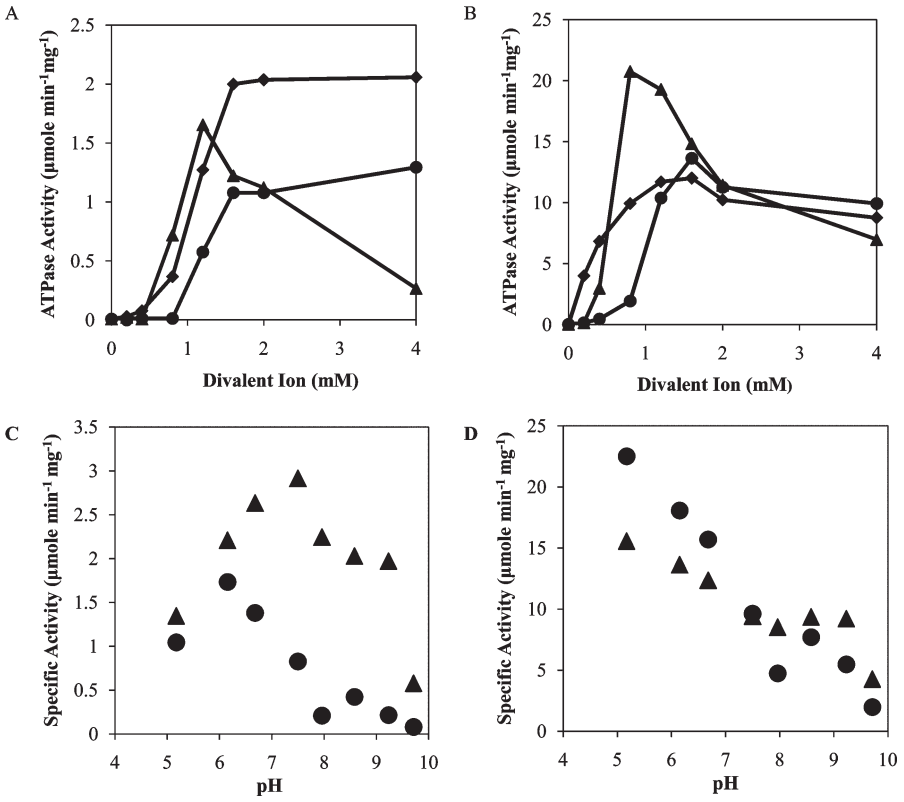

FIGURE 7: Dependence of the ATPase activities of chicken NTPDase8 and its ECD on divalent ions and pH. (A) The ATPase activity of chicken NTPDase8 was determined with $MgCl_2$ (◆), $CaCl_2$ (●), or $ZnCl_2$ (▲) at the following concentrations, 0, 0.2, 0.4, 0.8, 1.2, 1.6, 2, and 4 mM, in a 0.25 mL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM [γ-^{32}P] ATP, and 6.95 μg of membrane protein containing wild-type chicken NTPDase8. The reaction time was 10 min at 37 °C. (B) The same experiment was conducted with 1 μg of purified chicken NTPDase8 ECD. (C) The ATPase activity of the chicken NTPDase8 was determined with MgATP (▲) and MgADP (●) in a 0.25 mL reaction mixture containing 25 mM buffer, 2 mM substrates, and 5−10 μg of membrane protein. The reaction time was 10 min at 37 °C. The following buffers were used: sodium acetate (pH 5.17), imidazole (pH 6.15−6.68), Tris-HCl (pH 7.3−7.96), glycylglycine (pH 8.58−9.23), and glycine (pH 9.71). (D) The same experiment was conducted with 0.5−1 μg of purified chicken NTPDase8 ECD. Data shown were from one representative experiment.

Mg^{2+} at 4 mM total divalent ion concentration (in the presence of 1 mM EDTA). In contrast, maximal ZnATPase activity was greater than MgATPase and CaATPase activity for the chicken NTPDase8 ECD, and similar activities were obtained with all three divalent ions between 2 and 4 mM total divalent ion concentrations (in the presence of 1 mM EDTA) (Figure 7B). Calculation of free ion concentrations from the results of Figure 7A,B, using the Maxchelator program (http://www.stanford.edu/~cpatton/webmaxc/), indicated that the free Mg^{2+} and Ca^{2+} concentrations required to obtain half-maximal

Table 1: Comparison of Biochemical Characteristics of Chicken NTPDase8, Its ECD, and Truncated Mutants[a]

	membrane bound	ECD	ΔN-chicken NTPDase8	ΔN-ck-hu TMD2	ΔC-ck-hu TMD1	ck-hu TMD1,2
MgATPase/MgADPase (pH 7.5)	5.75 ± 0.89	1.02 ± 0.12	1.08 ± 0.12	0.89 ± 0.10	2.71 ± 0.15	1.44 ± 0.16
inhibition of MgADPase activity by 10 mM azide (%)	93.0 ± 0.0	59.0 ± 3.6	69.0 ± 2.1	66.0 ± 1.5	50.0 ± 6.0	91.7 ± 0.7
$K_{m,MgATP}$ (mM)	1.98 ± 0.40	0.22 ± 0.04	0.40 ± 0.06	0.47 ± 0.04	0.26 ± 0.08	0.39 ± 0.07
ATPase activity in the presence of 0.1% NP-40 (%)	119.7 ± 8.0	139.4 ± 17.2	76.5 ± 2.8	76.6 ± 7.8	98.9 ± 6.6	10.3 ± 3.3

[a]ATPase and ADPase assays were carried out with 5−50 μg of membranes prepared from HEK293 cells stably transfected with cDNAs of chicken NTPDase8, ck-hu TMD1,2 chimera, and the truncated mutants or 0.5−1 μg of purified ECD. MgATPase/MgADPase assays were performed in a 0.25 mL reaction mixture containing 25 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and 2 mM [γ-^{32}P]ATP or ADP. The amount of P_i released from ADP hydrolysis was determined colorimetrically. Azide inhibition was determined in a 0.25 mL reaction mixture containing 50 mM Mops, pH 6.4, 5 mM $MgCl_2$, and 5 mM ADP without and with 10 mM azide and membranes or ECD. $K_{m,MgATP}$ determinations were performed in a 0.25 mL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 0.05−0.80 mM MgATP, and the appropriate amounts of membrane protein and ECD. The reaction mixture was allowed to equilibrate at 37 °C for 5 min, and reaction was initiated by the addition of [γ-^{32}P]ATP and terminated after 1 min by the addition of 1.8 N $HClO_4$ (n = 3). The effect of NP-40 was determined using 1 mM [γ-^{32}P]ATP in the absence and presence of 0.1% NP-40.

activity were 2.6 and 41.3 μM for the chicken NTPDase8 ECD but 107 and 280 μM for the full-length chicken NTPDase8. Thus, the chicken NTPDase8 ECD had higher affinity for both Mg^{2+} and Ca^{2+} than the full-length enzyme.

The chicken NTPDase8 ECD also differed from the full-length enzyme with respect to the effect of pH. The pH optimum of MgATPase of the full-length chicken NTPDase8 was in the neutral pH range while the pH optimum of the MgADPase activity was more acidic at pH ~6.0 (Figure 7C). As a consequence, the MgATPase/MgADPase ratios of the full-length chicken NTPDase8 varied widely with pH. The MgATPase/MgADPase ratio was 5.75 ± 0.89 at pH 7.5 (Table 1) but 3.45 ± 0.53 at pH 6.15. In contrast, both MgATPase and MgADPase activities of the chicken NTPDase8 ECD were highest at the lowest pH tested (pH 5.0) and declined at higher pH values (Figure 7D). Similar MgATPase and MgADPase activities were obtained at all pH values tested so that the MgATPase/MgADPase ratio was 1.02 ± 0.12 at pH 7.5 (Table 1) and 0.77 ± 0.08 at pH 6.15.

As reported previously for human NTPDase8 ECD (*5*), inhibition of the MgADPase activity of the chicken NTPDase8 ECD by 10 mM azide, a common inhibitor of NTPDases that hydrolyze both NTP and NDP, was less than that of the full-length enzyme (Table 1). The K_m of the chicken NTPDase8 ECD for MgATP, 0.22 ± 0.04 mM, was 10-fold lower than that of the full-length enzyme, 1.98 ± 0.4 mM (Table 1).

Expression and ATPase Activity of Truncated Chicken NTPDase8 Mutants. The results described above for the chicken NTPDase8 ECD revealed marked differences in the biochemical properties between the soluble and the membrane-bound chicken NTPDase8. To determine if the properties of the native enzyme can be restored by anchoring the ECD to the membrane by a single TMD, we generated four constructs, which encode truncated chicken NTPDase8 proteins lacking either TMD1 or TMD2. In these mutants, the chicken NTPDase ECD is anchored to the membrane by its native TMD1 (ΔC-chicken NTPDase8), the TMD1 of the human NTPDase2 (ΔC-ck-hu TMD1), the native TMD2 (ΔN-chicken NTPDase8), or the TMD2 of the human NTPDase2 (ΔN-ck-hu TMD2) (Figure 1). ΔC-ck-hu TMD1 and ΔN-ck-hu TMD2 were included in this investigation to determine if anchorage of the chicken NTPDase8 ECD by a foreign TMD with different amino acid sequence will result in a different outcome. A chimeric protein, ck-hu TMD1,2, in which the chicken NTPDase8 ECD was anchored to the membranes by the TMD1 and TMD2 of the human NTPDase2 (Figure 1), was also generated as a control.

Table 2: ATPase Activities of HEK293 Cells Transiently Transfected with cDNAs of Chicken NTPDase8, Its Truncated Mutants, and the Ck-hu TMD1,2 Chimera[a]

cDNA	% WT ATPase activity	% protein expression	normalized activity
chicken NTPDase8	100	100	1
pcDNA3	0.87 ± 0.21	0	
ΔC-ck NTPDase8	1.04 ± 0.49	0	
ΔN-ck NTPDase8	5.50 ± 1.93	111.1 ± 6.1	0.045
ΔC-ck-hu TMD1	3.61 ± 0.0.76	8.8 ± 3.7	0.41
ΔN-ck-hu TMD2	7.41 ± 2.04	96.0 ± 3.2	0.077
Ck-hu TMD1,2	100.2 ± 10.6	97.7 ± 6.4	1.03

[a]HEK293 cells were transiently transfected with cDNA of chicken NTPDase8, pcDNA3 vector, ΔC-chicken NTPDase8, ΔN-chicken NTPDase8, ΔC-ck-hu TMD1, ΔN-ck-hu TMD2, and ck-hu TMD1,2 as described in Materials and Methods. ATPase activities of intact HEK293 cells were determined with 10−30 μg of cell protein for 10 min at 37 °C (n = 3). For determination of protein expression, the same amount of protein (2.5−15 μg) from cells transfected with the wild-type chicken NTPDase8, the truncated mutants, and the chimera was subjected to Western blot analysis as described in Materials and Methods. Protein expression was quantified by analyzing the densities on the X-ray films using Image J.

HEK293 cells transiently transfected with ΔC-ck-hu TMD1, ΔN-chicken NTPDase8, and ΔN-ck-hu TMD2 manifested cell surface ATPase activities that were lower than cells transfected with the wild-type chicken NTPDase8 cDNA and ck-hu TMD1,2. HEK293 cells transfected with ΔC-chicken NTPDase8 had negligible ATPase activity (Table 2). Western blot analysis indicated that expression of the ck-hu TMD1,2 and ΔN mutants was comparable to that of the wild-type chicken NTPDase8, whereas expression of the ΔC mutants was undetectable (ΔC-chicken NTPDase8) or low (ΔC-ck-hu TMD1) (Table 2). The normalized activity of the ΔN mutants was 5−8% of that of the full-length enzyme, and that of ΔC-ck-hu TMD1 was 41%. The decreased activities of the truncated mutants were not due to incomplete glycosylation or processing, which would result in misfolding or mistargeting of the mutant proteins. Protein bands of similar molecular mass to that of the untreated samples (Figure 8A, lanes 1, 3, 5, 7, and 9) were obtained upon treatment of the membranes by Endo H (Figure 8A, lanes 2, 4, 6, 8, and 10), indicating that the chimeric and truncated chicken NTPDase8

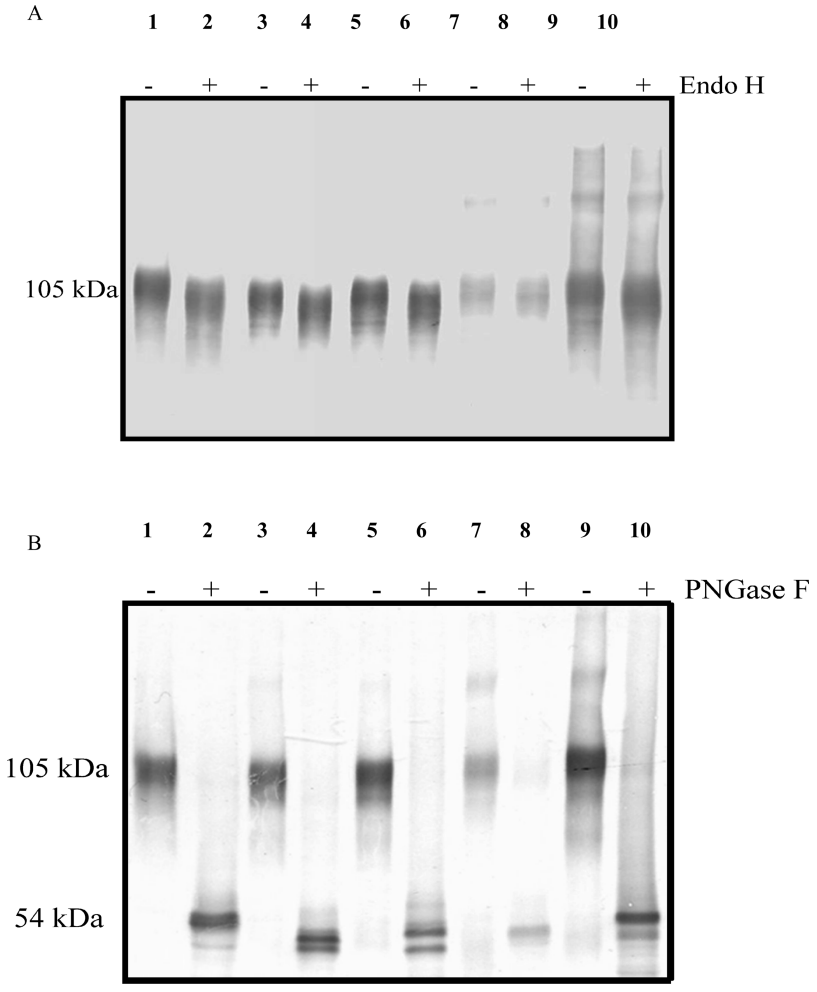

FIGURE 8: Effect of treatment of Endo H and PNGase F on the wild-type chicken NTPDase8, its truncated mutants, and the ck-hu TMD1,2 chimera. (A) Membranes (10 μg of protein) containing the wild-type chicken NTPDase8, ΔN-chicken NTPDase8, ΔN-ck-hu TMD2, ΔC-ck-hu TMD1, and ck-hu TMD1,2 were treated without (lanes 1, 3, 5, 7, 9) and with Endo H (lanes 2, 4, 6, 8, 10) as described in Materials and Methods. (B) The same five membranes were treated without and with PNGase F as described. The amounts of proteins used for Western blot analysis were 3.8 μg for wild-type NTPDase8 (lanes 1 and 2), 2.5 μg for ΔN-chicken NTPDase8 (lanes 3 and 4) and ΔN-ck-hu TMD2 (lanes 5 and 6), 7.4 μg for ΔC-ck-hu TMD1 (lanes 7 and 8), and 2.5 μg for ck-hu TMD1,2 (lanes 9 and 10). The bands above the 105 kDa bands in lanes 7 and 9 in both panel A and panel B are attributed to disulfide-linked dimers that formed spontaneously due to the presence of a cysteine residue in the TMD1 of human NTPDase2 (Figure 1), which constitutes the N-terminus of these two mutants.

mutants are properly processed as the wild-type enzyme. Treatment of the membranes containing the truncated mutants by PNGase F generated polypeptides (Figure 8B, lanes 4, 6, and 8) similar in sizes as those of the deglycosylated wild-type enzyme (Figure 8B, lane 2) and ck-hu TMD1,2 (Figure 8B, lane 10). The detection of two protein bands in the deglycosylated ΔN mutants suggests that these proteins may be more prone to proteolytic degradation, which may contribute to their lower activities.

The other possible cause of the lower activities of the truncated mutants is an inability to former oligomers, which has been proposed to explain the reduction of activity of many NTPDase mutants and the soluble NTPDases (*3*, *15*). The inability to form oligomers also applies to the chicken NTPDase8 ECD. While the wild-type chicken NTPDase8 formed a dimer after the membranes were treated with glutaraldehyde (Figure 6, lane 2), the chicken NTPDase8 ECD remained as a monomer under the same treatment (Figure 6, lane 4). In contrast, dimer formation was observed for all three truncated mutants and the ck-hu TMD1,2 chimera after glutaraldehyde treatment (Figure 6, lanes 6, 8, 10, and 12). These results support the previous proposal that only oligomers formed from interaction of heterologous TMDs express full activity (*3*). However, it is more likely that the lower activity of the truncated mutants is related to altered conformations of their extracellular domains as compared to that in the wild-type enzyme or the ck-hu TMD1,2 chimera.

The Truncated Chicken NTPDase8 Mutants Also Undergo Substrate Inactivation and Product Inhibition. Because the ΔC-chicken NTPDase8 mutant protein was not expressed, characterization of the truncated mutants was only carried out with ΔN-chicken NTPDase8, ΔN-ck-hu TMD2, and ΔC-ck-hu TMD1.

In contrast to the chicken NTPDase8 and the ck-hu TMD1,2 in which the chicken NTPDase8 ECD is anchored to the membrane by two TMDs, the ATPase activities of the three truncated mutants also declined with time (compare data in Figure 9 with that in the inset of Figure 3). Thus, the truncated mutants behaved like the soluble chicken NTPDase8. However, the decrease of activity of the ΔC-ck-hu TMD1 was 40% (inset in Figure 9) compared to 60% of the ΔN-chicken NTPDase8 and 75% of the ΔN-ck-hu TMD2 in a 10 min reaction period (Figure 9). The decrease of ATPase activity of the truncated mutants with time could also be attributed to substrate and product inhibition. Preincubation of ΔN-chicken NTPDase8 and ΔN-ck-hu TMD2 with ATP, ADP, and P_i resulted in a

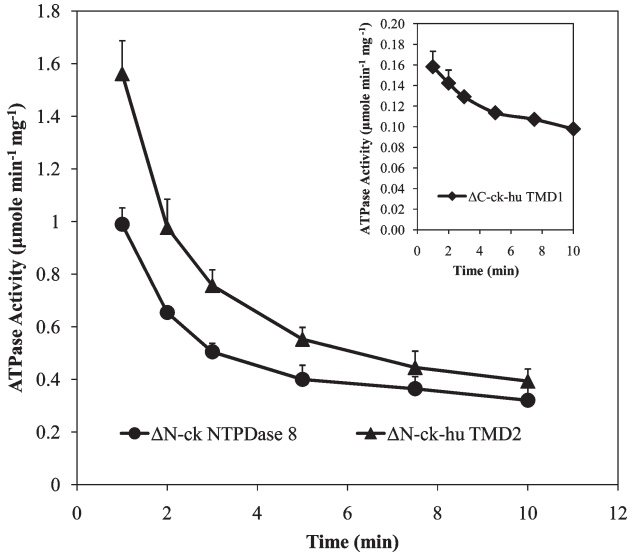

FIGURE 9: Time course of ATP hydrolysis of ΔN-chicken NTPDase8, ΔN-ck-hu TMD2, and ΔC-ck-hu TMD1 mutants at 37 °C. ATPase reactions were carried out in a 0.25 mL reaction mixture containing 25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 1 mM [γ-^{32}P]ATP with 15−30 μg of membrane proteins containing ΔN-chicken NTPDase8 (●), ΔN-ck-hu TMD2 (▲), and ΔC-ck-hu TMD1 (◆) for the indicated times at 37 °C (n = 3). Data obtained with ΔC-ck-hu TMD1 are shown in the inset.

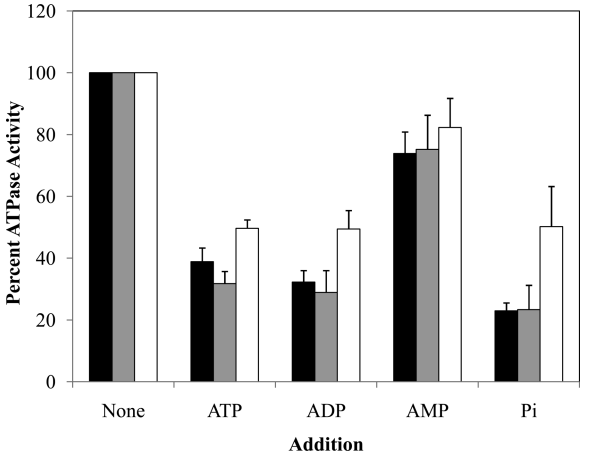

FIGURE 10: Effect of preincubation with substrates or products on the ATPase activities of the truncated chicken NTPDase8 mutants. Membranes containing ΔN-chicken NTPDase8 (black bars), ΔN-ck-hu TMD2 (gray bars), and ΔC-ck-hu TMD1 (white bars) were incubated in a 0.25 mL reaction mixture containing 50 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ in the absence or presence of 1 mM ATP, ADP, AMP, or P_i for 5 min at 37 °C. Reactions were initiated by the addition of 0.25−0.50 μmol of [γ-^{32}P]ATP. The reaction time was 1 min at 37 °C (n = 3).

60−80% decrease of activity, but the decrease of activity for ΔC-ck-hu TMD1 was only 50% (Figure 10), which agreed with the less severe loss of activity with time of this mutant shown in Figure 9. Preincubation of the truncated mutants with AMP resulted in little loss of activity (Figure 10).

Similar to the chicken NTPDase8 ECD (Figure 5A), the ATPase activities of the ΔN-chicken NTPDase8 and ΔN-ck-hu TMD2 mutants also decreased at temperatures higher than 25 °C, although less sharply (Figure 11). The ATPase activity of the ΔC-ck-hu TMD1 declined at temperatures higher than 37 °C (inset of Figure 11).

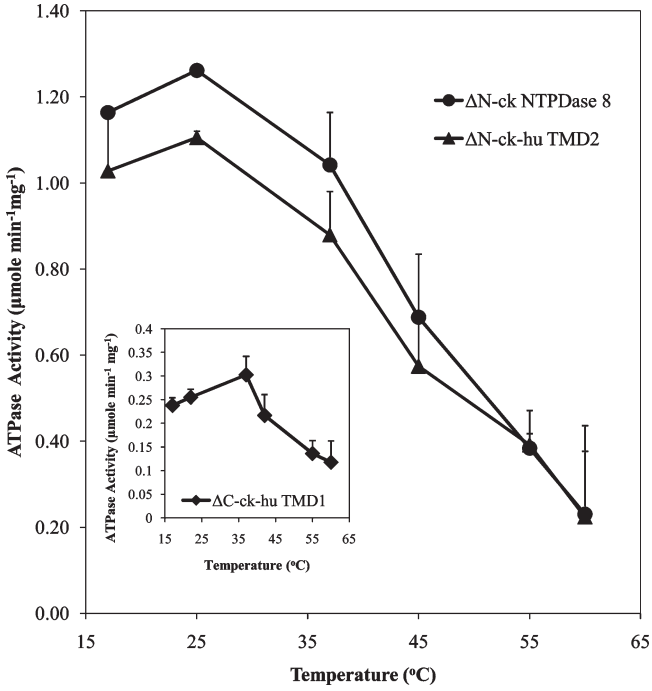

FIGURE 11: Effect of temperature on the ATPase activity of the truncated chicken NTPDase8 mutants. ATPase reactions were carried out in a 0.25 mL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and 2 mM [γ-^{32}P]ATP for 1 min at the indicated temperatures with 15−30 μg of membrane proteins containing ΔN-chicken NTPDase8 (●), ΔN-ck-hu TMD2 (▲), or ΔC-ck-hu TMD1 (◆) (n = 3). Data obtained with ΔC-ck-hu TMD1 are shown in the inset.

The MgATPase/MgADPase ratios of the ΔN-chicken NTPDase8 and ΔN-ck-hu TMD2 mutants were similar to that obtained with the chicken NTPDase8 ECD (Table 1), whereas the MgATPase/MgADPase ratio of the ΔC-ck-hu TMD1 was intermediate of that of the full-length enzyme and the ECD. Inhibition of the MgADPase activity by 10 mM azide and K_m values for MgATP of all three mutants were generally similar to that of the chicken NTPDase8 ECD (Table 1). Interestingly, in contrast to the full-length chicken NTPDase8 and its soluble ECD, whose activities were not affected by detergents or the ck-hu TMD1,2, whose activity was decreased to 10% by 0.1% NP-40, the activities of the ΔN mutants were moderately reduced by NP-40 (Table 1).

From the results described above, we conclude the following: (1) Unlike the full-length chicken NTPDase8, the truncated mutants are susceptible to substrate and product inactivation. Thus anchorage of the chicken NTPDase8 ECD by either an N- or a C-terminal TMD is insufficient to restore the properties of the full-length enzyme to the ECD. (2) The biochemical properties of the truncated chicken NTPDase8 mutants lacking the N-terminal TMD (ΔN-chicken NTPDase8 and ΔN-ck-hu TMD2) are more similar to the soluble chicken NTPDase8 than the mutant lacking the C-terminal TMD (ΔC-ck-hu TMD1).

DISCUSSION

The membrane anchorage of NTPDases is unusual among the membrane proteins. NTPDases 1−3, 4, 7, and 8 are anchored to plasma membrane or intracellular membranes by two TMDs near their N- and C-termini with very short cytoplasmic domains and NTPDases 5 and 6 are anchored to the ER or Golgi membranes by a single TMD near their N-termini (*1*). The extracellular domains

of the cell surface NTPDases and the intraluminal domains of the intracellular NTPDases, which are hydrophilic, contain conserved regions required for their catalytic activity. In view of the difficulty of crystallizing membrane glycoproteins, the potential utility of engineered water-soluble NTPDases in the determination of their three-dimensional structures is generally recognized. The structure of a bacterially expressed rat NTPDase2 was elucidated recently (*8*). However, it should be noted that unlike the native enzyme or enzyme expressed in mammalian cells, the bacterially expressed protein is not glycosylated. Crystallization of the soluble rat NTPDase1 expressed in insect cells has also been reported (*16*).

The first recombinant water-soluble NTPDase expressed was that of human CD39/NTPDase1 (*2*), the major NTPDase in vascular tissues (*17*) that plays an important role in thromboregulation (*18*). Successful application of the soluble human CD39 (solCD39) in inhibiting platelet aggregation *in vitro* (*2*) and in the animal model (*19*) has been reported. SolCD39 had an enhanced ADPase/ATPase ratio (1.5−2.8) compared to that of the native enzyme (∼1), and the specific ATPase activity of the purified solCD39 was ∼20 μmol min^{-1} mg^{-1} (*20*). While the native human CD39/NTPDase1 has not been purified, ATPase activity in the range of ∼1000 μmol min^{-1} mg^{-1} or greater has been reported for purified proteins of the NTPDase superfamily, e.g., rabbit and chicken NTPDase2 (*21*, *22*), chicken NTPDase8 (*11*, *12*), potato apyrase (*23*), and *Toxoplasma* NTPase (*24*); the last two are naturally occurring soluble proteins. The remarkably high nucleotide hydrolase activity, compared to the membrane-bound P-, V-, and F-type ATPases, is a unique characteristic of the NTPDases. In contrast, significantly lower CaATPase activity in the range of ∼10−70 μmol min^{-1} mg^{-1} was reported for partially purified soluble rat CD39/NTPDase1 (*3*), human NTPDase8 (*5*), and human NTPDase2 (*7*), besides solCD39. MgATPase activity of these soluble NTPDases was even lower (*5*, *7*, *20*). Somewhat higher activity, i.e., 40−200 μmol min^{-1} mg^{-1}, was obtained for the bacterially expressed ectodomains of rat NTPDase 1, 2, and 3 after refolding (*6*). Thus, the general consensus that the soluble NTPDases have lower activity was reached by comparing the activity of purified ECD (*2*−*7*) with purified membrane-bound NTPDases from other species (*11*, *12*, *21*, *22*). One of the four NTPDases purified from their native tissues, the chicken liver NTPDase8, has a specific activity of ∼1200 μmol min^{-1} mg^{-1} (*12*). In the present report, we showed that the specific activity of the soluble chicken NTPDase8 is ∼20 μmol min^{-1} mg^{-1}. The results from these two studies provided the first direct comparison of the activities of the chicken NTPDase8 without and with its two TMDs. Even taking into account that the chicken NTPDase8 ECD was ∼60% pure and that the activity obtained in a 10 min assay was ∼25% of that obtained in a 1 min assay, the maximal ATPase activity of the purified chicken NTPDase8 ECD would be ∼160 μmol min^{-1} mg^{-1}, which is at most 15% of the activity of the purified full-length enzyme (*12*). Thus, it can be concluded without ambiguity that the chicken NTPDase8 requires the presence of both its TMDs to achieve maximal activity. The same conclusion likely applies to other cell surface NTPDases.

It has been previously proposed that oligomeric NTPDases are more active than their monomeric forms (*3*, *15*, *25*−*27*). Disruption of the oligomers by detergents explains the decrease of activity of most membrane-bound NTPDases in the presence of detergents. The lower activity of the NTPDase ECDs has also been attributed to the failure of the ECD to form oligomers, which requires the interaction of TMD1 and TMD2 of two or more NTPDase monomers (*3*, *15*). While the issue of the existence of stable oligomers of the NTPDases is not completely resolved (*7*, *28*), the importance of intramolecular TMD interaction in regulating the catalytic activity in the ECD is now well established. Published reports showed that (1) TMDs of most cell surface NTPDases mediate the inhibitory effect of detergents (*3*, *7*, *13*, *29*), (2) restricted helical mobility by oxidative cross-linking of the two TMDs in the rat NTPDase1 and human NTPDase2 reduced their activity (*30*, *31*), and (3) modification of an intramembrane cysteine residue in the TMD1 of human NTPDase2 by *p*-(chloromercuri)benzenesulfonate resulted in inhibition of its activity (*31*). We further demonstrated that the strength of TMD interaction is isoform specific for human NTPDase2 and chicken NTPDase8 in studies using chimeras in which the TMDs of the two enzymes were exchanged (*7*, *13*, *32*) as well the data obtained with the ck-hu TMD1,2 chimera presented in this report. While the wild-type chicken NTPDase8 is resistant to detergent inactivation, the ck-hu TMD1,2 chimera in which the TMDs of the chicken NTPDase8 are substituted by those of the unstable human NTPDase2 suffered a 90% loss of activity in the presence of 0.1% NP-40 (Table 1).

Besides a marked decrease of catalytic activity, the soluble NTPDases also showed generally higher CaATPase than MgATPase activity, lower ATPase/ADPase ratios, and increased affinity for ATP when compared to the full-length enzymes (*5*−*7*). Mutation of the same residues in human CD39/NTPDase1 had a different effect on the soluble and full-length enzymes. While mutation of D54, S57, and D213 to alanine increased the CaADPase activity of the solCD39 up to 2-fold (*20*, *33*), mutation of the same residues decreased the CaADPase activity to less than 40% of that of the full-length human CD39/NTPDase1 (*34*). The most striking difference between soluble and membrane-bound NTPDase was demonstrated with human NTPDase2, which is primarily an NTPase. While the membrane-bound human NTPDase2 is susceptible to inactivation by NTPs and high temperature, the soluble human NTPDase2 is not (*7*). Thus it appears that the molecule composed only of the human NTPDase2 ECD is a "stable" protein albeit with lower activity. When the human NTPDase2 ECD is anchored to the membrane by its two TMDs, it assumes a conformation such that its V_{max} increases but it becomes inactivated by substrates and high temperature. In contrast, different results were obtained with the human NTPDase8. Both the full-length and soluble human NTPDase8 display a linear time course of ATP hydrolysis, and their activities increase with temperature up to 55 °C (*5*). The studies described above caution against applying the finding of one cell surface NTPDase to another. Indeed, the ECD derived from the uniquely stable chicken NTPDase8 yielded unexpected results.

The chicken NTPDase8 ECD, when freed from the restraints imposed by the TMDs, becomes susceptible to inactivation by substrates and inorganic phosphate in a temperature-dependent manner. It is not understood why such a regulatory mechanism occurs in the chicken NTPDase8 ECD but not the ECD of the human NTPDases 2 and 8 (*5*, *7*). Nevertheless, the results presented in this and other published studies suggest that the structures of the ECD of the NTPDases with and without the two TMDs are most likely different. While the three-dimensional structure of bacterially expressed rat NTPDase1 shows the overall protein fold and the atomic details of the ATP binding site (*8*), the effect of membrane anchorage on the catalytic and

regulatory mechanisms of the full-length cell surface NTPDases cannot yet be fully understood from such a structure.

We then determined (1) if the susceptibility of the chicken NTPDase8 ECD to substrate and product inactivation can be abolished if the ECD is attached to the membrane by a single TMD either at the N- or at the C-terminus and (2) if anchorage by a foreign TMD will make a difference. Thus, we generated the truncated chicken NTPDase8 mutants in which its ECD is anchored to the membrane either by its native TMD1 and TMD2 or by the TMD1 and TMD2 of the human NTPDase2. Truncated mutants of the rat CD39/NTPDase1 had previously been generated, and a decrease of activity was noted (*3*). However, the primary focus of the study by Wang et al. was on the response of the mutants to detergents and their ability to form oligomers (*3*). The present study on the truncated mutants of the chicken NTPDase8 focused on the comparison of their biochemical characteristics with both the full-length enzyme and its ECD. Importantly, we found that protein expression and characteristics were affected differently depending on whether the ECD was anchored to the membrane by TMD1 or TMD2. Mutants without a TMD2 were only poorly or not expressed, whereas expression of mutants lacking a TMD1 was comparable to that of the wild-type enzyme but with compromised activity. The three expressed truncated mutants of chicken NTPDase8 generally behaved like the ECD with respect to substrate and product inactivation. As a control for the ΔC-ck-hu TMD1 and ΔN-ck-hu TMD2, we generated the ck-hu TMD1,2 chimera in which the chicken NTPDase8 ECD is anchored to the membrane by the two TMDs of the human NTPDase2 and determined its biochemical properties. It is noteworthy that the chicken NTPDase8 is more tolerant of the grafting of foreign N- and C-terminal peptides with respect to protein expression and enzyme activity (*13*, *32*) compared to human NTPDase2 (*7*). The protein expression, activity, and resistance to substrate and product inactivation of ck-hu TMD1,2 are all similar to that of the wild-type chicken NTPDase8. These results further support the conclusion that anchorage of the chicken NTPDase8 ECD by a single TMD is insufficient in restoring the full activity and properties of the full-length enzyme to the ECD. ΔN-chicken NTPDase8 and ΔN-ck-hu TMD2, which lack TMD1, are generally more similar to the ECD than the mutant lacking TMD2, which may be related to their biogenesis. For ΔC-ck-hu TMD1, TMD1 is anchored in the membrane before the synthesis of the ECD. In contrast, the leader sequence necessary for the generation of the ECD and ΔN mutants is presumably cleaved, and the folding path of the chicken NTPDase8 ECD and the ΔN mutants in the ER lumen will be more or less identical even though the latter have a TMD at their C-termini.

In summary, the biochemical properties of the three truncated mutants exhibit very similar responses to the same modulators as that of the soluble chicken NTPDase8 with two notable differences: (1) protein expression was affected differently depending on which TMD was removed, and (2) ΔN-chicken NTPDase8 and ΔN-ck-hu TMD2 behaved more like the ECD than ΔC-ck-hu-TMD1 with respect to substrate and product inactivation. These results have provided further insight into the roles of the N- and the C-terminal TMD in the expression and function of chicken NTPDase8.

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