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Regulation of the Human NTPDase 2 Activity by Its Transmembrane Domains

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Chemistry

by

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The Dissertation of Wei-Chieh Chiang is approved, and it is acceptable in quality and form for publication on microfilm:

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2008

Dedication to my LORD Jesus Christ, my beloved wife and my whole family

for their love, prayers, support, patience, and guidance

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LIST OF ABBREVIATIONS

ACR	apyrase conserved region
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ConA	concanavalin A
Chaps	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMC	critical micelle concentration
CuP	copper phenanthroline
DMEM	Dulbecco's modified Eagle's media
DSS	disuccinimidyl suberate
ECD	extracellular domain
EDTA	ethylenediaminetetraacetic acid
E-NTPDase	ecto-nucleoside triphosphate diphosphohydrolase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Mops	3-(N-morpholino)-propanesulfonic acid
NBT/BCIP	nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate
NDPs	nucleoside diphosphates
NP-40	nonidet P-40
NTPs	nucleoside triphosphates
NTPDase	nucleoside triphosphate diphosphohydrolase
pCMB	*p*-chlomercuribenzoate

pCMPS	*p*-chloromercuriphenylsulfonate
PCR	polymerase chain reaction
PFO	pentadecafluorooctanoic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TMD	transmembrane domain
TMD1	N-terminal transmembrane domain
TMD2	C-terminal transmembrane domain
WT	wild-type

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FIELDS OF STUDY

Major Field: Biochemistry, Molecular Biology

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Professor Aileen F. Knowles

ABSTRACT OF THE DISSERTATION

Regulation of the Human NTPDase 2 Activity by Its Transmembrane Domains

by

Wei-Chieh Chiang

Doctor of Philosophy in Chemistry

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San Diego State University, 2008

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Nucleoside triphosphate diphosphohydrolases (NTPDases) are ubiquitous enzymes hydrolyzing NTPs and/or NDPs. The human NTPDase 2 is a cell-surface NTPDase containing a large extracellular domain (ECD), two transmembrane domains (TMDs), short cytoplasmic tails at the N- and C-termini, and five apyrase conserved regions (ACRs).

Human NTPDase 2, whether in the native tissue or expressed in tissue culture cells, displays several unique characteristics: inactivation by detergents, temperature, and substrate, and inhibition by pCMPS. These inhibitory effects are overcome by concanavalin A or glutaraldehyde that promote oligomerization.

Since detergents and high temperatures perturb membranes, their inhibitory effects on human NTPDase 2 activity are likely mediated through the TMDs. To investigate this possibility, chimeric NTPDase 2 and a soluble human NTPDase 2 consisting of only its ECD were generated. The soluble human NTPDase 2 was no

longer inactivated by membrane perturbation. These characteristics were also displayed by the hu-ck ACR1,5 chimera in which the ECD is anchored to the membrane by the two TMDs of the chicken NTPDase 8, which is not affected by membrane perturbation. These results indicate that the strength of interactions of the respective TMD pairs of the human NTPDase 2 and chicken NTPDase 8, which may be dictated by their amino acid sequences, determine their different responses to membrane perturbation.

The target of pCMPS modification is a free cysteine residue at position 26 in the N-terminal TMD. A mutant in which C26 was replaced by a serine was no longer inhibited by pCMPS. Inhibition of pCMPS may result from the combined effects of disturbance of TMD interaction and active site conformation, since C26 is situated close to ACR1.

While oligomer formation promoted by ConA or glutaraldehyde increases the human NTPDase 2 activity, oxidative cross-linking of wild-type enzyme and mutants containing a single cysteine residue in the C-terminal TMD is accompanied by reduction of ATPase activity. Similar reduction of activity is also obtained upon intramolecular disulfide formation of mutants containing a cysteine residue in both TMDs. These results indicate that mobility of TMDs is necessary for maximal catalysis.

General Introduction

Extracellular nucleotides and purinergic signaling

Adenosine 5'-triphosphate (ATP) is primarily known as the energy currency of the cell. The majority of ATP is produced in the mitochondria and the intracellular ATP concentration ranges from 1-10 mM. Upon its hydrolysis, ATP releases a substantial amount of free energy to power most of the energy-consuming activities of cells, such as active transport, muscle contraction, and anabolic reactions, e.g., biosynthesis of macromolecules and lipids. ATP is also used as a substrate by kinases that phosphorylate proteins and lipids involved in signal transduction, as well as by adenylate cyclase, which uses ATP to produce cyclic AMP, a second messenger.

A less well known role of ATP is its function as a signaling molecule. Extracellular ATP, whose concentration is usually in the nM range, elicits a multiplicity of receptor-mediated physiological responses (1, 2). The first unequivocal evidence that ATP affects a variety of tissues including heart, vasculature and smooth muscle was reported by Gillespie in 1934. He showed that upon ATP injection, the blood pressure of the animals increased temporarily (3). In 1972, Burnstock proposed that extracellular ATP was a transmitter involved in non-adrenergic, non-cholinergic nerve-mediated responses of the smooth muscle in the gut and bladder (4) and introduced the term "purinergic" (5).

Two types of purinergic receptors have been identified, P1 and P2 receptors (for adenosine and ATP/ADP, respectively). Four subtypes of P1 receptors have been cloned and characterized (6). All of them are members of the rhodopsin-like G

protein-coupled receptors. P2 receptors are subdivided into ionotropic P2X subtypes, that are ligand-gated ion channels, and metabotropic P2Y subtypes, that are G protein-coupled receptors (6). To date, seven P2X and eight P2Y receptor subtypes have been cloned and characterized. P2X receptors respond to ATP, whereas P2Y receptors can be activated by ATP, ADP, UTP, UDP, and UDP glucose/galactose, with variable agonist specificity (6, 7, 8). Both P2X and P2Y receptors are ubiquitously expressed. Depending on the tissues, the extracellular nucleotides activate immune responses, inflammation, pain, platelet aggregation, and regulate vasodilatation, cellular adhesion, cell proliferation, differentiation, and apoptosis (7-11).

The magnitude and type of purinergic signaling can be regulated by hydrolysis of extracellular nucleotides via ectonucleotidases. A considerable number of surface-located enzymes can potentially be involved, including ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) (12), ecto-nucleotide pyrophosphatases (E-NPP) (13), alkaline phosphatases (14), ecto-5'-nucleotidase (15, 16), and ectonucleoside diphosphokinase (E-NDPK) (17). These ecto-enzymes not only display overlapping tissue distrubutions, but also overlapping substrate specificities. Extracellular nucleoside tri- and -diphosphates may be hydrolyzed to nucleoside monophosphates by members of the E-NTPDase family, E-NPP family, and by alkaline phosphatases. Nucleoside monophosphates are subsequently subjected to hydrolysis by ecto-5'-nucleotidase, some members of the E-NPP family, and alkaline phosphatases. The end products, such as adenosine, can

subsequently activate the P1 receptors. Therefore, the ecto-nucleotidases may dictate a number of physiological responses by regulating purinergic receptor activation.

The E-NTPDases, members of the NTPDase family, are considered to be the dominant ectonucleotidases. Four members of this family, NTPDase 1, 2, 3 and 8, are relevant to P2 receptor signaling, since they are located at the cell surface and hydrolyze nucleotides in the range of concentration that activates P2 receptors (18-23) (Fig. G.1.). The physiological functions of the cell surface NTPDases are under active investigation. It has been demonstrated that CD39, an NTPDase 1, regulates platelet aggregation (24), and NTPDase 2 regulates the eye development in Xenopus laevis through P2Y receptors (25).

Molecular identification of NTPDases

The NTPDases, which include the E-NTPDases, constitute a novel ecto-nucleotidase family. They were previously referred to as E-type ATPases (26). The molecular identity of the prototypic member of the NTPDase family, NTPDase1, was not established until 1994. It was first cloned as a lymphocyte cell activation antigen, CD39, of undetermined function (27). Subsequently, Handa and Guidotti purified and cloned the potato tuber apyrase, a soluble enzyme that also hydrolyzes ATP and ADP. In their search of homologous sequences, they noted that this protein was related to the protozoan NTPases, NTP1 and NTP3 from *Toxoplasma gondii*, garden pea NTPase, *Saccharomyces cerevisiae* Golgi guanosine diphosphatase, and also human and murine CD39 (28). Sequence alignments

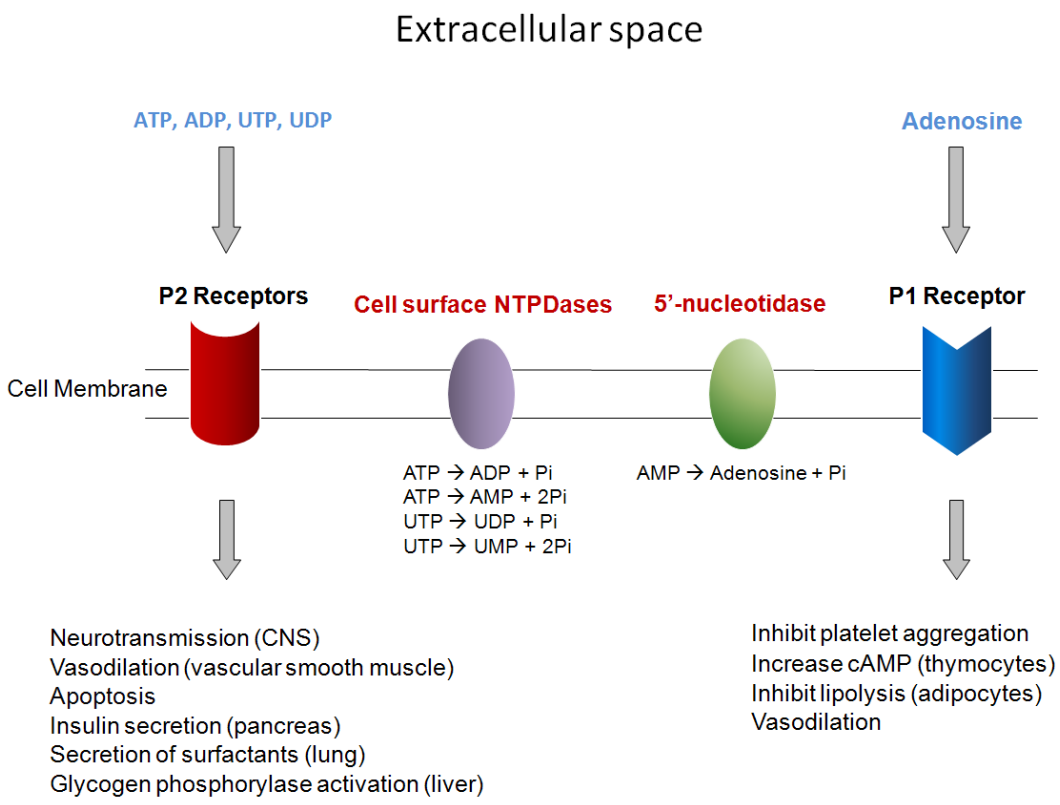

Figure G.1. Proposed physiological functions of cell surface NTPDases. The cell surface NTPDases regulate purinergic signaling by hydrolyzing the extracellular ATP, ADP, UTP, and UDP, with different substrate preferences. The 5'-nucleotidase can further hydrolyze AMP to produce adenosine, which is the ligand of P1 receptors.

of these proteins revealed four conserved regions, which were named ACRs (apyrase conserved regions). In 1996, Wang and Guidotti demonstrated by functional expression that human CD39 was, in fact, an ecto-apyrase (19).

To date, eight NTPDase subfamilies have been cloned from vertebrates. The tissue or subcellular distribution of the NTPDases, their previously used names, and their substrate preference are summarized in Table G.1. As mentioned

earlier, NTPDase 1, 2, 3, and 8 are associated with plasma membrane, whereas NTPDase 4-7 are intracellular. Except for NTPDase 7, the intracellular NTPDases prefer NDP, e.g., UDP and GDP, as substrate.

Three of the NTPDases were cloned in our laboratory: (i) the human NTPDase 2 from NCI H69 small cell lung carcinoma cells (29), (ii) the chicken NTPDase 8 from chicken liver (22), and (iii) the human NTPDase 8 from human liver (30).

Table G.1. Tissue and cellular distribution, previously used names, and substrate preferences of NTPDases.

Name	Previously used name	Distribution	Substrate preference
NTPDase 1	CD39, ecto-ATP diphosphohydrolase, ecto-apyrase	Vascular tissues, brain, lymphocyte	NTP ~ NDP
NTPDase 2	CD39L1, ecto-ATPase	Muscle, brain, tumor cells	NTP >>> NDP
NTPDase 3	CD39L3, HB6	Brain, digestive system	NTP > NDP
NTPDase 4	UDPase (hLALP70v), hLALP70	(Golgi)	UDP > GDP > CDP
NTPDase 5	CD39L4, ER-UDPase, PCPH	(ER)	UDP > GDP > CDP
NTPDase 6	CD39L2	(Golgi), heart, brain	GDP > IDP > UDP
NTPDase 7	LALP1, endo-apyrase	(Lysosome)	UTP, GTP, CTP
NTPDase 8	ecto-ATPDase	Liver, oviduct, stomach	NTP > NDP

Structural features of NTPDases

The four cell surface NTPDases, NTPDase 1, 2, 3, and 8, have unusual membrane topology in that a large extracellular domain containing the active site is anchored to the plasma membranes by two transmembrane domains near the N- and C-termini flanked by short cytoplasmic peptides. In contrast, NTPDases 4-7 are anchored to the membranes of intracellular organelles by one (NTPDases 5 and 6) or two (NTPDases 4 and 7) transmembrane domains and their catalytic site faces the lumen of intracellular compartments such as the Golgi apparatus and the endoplasmic reticulum (Fig. G.2.). NTPDase 5 and 6 can also be secreted as soluble enzymes following proteolytic cleavage.

The eight NTPDases share the five apyrase conserved regions (ACRs) that are present in the potato apyrase and other NTPases (28, 31). The ACRs have been shown to be essential for catalytic activity (32, 33). The cell surface NTPDases also contain four other conserved regions (CRs) (34), and 10 conserved cysteine residues in the extracellular domain. The conserved motifs are highlighted in the human NTPDase 2 sequence (Fig. G.3). Glycosylation of the different NTPDases is variable. Therefore, the molecular masses of the mature cell surface NTPDases range from 66 to 85 kDa, even though the polypeptide chains deduced from their cDNA sequences have a similar size, i.e., 54 kDa. Apart from the ACRs and the CRs, the sequences of the different NTPDases vary greatly.

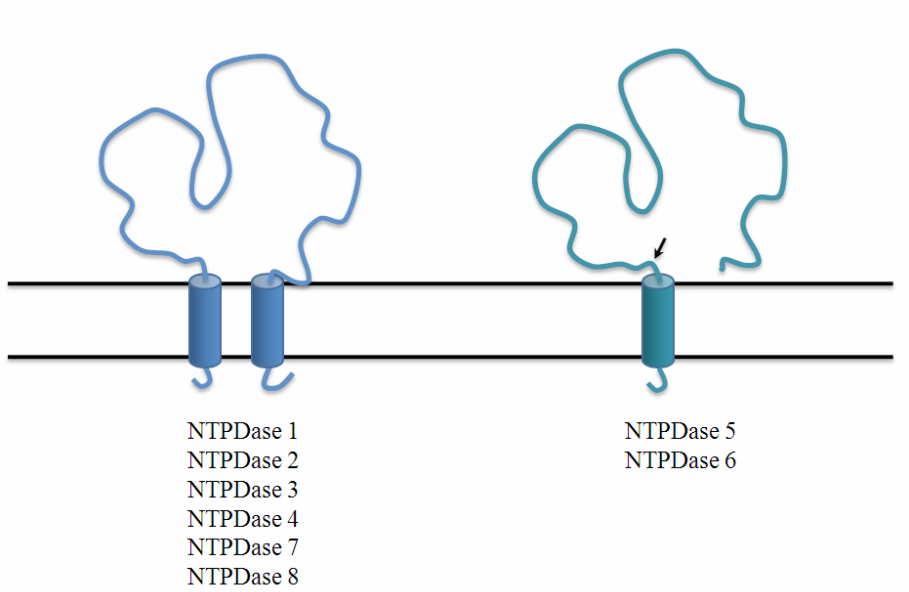

Figure G.2. Predicted membrane topography of NTPDases. NTPDase 1, 2, 3, 4, 7, and 8 contain an N-terminal and C-terminal transmembrane domain, respectively. NTPDase 5 and NTPDase 6 lack the C-terminal transmembrane domain and may be cleaved close to the N-terminus (black arrow) to form soluble proteins.

```
  1 MAGKVRSLLPPLLLAAAGLAGLLLLCVPTRDVREPPALKYGIVLDAGSSH
 51 TSMFIYKWPADKENDTGIVGQHSSCDVPGGGISSYADNPSGASQSLVGCL
101 EQALQDVPKERHAGTPLYLGATAGMRLLNLTNPEASTSVLMAVTHTLTQY
151 PFDFRGARILSGQEEGVFGWVTANYLLENFIKYGWVGRWFRPRKGTLGAM
201 DLGGASTQITFETTSPAEDRASEVQLHLYGQHYRVYTHSFLCYGRDQVLQ
251 RLLASALQTHGFHPCWPRGFSTQVLLGDVYQSPCTMAQRPQNFNSSARVS
301 LSGSSDPHLCRDLVSGLFSFSSCPFSRCSFNGVFQPPVAGNFVAFSAFFY
351 TVDFLRTSMGLPVATLQQLEAAAVNVCNQTWAQLQARVPGQRARLADYCA
401 GAMFVQQLLSRGYGFDERAFGGVIFQKKAADTAVGWALGYMLNLTNLIPA
451 DPPGLRKGTDFSSWVVLLLLFASALLAALVLLLRQVHSAKLPSTI
```

Figure G.3. Domain structure of the human NTPDase 2. The primary structure of the human NTPDase 2 is shown. The two transmembranous domains are shaded in light gray. The five apyrase conserved regions (ACR) are shaded in green. The four conserved regions (CR) are shaded in yellow. Ten conserved cysteine residues are shown in red.

Thus far, the three-dimensional structures and active sites of the NTPDases have not been elucidated. The most common ATP binding motifs among ATPases are the Walker A and B sequences, which are $(G,A)X_4(G,A)K(S,T)$ and $(R,K)X_{1-4}GX_{2-4}\phi X\phi(D,E)$, respectively, where ϕ is a hydrophobic amino acid, and X is any amino acid (35). Both motifs contain the positively charged lysine and arginine residues. However, the NTPDases lack the consensus Walker ATP binding sequences; instead, the deduced amino acid sequences of the NTPDases have been shown to share two common sequence motifs with members of the actin-HSP70-hexokinase superfamily (36). Based on the X-ray structures of actin, HSP70, and hexokinase, five sequence motifs in this superfamily are shown to be involved in ATP binding. These five sequence motifs consist of approximately 20 amino acids each and are termed phosphate 1, connect 1, phosphate 2, adenosine, and connect 2. The sequence of the phosphate 1 motif [(I/L/V)X(I/L/V/C)DXG(T/S/G)(T/S/G)XX (R/K/C)] (37), which contains a DXG sequence that is strictly conserved, can be found in the ACR1 of all the NTPDases. The phosphate 2 motif, which also contains a DXG conserved sequence, corresponds to ACR4 in these proteins (28, 31-33). Table 2 shows the sequence alignment of the phosphate 1 and phosphate 2 motif sequences of the actin-HSP70-hexokinase superfamily with human NTPDase 2 and 8 and chicken NTPDase 8. Smith and Kirley showed that mutations of the conserved aspartate and glycine residues in both ACR1 and 4 of human brain NTPDase 3 dramatically reduced ATP and ADP hydrolysis rates (32).

In the absence of a crystal structure of an NTPDase, Ivanenkov et al.

generated a computational 3-D model of the extracellular domain of the human NTPDase 3 (38), based on their designation of the disulfide bonds (38) and the recently published crystal structure of the bacterial exopolyphosphatase/guanosine pentaphosphate phosphohydrolase (PPX/GPPA) (39), another member of the actin-HSP70-hexokinase superfamily. In this model, ACR1 and ACR 4 line the cleft of the putative active site of the human NTPDase 3. The computational 3-D model of the extracellular domain of NTPDase 2 is also similar and shown in Fig. G.4.

Table G.2. Alignment of the phosphate 1 and phosphate 2 motif sequences of the actin-HSP70-hexokinase superfamily. Apyrase conserved regions 1 and 4 from human NTPDase 2, human NTPDase 8, and chicken NTPDase 8 are aligned with the phosphate 1 and phosphate 2 motifs of the human actin, HSP 70, and hexokinase, respectively. Strictly conserved residues are in bold. The consensus sequence of the phosphate 2 binding motif for all of the sugar kinases, i.e., hexokinase, is GT. The consensus sequence for actin, HSP70, and the NTPDases is DXG. Note that all other cloned NTPDases also contain the conserved DXG in both ACR1 and ACR4.

Protein	Phosphate 1 binding motif	Phosphate 2 binding motif
Human actin	7 ALVC**D**N**G**SGLVK	150 GIVL**D**S**G**DGVTH
Human HSP 70	6 AVGI**D**L**G**TTYSC	195 VLIF**D**L**G**GGTFD
Human hexokinase	528 FLAL**D**L**G**GTNFR	675 GLIV**GT**GSNACY
Human NTPDase 2	41 GIVL**D**A**G**SSHTS	197 LGAM**D**L**G**GASTQ
Human NTPDase 8	44 GIVF**D**A**G**SSHTS	201 VGAL**D**M**G**GASTQ
Chicken NTPDase 8	42 GLVF**D**A**G**STHTA	199 LGAL**D**L**G**GASTQ

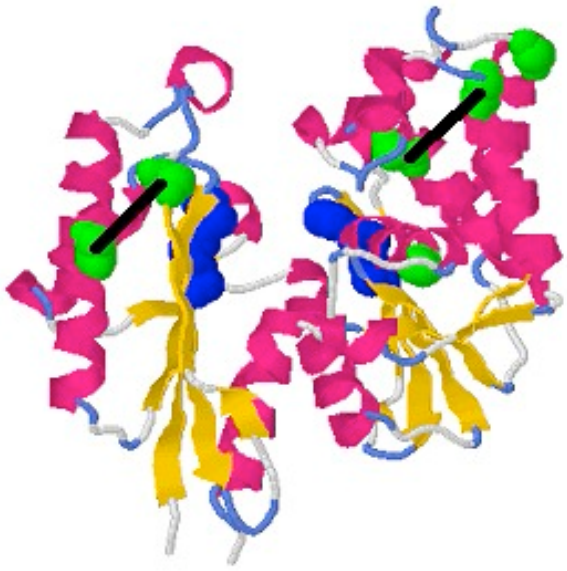

Figure G.4. The 3-D model of the extracellular region of NTPDase 2. The 3-D model was generated by the FUGUE program based on the bacterial PPX/GPPA structure. The model consists of the peptide backbone only and does not contain the amino acid side chains. Helices are represented by red ribbons and beta sheets by yellow arrows. The backbone locations of the NTPDase2 cysteine residues are indicated in the model by space-filling mode (green), as are the "DXG" motifs found in ACR1 and ACR4 (blue). The disulfide bonds are indicated in black lines. The putative active site is located in the cleft between ACR1 and ACR4. [Reprinted with permission from *Biochemistry*. **2005**, *44*, 8998-9012. Copyright 2005 American Chemical Society.]

Enzymatic properties of cell surface NTPDases

Cell surface NTPDases (NTPDases 1, 2, 3 and 8) hydrolyze a variety of nucleoside triphosphates (e.g. ATP and UTP) and diphosphates (e.g., ADP and UDP) in the presence of divalent cations. They all require Ca^{2+} or Mg^{2+} ions in the millimolar range for maximal activity but can be differentiated according to substrate preference. NTPDase 1 hydrolyzes ATP and ADP equally, while NTPDase 2 stands out for its high preference for nucleoside triphosphates, with ADPase/ATPase ratios of ~0.1. NTPDase 3 and NTPDase 8 are functional

intermediates between NTPDases 1 and 2 (Table G.1.). Only the chicken gizzard smooth muscle NTPDase 2 (40), the rabbit transverse tubules NTPDase 2 (41), and the chicken liver and oviduct NTPDase 8 (22, 42) have been purified to homogeneity. Their high turnover numbers, 1000-7000 μmol min^{-1} mg $protein^{-1}$, exceed that of the membrane bound P-, V-, F-type ion-motive ATPases.

Identification of Human NTPDase 2

The presence of a cell membrane associated ATPase was first revealed by cytochemical staining. Novikoff was the first to demonstrate that such an ATPase activity was abundant in the cell membranes of rat liver tumor cells (43). Subsequently, Karasaki showed that the cell surface ATPase activity in the normal rat liver was localized at the bile canaliculi; whereas the ATPase activity increased and was spread over the entire surface of the tumor cells in the N,N'-dimethylaminoazo-benzene-induced rat hepatoma (44). In addition, the staining of the cell surface ATPase activity in the rat hepatoma cells was abolished by 10 mM *p*-chlomercuribenzoate (pCMB), whereas that in the normal rat hepatocytes was not (44, 45). The differential effects of pCMB on the staining of ATPase activity provided the first indication that the ATPases in the normal rat liver and rat hepatoma are different. However, these ATPase activities were not further characterized by these investigators.

In 1984, Knowles and Leng partially purified and characterized a low affinity $Mg^{2+}(Ca^{2+})$-ATPase activity from the plasma membranes of human oat cell carcinoma (small cell lung carcinoma) xenograft from the nude mice (46). This

enzyme was activated by either magnesium or calcium, had broad substrate specificity, was not inhibited by any of the specific inhibitors of the P-, V-, and F-type ATPases, but was inhibited by a mercurial, i.e., *p*-chloromercuriphenylsulfonate (pCMPS). The mercurial-sensitive ATPase lost its activity in the presence of low concentration of several detergents, e.g., NP-40, triton X-100, and deoxycholate. This unusual sensitivity to detergents distinguishes this ATPase from other membrane-bound ATPases, and has made its purification a difficult task. The presence of the mercurial-sensitive ATPase activity was further demonstrated using intact human hepatoma Li-7A cells (47) and three lines of human small cell lung carcinoma cells (48), thus establishing the cell surface location of the ATPase.

The mercurial-sensitive ecto-ATPase was later cloned from the human small cell lung carcinoma cells, NCIH69 (29), and identified as NTPDase 2 (previously named ecto-ATPase). The human NTPDase 2 contains 495 amino acid residues and has a molecular mass of 66 kDa (Fig. G.3.). The schematic representation of the human NTPDase 2 is shown in Fig. G.5. Besides the five ACRs, there are also four other conserved regions (CRs), and ten conserved cysteine residues (C75, C99, C242, C265, C284, C310, C323, C328, C377, and C409) in the extracellular domain, that are probably involved in disulfide bond formation. Human NTPDase 2 also contains six putative N-glycosylation sites, N64, N88, N129, N294, N378, and N443, in which N378 was found to be not utilized when expressed in HEK293 cells (34).

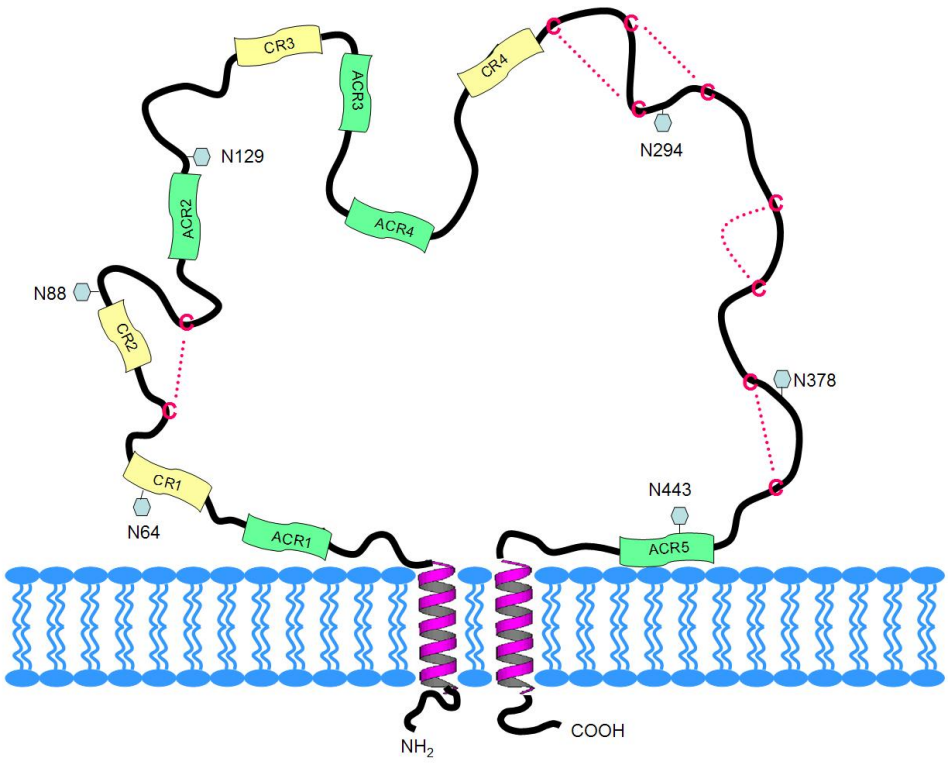

Figure G.5. Schematic representation of the human NTPDase 2. The human NTPDase 2 is a cell surface glycoprotein with one extracellular domain, two transmembrane domains (TMDs), five apyrase conserve regions (ACRs), four conserved regions (CRs), six putative N-glycosylation sites, and ten conserved cysteine residues which form five disulfide bonds.

Subject matter of this dissertation

Previous studies on NTPDase 2 in their native membranes from different species showed that their activities were modulated by a variety of compounds with different chemical structures (46, 48-53). The most interesting of these are detergents, e.g., NP-40 and Triton X-100, which inactivate the enzyme, and cross-linking reagents, e.g., concanavalin A (ConA), disuccinimidyl suberate (DSS), and glutaraldehyde, which increase the activity. After the human NTPDase 2 cDNA

was obtained, it was expressed in HeLa and HEK293 cells. Chapter 1 describes the enzymatic characteristics and the effects of these modulators on the expressed human NTPDase 2. Besides inhibition by detergents, the activity of the human NTPDase 2 was also decreased at temperature higher than 37^{o}C. Since detergents and high temperature increase membrane fluidity, these results indicate that the activity of the human NTPDase 2 is diminished under conditions that promote dissociation of membrane proteins or disruption of the interaction of transmembrane domains.

Interestingly, the chicken NTPDase 8 is neither inhibited by NP-40 nor higher temperature (22, 42, 54), indicating that the ATPase activity of the chicken NTPDase 8 is not susceptible to membrane perturbation. Since the sequences of the TMD of the human NTPDase 2 and chicken NTPDase 8 are different, it was of interest to determine if the interaction of the respective TMDs in the human NTPDase 2 and chicken NTPDase 8 are responsible for their different stabilities to membrane perturbation. To address this question, four constructs of the human NTPDase 2 were generated and characterized in Chapter 2. These constructs are a soluble human NTPDase 2, consisting of only the extracellular domain (ECD) of the enzyme, and three chimeric constructs, which are (i) hu-ck ACR1 chimera, (ii) hu-ck ACR5 chimera, and (iii) hu-ck ACR1,5 chimera, in which the sequence of N-terminus to ACR1 or the sequence of ACR5 to C-terminus or both of the human NTPDase 2 were substituted by the corresponding regions of the chicken NTPDase 8. I showed that the soluble human NTPDase 2 ECD and hu-ck ACR1,5 chimera

were no longer inactivated by detergents and high temperature, indicating that the inhibitory effect of membrane perturbation is mediated through TMDs and the strengths of interaction of the respective TMD pairs of the human NTPDase 2 and chicken NTPDase 8 determine their different responses to membrane perturbation as well as substrate inactivation.

The study described in Chapter 3 is divided into two parts. In the first part, the inhibitory effect of pCMPS, a sulfhydryl reagent that covalently modified free cysteine residues, on the ATPase activity of the human NTPDase 2 was investigated. The results revealed that the free cysteine residue at position 26 in the N-terminal TMD, which is a distance away from the putative active site, is the target of pCMPS modification. The mutant in which C26 was replaced by serine was no longer inhibited by pCMPS; however, mutants in which cysteine residues were placed in the C-terminal transmembrane domain near the extracellular surface were still modified by pCMPS, but inhibition of the ATPase activity of these mutants was significantly less. It is proposed that the bulky side group of pCMPS linked to C26 inhibited the activity by disturbing the TMD interaction of the human NTPDase 2 as well as affecting the conformation of the active site.

Since an important determinant in enzyme stability and activity is the strength of the interaction of the TMD (ref 54 and Chapter 2), I further generated mutants containing two cysteine residues, one in each of the two TMDs, and determined the effect of oxidative cross-linking of these cysteine residues on enzyme activity. My data which are summarized in the second part of the Chapter 3,

showed that inter- and intramolecular cross-linking of the cysteine residues in the TMD resulted in marked decrease of the human NTPDase 2 activity. Therefore, the mobility of the TMDs is critical for enzyme activity. However, immobilization of the TMD helices due to intra- and inter-molecular disulfide bond formation reduces or abolished inactivation of the residual activity of human NTPDase 2 by detergent and higher temperatures.

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Chapter 1

Regulation of the Enzymatic Activity of

Human Ecto-ATPase/NTPDase 2

Abstract

Human ecto-ATPase (NTPDase 2) is a cell surface integral membrane glycoprotein. Its activity is inhibited by parameters that decrease membrane protein interaction, i.e., detergents and high temperatures. These inhibitory effects are overcome when membranes are pretreated with concanavalin A or chemical cross-linking agents that increase the amounts of NTPDase 2 oligomers. Cross-linking agents also abrogate substrate inactivation of the NTPDase 2, a unique characteristic of the enzyme. These effects indicate that the magnitude of negative substrate regulation is dependent on quaternary structures of the protein, which likely involves interaction of transmembrane domains. The importance of transmembrane domains of the NTPDase 2 in activity modulation is further demonstrated by the stimulatory effect of digitonin, a steroid glycoside that preferentially interacts with cholesterol in the membranes but does not promote oligomer formation. These results indicate that NTPDase 2 activity is regulated by a multitude of mechanisms, some of which may have physiological significance.

Introduction

A nucleotide phosphohydrolase in the plasma membranes of a human small cell lung carcinoma xenograft in nude mice was previously characterized. The enzyme hydrolyzed nucleoside triphosphates (NTPs) other than ATP, but was less active with nucleoside diphosphates (NDPs) (1, 2). It was activated by ConA (1-3), inhibited by the sulfhydryl reagent, p-chloromercuriphenylsulfonate (pCMPS) (2, 3), but was not affected by high concentrations of azide, an inhibitor of a membrane-bound ATP diphosphohydrolase (2, 4). The enzyme was inactivated by most non-ionic detergents and bile salts and could only be partially solubilized in an active form by digitonin (5, 6). In addition to pCMPS, the partially purified enzyme was also inhibited after prolonged incubation with p-fluorosulfonylbenzoyl-5'-adenosine, an ATP analogue, and dithiothreitol, which reduces disulfide bonds (5). Subsequently, it was shown that this membrane-bound nucleotide phosphohydrolase with broad substrate specificity is a manifestation of a cell surface ATPase using several human small cell lung carcinoma (SCLC) cell lines (6). An ecto-ATPase with similar characteristics was also shown to be the major ecto-nucleotidase in the cells of a human hepatoma cell line, Li-7A (7, 8). Molecular identification of the protein contributing to these catalytic activities has been hampered by difficulties in obtaining purified enzyme from human tumor tissues because the ecto-ATPase is only present in small amount and the quantity of the tumor was limited. Without purified ecto-ATPase, which is necessary for

obtaining amino acid sequence information, molecular cloning has been difficult to accomplish.

The first related sequence, human CD39L1, was cloned from a cDNA library enriched for transcripts from human chromosome 9q (9) (GenBank Accession No.U91510). CD39L1 encodes a 472 amino acid protein, which displays more than 60% of sequence homology with the chicken muscle ecto-ATPase (10). Northern blot analysis showed that the CD39L1 transcript is abundant in brain and muscle, which correlates with the abundance of the ecto-ATPase activity in these tissues from biochemical determination. However, the biochemical characteristic of CD39L1 was not determined. Based on the sequence of CD39L1, Mateo et al. obtained cDNA from human ECV304 cells (GenBank Accession No.AF144748). The 495 amino acid protein was expressed in NIH3T3 cells and the transfected cells displayed ecto-ATPase activity (11). Subsequent studies indicated that the sequence of CD39L1, reported by Chadwick & Frischauf, encodes a splice variant of the human ecto-ATPase, which is not functional (12). Using the forward and reverse primers corresponding, respectively, to the 5'- and 3'-ends of the published sequence of human CD39L1, our lab cloned a human ecto-ATPase cDNA from human SCLC NCIH69 cells by RT-PCR (13). Its nucleotide sequence (GenBank Accession No. EF495152) is identical to that cloned by Mateo et al.

In this chapter, the human SCLC ecto-ATPase/NTPDase 2 was expressed in HeLa or HEK293 cells and characterized. The result shows that the expressed NTPDase 2 retains the same characteristics as the enzyme in its native membrane.

Importantly, the activity of NTPDase 2 is negatively regulated by substrates and parameters that decrease membrane protein interaction, while stimulated by protein cross-linking agents and digitonin. These findings suggest that the activity of NTPDase 2 can be modulated *in vivo* by parameters that affect its quaternary structure.

Materials and Methods

Materials

Dulbecco's modified Eagle's media (DMEM), OptiMEM, fetal bovine serum, antibiotics, and Lipofectamine were purchased from Gibco Life Technologies, Inc. (now Invitrogen). Newborn calf serum was purchased from Gemini Bio-Products Inc. Goat anti-rabbit Ig conjugated with alkaline phosphatase was purchased from Promega. Nucleotides and other biochemical reagents were purchased from Sigma Chemical Co. Dissucinimidyl suberate (DSS) was purchased from Pierce. Pentadecafluorooctanoic acid (PFO) was purchased from Oakwood Products, Inc. (West Columbia, SC). Bio-Rad *Dc* protein assay reagents were purchased from Bio-Rad Laboratories.

Transient and stable transfection

HeLa cells were grown in DMEM containing 10% fetal calf serum and HEK293 cells were grown in DMEM containing 5% fetal calf serum and 5% newborn calf serum. Cells were plated in either 6-well plates or 10 cm plates and were

used for transfection after reaching 50-70% confluence. For transfection in 6-well plates, the cells were washed twice with 1 ml OptiMEM and then layered with 1 ml OptiMEM containing DNA (1 μg each well) and Lipofectamine (5 μl each well) that had been premixed and incubated according to the manufacturer's instruction. After 5 hours, 1 ml of DMEM containing 10% fetal calf serum (for HeLa cells) or DMEM containing 5% fetal calf serum and 5% newborn calf serum (for HEK293 cells) was added to the wells. Twenty-four hours after transfection, the media were replaced by fresh DMEM- 10% fetal calf serum (for HeLa cells) or DMEM- 5% fetal calf serum and 5% newborn calf serum (for HEK293 cells). After another 24-48 hours, cells were harvested by trypsinization, washed in isotonic buffer (0.1 M NaCl, 0.01 M KCl, and 25 mM TrisCl, pH 7.4), and resuspended in 0.2-0.4 ml of the buffer. Enzyme activities were determined using 10-50 μl cell suspension (20-100 μg cell protein).

To obtain stably transfected cells, HeLa cells or HEK293 were first transfected in 6-well plates with the human ecto-ATPase/NTPDase 2 cDNA (in pcDNA3) as described above. Two days after transfection, cells were harvested, plated into a T-25 flask, and allowed to attach overnight. Geniticin was then added gradually to 400 μg/ml. Media were replaced every three days. The established geniticin-resistant clones, which displayed an ecto-ATPase activity ~10 fold higher than that of the transiently transfected cells, were propagated and used for membrane preparation.

Membrane preparation

Membranes were prepared from 10-15 10-cm plates of HeLa cells or HEK293 stably transfected with the human NTPDase 2 cDNA. Cells were harvested by trypsinization and washed once with isotonic buffer to remove residual serum and trypsin. The cell pellet was homogenized in homogenization buffer (3 ml for each 10-cm plate of cells) containing 0.25 M sucrose, 30 mM Mops, pH 7.4 and 2 mM EDTA with a Potter-Elvejem homogenizer. The homogenate was centrifuged at 48,000 rpm for 60 min in a Beckman 65 rotor. The membrane pellet was resuspended in homogenization buffer (1 ml for each 10-cm plate of cells) and 3 ml of the membranes were layered on top of 8 ml sucrose solution containing 40% sucrose (w/v) and 20 mM Mops, pH 7.4 in a Beckman SW41 tube and centrifuged at 14,000 rpm for 1 hr. Membrane fraction that partitioned at the interface of the two sucrose layers was enriched in plasma membranes with a specific ATPase activity 4-5 times greater than the membrane fraction that sedimented at the bottom of the 40% sucrose layer. The plasma membrane enriched fraction was collected (~ 1-3 mg protein/ml) and stored at -20^{o}C.

Antibodies

A polyclonal antibody to the human NTPDase 2 was generated in rabbits by DNA immunization (14) using recombinant pcDNA3 containing human NTPDase 2 cDNA as the antigen. Antibody production was carried out in the laboratory of Dr. Jean Sévigny of Laval University (Quebec, Canada). This antibody only binds un-

reduced NTPDase 2 protein in immunoblotting, presumably because reduction of disulfide bonds in the protein destroys the epitopes.

Gel electrophoresis and Western blot analysis

SDS-PAGE was performed in 7.5% polyacrylamide gel in a mini-gel apparatus (Bio-Rad). Protein samples to be analyzed were mixed with 4X SDS sample buffer without reducing agents and heated at 100^{o}C for 3 min.

Proteins separated by SDS-gel electrophoresis were electrophoretically transferred to PVDF membranes (NEN^{TM} Life Science Products, Inc.). Molecular mass markers on the PVDF membrane after transfer of the PFO gel were detected by Amido black staining. For immunoblotting, the membranes were blocked in Tris-buffered saline (TBS) containing 5% non-fat milk overnight, incubated with primary antibody (1000 fold dilution) for 2 hr at room temperature, washed 4 times with TBS containing 0.05% Tween 20, and then incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (5000 fold dilution) for 1 hr. After washing 3 times with TBS containing 0.05% Tween 20 and a final wash with TBS, the immunoreactive proteins were detected by treating the PVDF membrane with alkaline phosphatase substrates (Bio-Rad).

Enzyme assays

ATPase assays (duplicates) were carried out in 0.5 ml reaction mixture containing 25 mM TrisCl, pH 7.5, 5 mM $MgCl_2$ and 5 mM ATP with 2-5 μg membrane proteins. Reactions were initiated by the addition of 25 μl 0.1 M ATP and allowed to proceed at 37^{o}C for 10 min (standard condition) or the indicated

times, then terminated by the addition of 0.1 ml 10% trichloroacetic acid. After centrifugation to remove denatured proteins, an aliquot of the supernatant solution was used for determination of Pi using a colorimetric reagent consisting of 10 mM ammonium molybdate, 5 N H_2SO_4, and acetone (1:1:2) as described (15). Alternatively, [γ-^{32}P]ATP (~2 x 10^5cpm/μmol) was used as the substrate. After termination of reaction by trichloroacetic acid, ^{32}Pi and [γ-^{32}P] ATP in an aliquot of the reaction mixture were separated by extraction with isobutanol-benzene (7). Radioactivity in an aliquot of the isobutanol-benzene phase containing ^{32}Pi was determined by Cerenkov counting. All experiments were conducted at least three times. Results presented were from representative individual experiments.

Results

General characteristics of expressed human NTPDase 2

The NTPDase 2 cDNA cloned from NCIH69 cells was expressed in HeLa and HEK293 cells. HeLa cells transiently transfected with the human NTPDase 2 cDNA displayed an ecto-ATPase activity (0.25 μmol min^{-1} mg $protein^{-1}$) that was approximately 30 fold greater than HeLa cells transfected with the pcDNA3 vector (0.008-0.01 μmol min^{-1} mg $protein^{-1}$). However, HEK293 cells transiently transfected with the human NTPDase 2 cDNA displayed a higher ecto-ATPase activity (2.5 μmol min^{-1} mg $protein^{-1}$). We then generated stably transfected HeLa and HEK293 cell lines, and prepared plasma membrane enriched fractions from

these cells by differential and sucrose gradient centrifugation. Specific ATPase activities in these membranes are typically ~10-20 μmol min^{-1} mg $protein^{-1}$ (for HeLa cell line) and ~25-45 μmol min^{-1} mg $protein^{-1}$ (for HEK293 cell line) when assayed under standard conditions, and is one order of magnitude higher than the activity in plasma membrane fractions isolated from human small cell lung carcinoma xenograft (5, 6). Similar to the enzyme in its native membranes (5), the expressed NTPDase 2 hydrolyzes ATP in the presence of either Mg^{2+} or Ca^{2+} (data not shown). Km for ATP is 0.21 ± 0.022 mM with 5 mM Mg^{2+}. Hydrolysis of UTP, a physiological substrate of cell surface E-NTPDases, is similar to that of ATP, whereas ADPase activity is less than 5% of either ATPase or UTPase activities (Table 1.1.).

Table 1.1. Effect of modulators on hydrolysis of ATP, ADP, and UTP by expressed human NTPDase 2. ATPase reactions were carried out with 2.5 μg membranes prepared from HeLa cells stably transfected with human NTPDase 2 cDNA as described in "Materials and Methods". The membranes were preincubated in the reaction mixture with the indicated concentrations of the modulators for 5 min at room temperature before the reactions were initiated by the addition of substrates. After 10 min at 37°C, the reaction was terminated by the addition of 0.1 ml 10% trichloroacetic acid. The amount of Pi released was determined colorimetrically as described in "Materials and Methods". Data presented are the average from three separate experiments with the same membrane preparation.

Addition	ATP	ADP	UTP
	(μmol min^{-1} mg $protein^{-1}$)		
None	22.0 ± 1.1	1.00 ± 0.092	23.8 ± 1.57
NP-40 (0.01%)	1.09 ± 0.19	0.22 ± 0.05	0.66 ± 0.29
Digitonin (0.1%)	57.0 ± 4.07	1.21 ± 0.092	50.2 ± 7.99
ConA (50 mg)	46.7 ± 2.88	1.41 ± 0.051	44.0 ± 3.57
pCMPS (0.1 mM)	7.15 ± 0.49	0.72 ± 0.064	8.88 ± 0.5

Effect of modulators on expressed human NTPDase 2 activity

Table 1.1. also shows that the expressed NTPDase 2 activity is affected by compounds that either decreased or increased the activities in the native human tumor cell membranes. (i) ATPase activity was almost completely abolished by low concentrations (0.01%) of the non-ionic detergent NP-40, which dissolves into the lipid bilayer. (ii) In contrast to NP-40, digitonin, increased the ATPase activity more than two fold. (iii) ATPase activity was increased ~2 fold if the membranes were preincubated with the tetravalent lectin, ConA, which cross-links glycoproteins. (iv) ATPase activity was markedly inhibited by the sulfhydryl reagent, pCMPS. UTP hydrolysis was similarly affected by these compounds. The identical responses of the expressed NTPDase 2 activity to NP-40, digitonin, ConA, and pCMPS as the enzyme in the native membranes (5, 6) indicated that these characteristics are inherent in the protein encoded by the human NTPDase 2 cDNA and is not dependent on a particular membrane environment.

ConA and other cross-linking reagents prevent inactivation of the human NTPDase 2 by high temperature and NP-40

The stimulating effect of ConA on the expressed human NTPDase 2 activity was more pronounced at higher temperatures. Fig. 1.1. shows that ATPase activity increased with temperature up to 30-37^{o}C but declined at higher temperatures. In contrast, the ATPase activity of membranes pre-treated with ConA increased with temperature up to 48^{o}C and showed a slight decrease only at 55^{o}C. As a consequence, ConA stimulation of ATPase activity increased from two to six fold

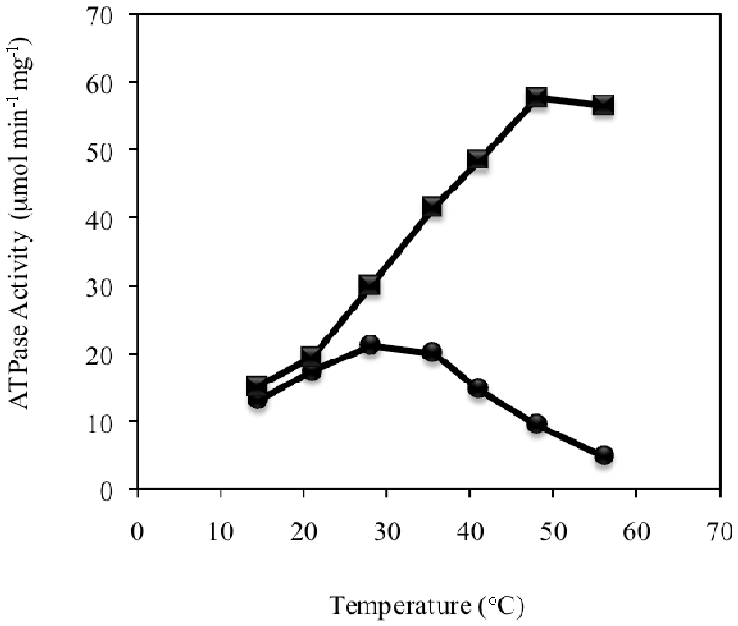

Figure 1.1. Effect of temperature on the activities of untreated membranes and membranes preincubated with ConA. Membranes (53 μg) prepared from HeLa cells stably transfected with human NTPDase 2 cDNA were preincubated with either 150 μg of BSA (●) or ConA (■) in a solution (150 μl) containing 20 mM Mops, pH 7.4 and 5 mM $MgCl_2$ for 20 min at room temperature. Aliquots of the membrane (2.5 μg) were used for ATPase assays at the indicated temperatures for 10 min.

when assay temperature increased from 35.5°C to 48°C. Besides ConA, the human NTPDase 2 activity was also significantly increased if the membranes were pre-treated with two lysine-specific chemical cross-linking reagents, DSS and glutaraldehyde, the enhancement being particularly noticeable at 50°C (Fig. 1.2.).

Treatment of the membranes by ConA, DSS, and glutaraldehyde also markedly reduced inactivation of the activity of the human NTPDase 2 by NP-40. This was most obvious with glutaraldehyde-treated membranes. Fig. 1.3. shows

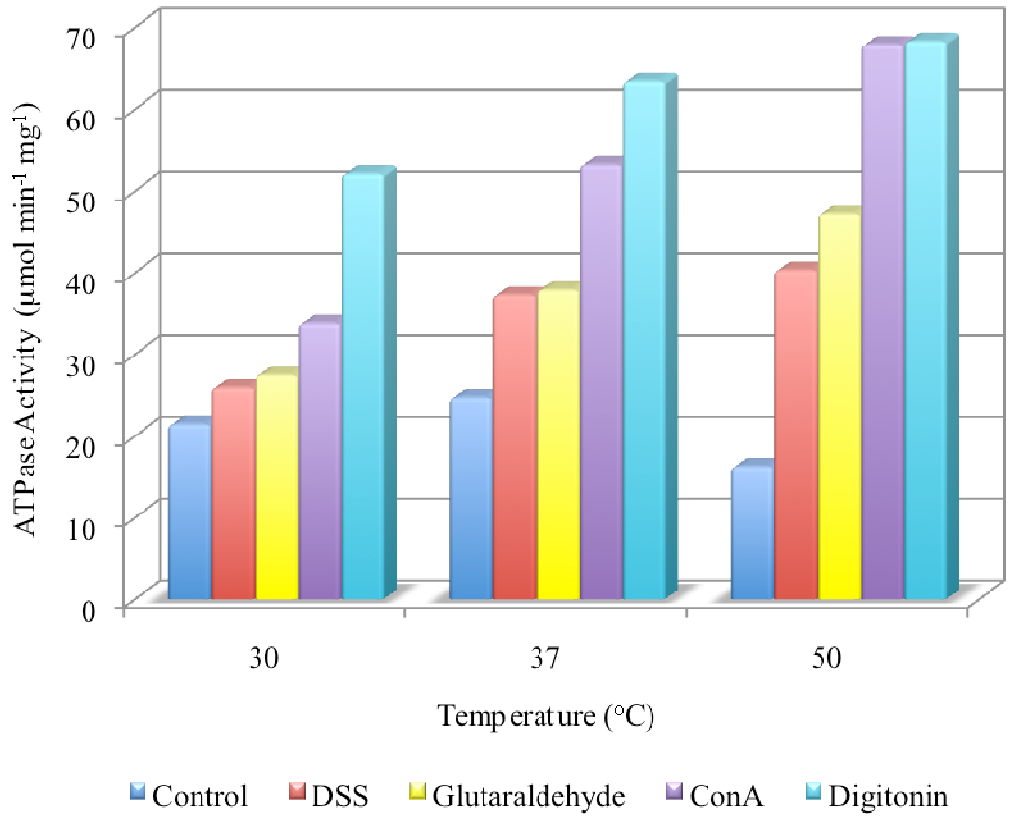

Figure 1.2. Treatment of membranes by cross-linking reagents and digitonin increases the activity of the human NTPDase 2 at different temperatures. Membranes (35 µg) were preincubated in 0.1 ml solution containing 20 mM Mops, pH 7.5 and 5 mM $MgCl_2$, without or with 150 µg ConA, 1 mM DSS, or 10 mM glutaraldehyde for 20 min at room temperature, after which 10 µl of 0.25 M lysine was added. Aliquots containing 2.5 µg of membrane proteins were used for enzyme assays at the indicated temperatures for 10 min. For ATPase assays in the presence of digitonin, the reaction mixtures contained 0.05% digitonin.

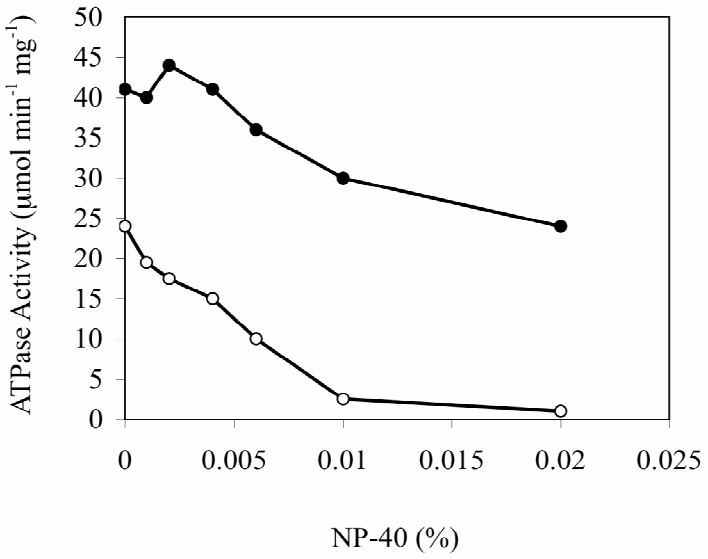

Figure 1.3. NP-40 inhibition of the human NTPDase 2 activity is diminished in membranes preincubated with glutaraldehyde. Membranes (35 µg) were preincubated in 0.1 ml solution containing 20 mM Mops, pH 7.4, 5 mM $MgCl_2$, without (○) or with (●) 10 mM glutaraldehyde for 20 min at room temperature, after which 10 µl of 0.25 M lysine was added to stop the reaction. Membranes were transferred to 4°C and aliquots containing 2.5 µg membrane proteins were used for ATPase assays in the absence or presence of the indicated concentrations of NP-40for 10 min at 37°C.

that 0.01% NP-40 completely abolished ATPase activity of the untreated membranes while ATPase activity of glutaraldehyde-treated membranes was only reduced by ~25%. Even in the presence of 0.02% NP-40, the glutaraldehyde treated membranes displayed activity similar to that of untreated membranes in the absence of NP-40. ConA and DSS treatment also afforded protection against NP-40 inactivation, but were less effective than glutaraldehyde (Fig. 1.4.). The effect of NP-40 was reversible. Addition of ConA to NP-40 treated membranes restored the ATPase activity to the same level as that when ConA was added before NP-40 (data not shown).

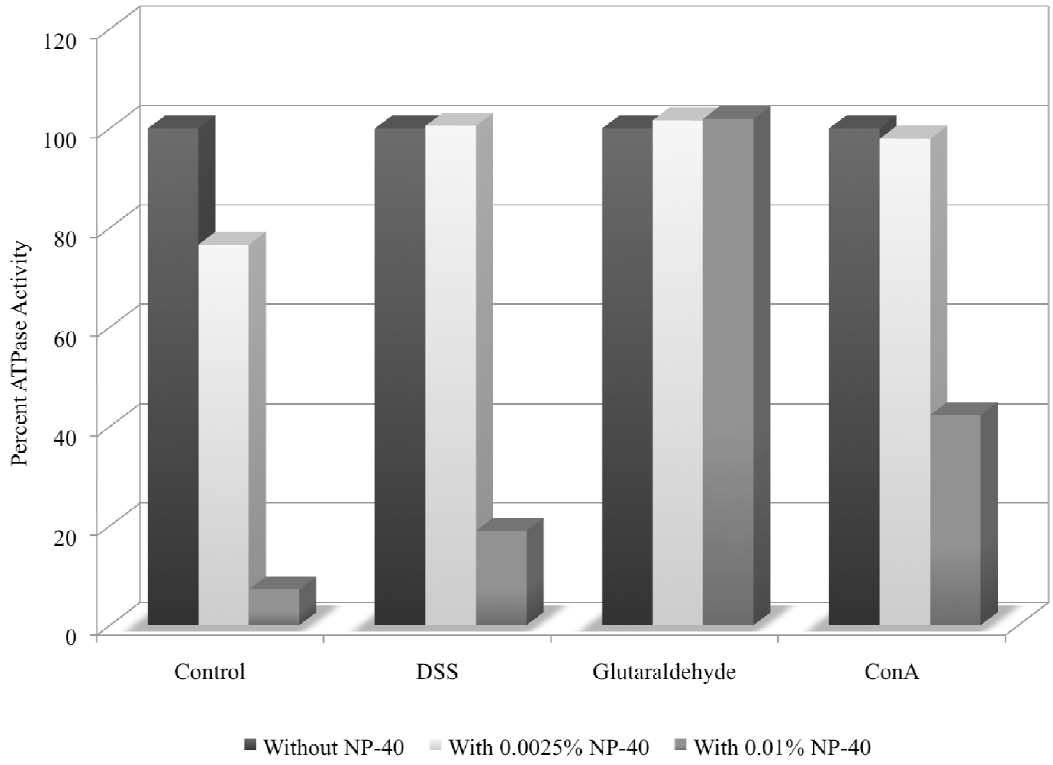

Figure 1.4. Treatment of membranes by cross-linking agents protects the activity of the human NTPDase 2 against inhibition by NP-40. Preincubation of the membranes with ConA, DSS and glutaraldehyde was carried out as described in the legend of Fig. 1.2. Membrane proteins (2.5 μg) were used for ATPase assays in the absence and presence of 0.0025% and 0.1% NP-40 for 10 min at 37^{o}C

The results described above indicated that the activity of the human NTPDase 2 is decreased under conditions that reduce membrane protein interaction, e.g., high temperature and treatment with most detergents at concentrations below that for effective solubilization of membrane proteins. However, the activity of the human NTPDase 2 is increased under conditions that promote oligomer formation, e.g., after treatment by cross-linking agents. The greater protective effect of glutaraldehyde against NP-40 inactivation may be related to its ability to cross-link a larger proportion of monomers. The human NTPDase 2 monomer is a 67 kDa protein (Fig. 1.5., lane 1). Cross-linking by the bifunctional reagent, DSS, resulted in the formation of dimers, but a significant amount of monomers remained (Fig. 1.5., lane 2). In contrast, the amount of monomers in the glutaraldehyde-treated sample was greatly reduced (Fig. 1.5., lane 3). The weaker staining of the human NTPDase 2 oligomers is probably due to the loss of antigenicity in the cross-linked protein.

Cross-linking reagents prevent inactivation of the human NTPDase 2 by ATP

The stimulating effects of cross-linking agents are related to their ability to prevent ATP inactivation of the human NTPDase 2. Fig. 1.6. and Table 1.2. show that the rate of ATP hydrolysis by the expressed human NTPDase 2 decreased with time, so that specific activity of ATP hydrolysis obtained in a 10-min reaction was ~30% of that obtained in a 1-min reaction. This was also observed when UTP was used as a substrate (not shown). The decrease of ATP hydrolysis rate is not due to

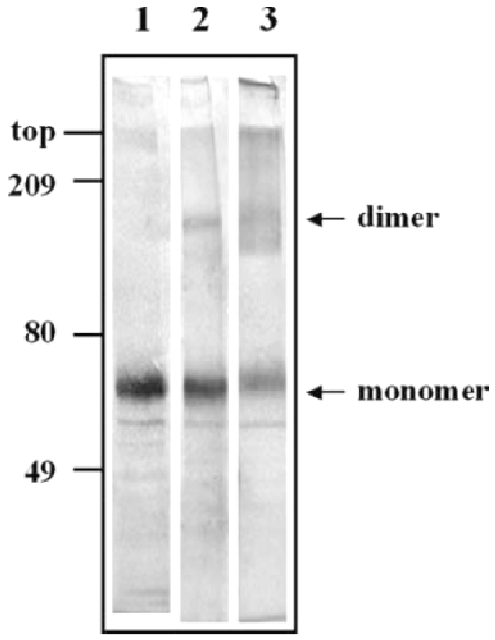

Figure 1.5. Western blot analysis of the human NTPDase 2 without and with cross-linking by DSS and glutaraldehyde. Membranes (35 μg) were preincubated in 0.1 ml solution containing 20 mM Mops, 5 mM $MgCl_2$, without or with 1 mM DSS or 10 mM glutaraldehyde for 20 min at room temperature, after which 10 μl of 0.25 M lysine was added to stop the reactions. The untreated and treated membranes were mixed with 4X gel sample buffer without reducing agent and heated. Samples containing 6.5 μg proteins were subjected to SDS-PAGE and Western blot analysis with the human NTPDase 2 antibody (KW3) obtained by DNA immunization. Lane 1, untreated membranes; lane 2, membranes treated with DSS; lane 3, membrane treated with glutaraldehyde.

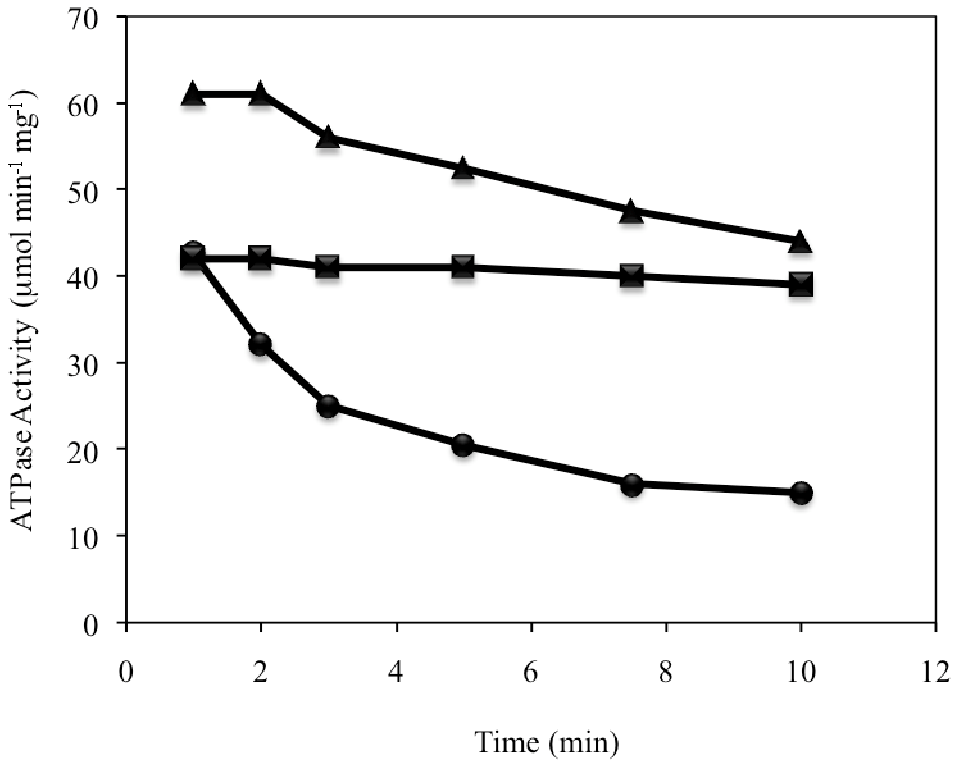

Figure 1.6. Time course of ATP hydrolysis by the human NTPDase 2. Preincubation of the membranes with glutaraldehyde was carried out as described in the legend of Fig. 3. ATPase activities of the membranes were determined for the indicated times, (●), ATP hydrolysis by untreated membrane; (■), ATP hydrolysis by glutaraldehyde-treated membranes; (▲) ATP hydrolysis by untreated membrane in the presence of 0.05% digitonin.

inactivation of the enzyme at 37°C. Membranes that were preincubated in the reaction mixture for 37°C for 10 min without substrate did not suffer any loss of activity. Nor is it due to product inhibition. Inclusion of 2 mM Pi or ADP in the reaction mixture, equivalent to 40% substrate hydrolysis, did not exacerbate the decline of enzyme activity during the 10-min reaction (Table 1.2.). We conclude that decrease of ATPase activity is due to a negative regulatory effect of substrate on the enzyme. Importantly, decrease of ATPase activity was negligible when the membranes were pretreated with 10 mM glutaraldehyde. Fig. 1.6. shows that specific activity of ATP hydrolysis of glutaraldehyde-treated membrane remained constant during a 10-min reaction, resulting in an apparent stimulation of the ATPase activity by more than two fold at 37°C. Pretreatment of the membrane by DSS and ConA also prevented inactivation by substrate (data not shown) with DSS being less effective than glutaraldehyde or ConA. These results explain the general activating effects of cross-linking agents on NTPDase 2 activity. We conclude that alteration of quaternary structure of the NTPDase 2 by cross-linking agents protects the enzyme from inactivation by substrates.

Effect of digitonin on the activity of the human NTPDase 2

Digitonin, a steroid glycoside, was the only detergent that did not inactivate the activity of the human NTPDase 2. On the contrary, it stimulated the ATPase activity and was used in its solubilization and partial purification (5, 6). Fig. 1.2. shows that the stimulatory effect of digitonin on the expressed NTPDase 2 was

Table 1.2. The decrease of NTPDase 2 activity with time is the same in the absence and presence of the reaction products, ADP and Pi. ATPase assays were conducted at 37°C with 1.8 μg membrane protein in 0.5 ml reaction mixture containing 25 mM TrisCl, pH 7.4, 5 mM $MgCl_2$, 5 mM ATP, and 0.1 mM azide in the absence or presence of 2 mM ADP or 2 mM sodium phosphate. The reaction was initiated by the addition of 25 μl of 0.1 M [γ-^{32}P]ATP (1.5 x 10^5cpm/μmol) and stopped with 0.1 ml trichloroacetic acid after the indicated reaction times. An aliquot (0.2 ml) of the reaction mixture was used for extraction of ^{32}Pi as described in" Materials and Methods". Data presented are the average of three separate experiments with the same membrane preparation.

Addition	1 min	2.5 min	10 min
	(μmol Pi released min^{-1} mg $protein^{-1}$)		
None	30.13 ± 2.26	21.43 ± 0.99	10.7 ± 0.72
2 mM ADP	29.15 ± 2.34	22.1 ± 1.92	10.91 ± 1.04
2 mM Pi	30.48 ± 3.54	22.13 ± 2.04	11.31 ± 1.74

obtained even at higher temperature. At 50°C, 0.1% digitonin stimulated the NTPDase 2 activity by 5 fold, exceeding the ATPase activities of membranes pre-treated with all three cross-linking agents. The stimulatory effect was partly attributed to a significant reduction of substrate inactivation (Fig. 1.6.). However, in contrast to glutaraldehyde, the activating effect of digitonin was apparent in the first minute of the reaction resulting in a specific activity that was 50% greater than that obtained with either of the untreated or glutaraldehyde-treated membranes in a 1-min reaction (Fig. 1.6.). That the mechanisms of activation by digitonin and the cross-linking agents are different is supported by results obtained with PFO-gel

electrophoresis. The human NTPDase 2 protein was present mostly as monomers in the digitonin-treated sample and the weak staining in the high molecular weight area of the gel seen in the untreated sample had disappeared (data not shown).

Discussion

Previous characterization of the human NTPDase 2 was conducted with plasma membrane preparations of human SCLC cells (5, 6) before molecular cloning of the enzyme. Molecular cloning of both human and mouse NTPDase 2 cDNA from tumor cells (11, 13, 16) indicates that the enzyme is highly expressed in some tumor tissues. However, NTPDase 2 is not tumor specific. Enzymes with similar characteristics are present in the membranes of skeletal, smooth, and heart muscles (17-21) and brain (19, 22-24), and the chicken and rat NTPDase 2 cDNA were respectively cloned from smooth muscle (10) and brain (25, 26). Thus, NTPDase 2 is normally distributed in excitable tissues and its expression in some tumors may be a result of tumorigenesis.

Published studies on NTPDase 2 in their native membranes from different species showed that their activities are modulated by a variety of compounds with different chemical structures (5, 8, 17, 19, 27-29). The most interesting of these are detergents, e.g., NP-40 and Triton X-100, which inactivate the enzyme and cross-linking agents, which stimulate the activity. In the study described in this chapter,

the effects of these modulators on the expressed human NTPDase 2 were investigated.

In addition to inhibition by detergents, the activity of the human NTPDase 2 is also decreased at higher temperatures (Figs. 1.1. and 1.2.). Since detergents and high temperature increase membrane fluidity, these results indicate that the activity of the human NTPDase 2 is diminished under conditions that promote dissociation of membrane proteins. This conclusion is supported by the observation that decrease of ATPase activity in the presence of NP-40 or at higher temperatures does not occur if the membranes are pre-treated by the cross-linking reagents, i.e., ConA, DSS, and glutaraldehyde (Figs. 1.1.-1.4.), that promote oligomer formation of the human NTPDase 2 protein (Figs. 1.5.). These results can be interpreted to mean that the oligomeric human NTPDase 2 possesses higher activity than the monomeric human NTPDase 2, which has been proposed for the NTPDase 2 of other species (17, 27-29). Revision of this model is now required in view of the observed effect of cross-linking agents on preventing substrate inactivation. ATP hydrolysis activity of the expressed human NTPDase 2 rapidly decreases after the start of the reaction, so that specific activity obtained at 10 min is usually 30% of that obtained at 1 min (Fig. 1.1. and Table 1.2.). This loss of activity cannot be attributed to product inhibition since time courses of the reaction obtained in the absence and presence of ADP and Pi were identical (Table 1.2.). These results suggest that a conformational change occurs in the protein after the onset of ATP hydrolysis, which shifts the enzyme into a less active state. However, ATP inactivation is completely abolished when the

membranes were pre-treated by cross-linking agents, suggesting that dimeric or oligomeric human NTPDase 2 are more resistant to the shift to the less active conformation. On the other hand, such a shift may be accelerated in the presence of detergents or at high temperatures, thus explaining their inhibitory effect. At present, the mechanism of ATP inactivation of human and other NTPDase 2 (17, 21, 23, 30, 31) is not known. In contrast to the chicken transverse tubule and smooth muscle NTPDase 2 for which a low and a high Km values for ATP have been determined (29, 32) and an ATP regulatory site has been proposed (18, 29), the expressed human NTPDase 2 demonstrates typical Michaelis-Menten kinetics with respect to ATP when data points were collected with a 1-min reaction time. It has also been proposed that ATP-induced inactivation of the rat brain NTPDase 2 is mediated partially by phosphorylation of membrane proteins (23). This seems unlikely in the case of the human NTPDase 2 since inactivation of the enzyme was also obtained with UTP, which does not serve as a substrate for most protein kinases. Regardless of the specific mechanism, inactivation of the human NTPDase 2 by substrate and its activation by binding to naturally occurring lectins present themselves as unique regulatory mechanisms which may be of physiological significance for the human NTPDase 2 *in vivo*.

Three other NTPDases have been studied with respect to effects of detergents and cross-linking agents. The most interesting among these is the chicken ecto-ATPDase (an NTPDase 8), which displays entirely different responses to parameters that affect the human NTPDase 2 in that it is not susceptible to inactivation by ATP,

temperature, and detergent, and is not stimulated by cross-linking agents (33, 34), suggesting that its activity is not regulated by quaternary structures. On the other hand, a human ecto-ATPDase (NTPDase 3/HB6) shows a non-linear time course and is stimulated by DSS and ConA (35) and may be susceptible to the same regulation as the human NTPDase 2. The rat NTPDase 1/CD39 is inactivated by Triton X-100 (36), but its enzyme kinetics and the effect of cross-linking agents have not been investigated. However, Wang et al. did show that an engineered soluble form of rat CD39 was no longer inhibited by Triton X-100 and suggested that the transmembrane domains of CD39 mediates the inhibitory effect of detergents. These investigators further demonstrated that interaction of heterologous transmembrane domains of rat CD39 is required for oligomer formation (36).

In contrast to Triton X-100 and NP-40, digitonin has a marked stimulatory effect on the expressed human NTPDase 2. While digitonin also prevents loss of the human NTPDase 2 activity at higher temperatures (Fig. 1.2.) and inactivation by substrate (Fig. 1.6.), the mechanism of action of digitonin is most likely different from that of the cross-linking agents. In contrast to these agents, the stimulatory effect of digitonin is apparent in the first minute of the reaction (Fig. 1.6.) and the human NTPDase 2 is present mostly as monomers in digitonin-treated membranes according to analysis by PFO gel electrophoresis (data not shown). Since digitonin is known to preferentially bind membrane cholesterol (37), which together with glycolipids, are major components in membrane rafts (38), its effect on the human NTPDase 2 suggests the interesting possibility that human NTPDase 2 may be

located in these rafts. Association of human CD39/NTPDase 1 with caveolae has been reported previously (39) but the effect of digitonin on kinetics of the enzyme has not been investigated. In the case of the human NTPDase 2, sequestration of cholesterol by digitonin may alter the interaction of the transmembrane domains of the human NTPDase 2 with other lipids in these rafts, resulting in activation of the enzyme. It is interesting to note that stimulation of the chicken smooth muscle NTPDase 2 by sphingosine has been observed (40).

The responses of the human NTPDase 2 to detergents, high temperatures, and substrate are unusual among membrane enzymes. These results have prompted investigation of the role of the transmembrane domains of the human NTPDase 2 in determining its unique enzyme properties, which is discussed in Chapter 2 of this dissertation.

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Chapter 2

Transmembrane Domain Interactions Affect the Stability of the Extracellular Domain of the Human NTPDase 2

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Transmembrane domain interactions affect the stability of the extracellular domain of the human NTPDase 2 ☆

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Abstract

Human NTPDase2 and chicken NTPDase8 are cell surface nucleotidases that contain two transmembrane domains (TMD) and five apyrase conserved regions (ACRs). ACR1 is located near the N-terminal TMD whereas ACR5 is located near the C-terminal TMD. The human NTPDase2 activity is decreased by low concentration of NP-40 and at temperatures higher than 37 °C, and undergoes substrate inactivation, whereas the chicken NTPDase8 activity is not. When freed from membrane anchorage, the soluble human NTPDase2 is no longer inactivated by detergents, high temperature, and substrate. These characteristics are retained in the hu-ck ACR1,5 chimera in which the extracellular domain is anchored to the membrane by the two TMDs of the chicken NTPDase8. The hu-ck ACR1,5 chimera is the first chimeric NTPDase reported that shows a resistance to membrane perturbation and substrate inactivation. Our results indicate that the strengths of interaction of the respective TMD pairs of the human NTPDase2 and chicken NTPDase8 determine their different responses to membrane perturbation and substrate.

Keywords: Human NTPDase2; Human ecto-ATPase; NTPDase chimeras; Transmembrane domain; Extracellular domain; Detergent effect; Temperature effect; Substrate inactivation

Nucleoside triphosphate diphosphohydrolases (NTPDases[1]) are ubiquitous enzymes that have an extraplasmic orientation and hydrolyze NTPs and NDPs. Of the eight subfamilies of NTPDases that differ in tissue and subcellular distribution, substrate preference, and the modes of anchoring to the membranes, NTPDase 1, 2, 3, and 8 are cell surface enzymes, while NTPDase 4–7 are found in intracellular organelles. NTPDase 5 and 6 can also be secreted [see review in [1]]. The cell surface NTPDases have a large extracellular domain (ECD), two transmembrane domains (TMDs) and short cytoplasmic tails at the N- and C-termini. They are variably glycosylated and contain ten conserved cysteines, five apyrase conserved regions (ACRs) [2,3], and four additional conserved regions [4]. NTPDase 1, 3, and 8 hydrolyze both nucleoside triphosphate and nucleoside diphosphate in the presence of Mg^{2+} or Ca^{2+} with ADPase/ATPase ratios ranging from 0.25 to 1 at pH 7.5 [5–9], while NTPDase 2 preferentially hydrolyzes NTP with an ADPase/ATPase ratio of ~0.1 [9–13].

Our laboratory has cloned and characterized two of the cell surface NTPDases, i.e., the human ecto-ATPase, an NTPDase 2 (GenBank Accession No. EF495152) [10], and the chicken ecto-ATP-diphosphohydrolase, an NTPDase 8 (GenBank Accession No. AF041355) [14]. Both have been expressed in human cells, i.e., HeLa and HEK293 cells, and characterized. In spite of the presence of similar conserved elements in these proteins, the two enzymes differ in their responses to detergents, high

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[1] *Abbreviations used:* NTPDase, nucleoside triphosphate diphosphohydrolase; ECD, extracellular domain; TMD, transmembrane domain; ACR, apyrase conserved region; NP-40, nonidet P-40; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Mops, 3-(*N*-morpholino)-propanesulfonic acid, Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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temperatures, and cross-linking reagents, such as concanavalin A and glutaraldehyde [10,14,15]. The human NTPDase 2 activity is decreased by low concentrations of NP-40 (~0.01%) and at temperatures above 37 °C [10], while the chicken NTPDase 8 is not [14,15]. The human NTPDase 2, as well as NTPDase 2 of other species, displays the unusual property of inactivation by substrate [10,11,16,17] but the chicken NTPDase 8 does not [15]. Moreover, ATP-induced inactivation and decrease of activity due to NP-40 and high temperatures of the human NTPDase 2 can be prevented by treating the membrane with cross-linking reagents [10], but the same reagents have no effect on the activity of the chicken NTPDase 8 [15].

Since detergents and high temperature can increase membrane fluidity, their effects on the human NTPDase 2 are likely to be mediated by the TMDs of the enzyme. That both TMDs are required to mediate the inhibitory effect of Triton X-100 was first demonstrated in rat NTPDase 1/CD39. Wang et al. generated CD39 constructs lacking either the N- or C-terminal TMD and showed that the expressed proteins were no longer inhibited by Triton X-100 [18]. It should be noted, however, that the membrane topology was altered in these engineered proteins compared to the native CD39 since one of the two TMDs was removed. To demonstrate the importance of TMD interaction in determining sensitivity to detergent inhibition while maintaining the membrane topology of the NTPDase, we previously generated chimeras of the chicken NTPDase 8 in which its N- or C-terminal TMD or both were substituted by that of the human NTPDase 2 [15,19]. In contrast to the wild-type chicken NTPDase 8, the activity of which is not affected by detergents, the activities of all three chimeric proteins are inhibited by NP-40. Furthermore, the chimeras respond to higher temperatures differently than the wild-type enzyme [15,19]. Thus substitution of either of the TMD of the chicken NTPDase 8 by that of the human NTPDase 2 results in altered interactions of the chimeric TMD pairs, which negatively affect catalysis in the extracellular domain of the chicken NTPDase 8.

If the TMD of the unstable human NTPDase 2 could render the chicken NTPDase 8 chimeras sensitive to detergent inhibition, is it possible to generate a detergent-resistant human NTPDase 2 by substituting one or both of its TMD by that of the chicken NTPDase 8? To answer this question, four constructs of the human NTPDase 2 were generated and characterized. These are a soluble human NTPDase 2, consisting of only the extracellular domain (ECD) of the enzyme, and three chimeric constructs, which are (i) hu-ck ACR1 chimera, (ii) hu-ck ACR5 chimera, and (iii) hu-ck ACR1,5 chimera, in which the sequence of N-terminus to ACR1 or the sequence of ACR5 to C-terminus or both of the human NTPDase 2 are substituted by the corresponding regions of the chicken NTPDase 8 (Fig. 1).

Our results show that the soluble ECD of the human NTPDase 2 is not inhibited by detergent nor substrate and its activity increases with temperature; thus providing definitive evidence that TMDs in the full-length human NTPDase 2 mediate these inhibitory effects. The resistance to inhibition by detergent, substrate, and high temperature is maintained in the hu-ck ACR 1,5 chimera but not the hu-ck ACR 5 chimera. Thus, substitution of both TMDs of the human NTPDase 2 by those of the chicken NTPDase 8 converts the human NTPDase 2 to a more stable enzyme.

Materials and methods

Materials

Dulbecco's modified Eagle's media (DMEM), OptiMEM, fetal bovine serum, antibiotics, trypsin-EDTA, subcloning efficiency DH5α cells, Top 10 *Escherichia coli* cells, pcDNA3, pcDNA3.1/V5-His, and pSecTag 2 vectors, *Taq* polymerase, Platinum PCR Supermix, Lipofectamine, anti-V5 antibody, and anti-V5 and anti-His antibodies conjugated to alkaline phosphatase were purchased from Invitrogen. Anti-mouse antibody conjugated to horseradish peroxidase was purchased from GE Healthcare. Newborn calf serum was purchased from Gemini Bio-Products Inc. (Calabasas, CA). *Pfu* Turbo DNA polymerase was purchased from Stratagene. Restriction enzymes were purchased from New England Biolabs. DNA miniprep kit and QIA gel extraction kit were purchased from QIAGEN. Affi-gel Blue gel, SDS–PAGE reagents and Bio-Rad DC protein assay kit were purchased from Bio-Rad. PVDF membrane and [γ-^{32}P]ATP were purchased from Perkin-Elmer Life Sciences Inc. Alkaline phosphatase substrate tablets (NBT/BCIP) were purchased from Roche. Sephacryl S-200-HR, nucleotides, detergents, and all other biochemical

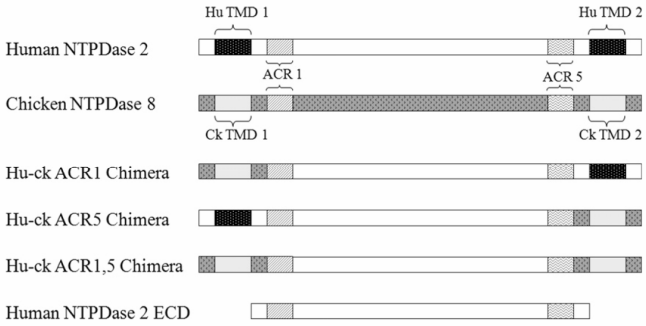

Fig. 1. Construction of the human NTPDase 2 chimeras and its ECD. Schematic representation of the cDNAs of the wild-type human NTPDase 2, the wild-type chicken NTPDase 8, the hu-ck ACR1, the hu-ck ACR5, and hu-ck ACR1,5 chimeras, and the human NTPDase 2 ECD.

reagents were purchased from Sigma Chemical Co. Ultrafree-0.5 centrifugal filter device with Biomax-5K NMWL membrane was purchased from Millipore. Oligonucleotides used as primers for PCR and sequencing were synthesized at the San Diego State University Microchemical Core Facility. DNA sequencing service was provided by the same facility.

Molecular cloning of the extracellular domain (ECD) of human NTPDase 2 and construction of the hu-ck chimeras

A 1.3 kb DNA coding for the extracellular domain of human NTPDase 2 was obtained by using the full length human NTPDase 2 cDNA (1.5 kb) in pcDNA3 [10] as the template in a PCR with *Pfu* Turbo DNA polymerase and the forward primer, gcatactgcaagctt*cccacccgcgacgtccg*, which contains a HindIII site (underlined) upstream of the sequence that anneals to nucleotide 82–98 (italicized) of the human NTPDase 2 cDNA and the reverse primer, gctcgaattcgcggccgcg*gagctgaagtctgtgcc*, which contains a NotI site (underlined) downstream of the sequence that anneals to nucleotide 1372–1389 (italicized) of the human NTPDase 2 cDNA. The 1.3 kb PCR product was digested with HindIII and NotI and ligated to pSecTag2B plasmid DNA that had been digested with the same restriction enzymes. After transformation in Top10 *E. coli* cells, the bacterial colony that contained plasmid with the ECD cDNA insert was propagated and DNA isolated.

The hu-ck ACR5 chimera was constructed by combining amino acid residues 1–449 of human NTPDase 2 with amino acid residues 452–493 of the chicken NTPDase 8 using two-step PCR amplification. Two overlapping DNA fragments were generated separately in the first step. The DNA fragment containing the N-terminal portion of the human NTPDase 2 was produced by PCR using the human NTPDase 2 cDNA (in pcDNA3) as the template, a pcDNA3 forward primer annealing to the vector upstream of the start codon and including a BamHI site, and a hu-ck ACR5 reverse chimeric primer annealing to ACR5. The sequence of the hu-ck ACR5 reverse primer is 5′-catgttggtgAGGTTCAGCATG*tagccg*-3′ in which the nucleotides in lower case are from the chicken NTPDase 8 sequence, nucleotides in italicized lower case are from the human NTPDase 2 sequence, and the underlined nucleotides in capital letters are those that are identical in both sequences. The DNA fragment containing the C-terminal portion of the chicken NTPDase 8 was obtained by PCR using chicken NTPDase 8 cDNA (in pcDNA3) [14] as the template, a hu-ck ACR5 forward chimeric primer, 5′-*cggcta*CATGCTGAACCTcaccaacatg-3′, annealing to ACR5, and a ck-XhoI reverse primer, 5′-gctcgaattcctc gagctatttggatttccagaaacac-3′, annealing to the C-terminal end of chicken NTPDase 8 cDNA and including an XhoI site (underlined). The entire hu-ck ACR5 chimeric cDNA was obtained in the second PCR using the two overlapping DNA fragments generated above as templates, the pcDNA3 forward primer, and the ck-XhoI reverse primer. PCR conditions for generating the overlapping DNA fragment and the chimera were similar to that described previously [15]. The PCR product was purified, double digested by BamHI and XhoI, and ligated with the pcDNA3 vector digested by BamHI and XhoI.

Similar strategy was used to generate the hu-ck ACR1 chimera, in which amino acid residues 1–50 of the chicken NTPDase 8 were combined with amino acid residues 50–495 of the human NTPDase 2. The DNA fragment containing the N-terminal portion of the chicken NTPDase 8 was produced by PCR using the chicken NTPDase 8 cDNA (in pcDNA3) as the template, the same pcDNA3 forward primer containing a BamHI site as described above, and a hu-ck ACR1 reverse chimeric primer, 5′-*ataaacatgga*CGTGTGcgtggagccggc-3′, annealing to ACR1. The DNA fragment containing the C-terminal portion of the human NTPDase 2 was obtained by PCR using the human NTPDase 2 cDNA (in pcDNA3) as the template, a hu-ck ACR1 forward chimeric primer, 5′-gccggctc cacgCACACG*tccatgtttatc*-3′, annealing to ACR1, and the same pcDNA3 reverse primer containing a XhoI site as described above. The entire hu-ck ACR1 chimeric cDNA was obtained by PCR using the two overlapping DNA fragments as templates and the pcDNA3 forward and reverse primers. The PCR product was purified, double digested by BamHI and XhoI, and ligated with the pcDNA3 vector digested by BamHI and XhoI.

The hu-ck ACR1,5 chimera was constructed by combining the amino acid residues 1–50 of the chicken NTPDase 8 with amino acid residue 50–449 of the human NTPDase 2 followed by amino acid residue 452–493 of the chicken NTPDase 8. The DNA fragment coding for the N-terminus of the chicken NTPDase 8 extending to ACR1 followed by ACR1 to ACR5 of human NTPDase 2 was produced by PCR using the hu-ck ACR1 chimeric cDNA (in pcDNA3) as the template and the pcDNA3 forward primer and the hu-ck ACR5 reverse chimeric primer as described above. The DNA fragment coding for the C-terminal portion of the chicken NTPDase 8 was obtained by PCR as described above. The entire hu-ck ACR1,5 chimeric cDNA was obtained by PCR using the two overlapping DNA fragments obtained above as templates, the pcDNA3 forward primer, and the ck-XhoI reverse primer. The PCR product was purified, double digested by BamHI and XhoI, and ligated with the pcDNA3 vector digested by BamHI and XhoI.

Because the chimeric proteins, except hu-ck ACR5, have diminished cross reactivity with KW3, a polyclonal antibody obtained by DNA immunization of the human NTPDase 2 cDNA [10,20], we also generated the V5 epitope and poly-his tagged chimeric proteins by inserting the chimeric cDNAs obtained by PCR into the pcDNA3.1/V5-his vector through TA cloning. DH5α cells were used for transformation by the chimeric cDNAs in pcDNA3 and Top 10 *E. coli* cells were used for transformation by the chimeric cDNAs in pcDNA3.1. Bacterial colonies containing recombinant plasmid DNA were propagated and plasmid DNA were isolated. DNA sequencing showed that there was no unintended mutation in all the recombinant DNA constructs.

Expression of the human NTPDase 2 ECD and the hu-ck chimeras

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing 5% fetal bovine serum, 5% newborn calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Transfection of HEK293 cells by the human NTPDase 2 ECD in pSecTag2B and the hu-ck chimeras in pcDNA3 or pcDNA3.1 using Lipofectamine was as described previously [4]. The cells transfected with the hu-ck chimeric cDNA were harvested 48 h after transfection for ATPase activity determination. To obtain stably transfected cells, aliquots of transfected cells were plated into T-25 flasks after 48 h, and allowed to attach overnight. Geneticin was then added up to 300 μg/ml over a period of two to three weeks. Media were replaced every two to three days. The established geneticin-resistant cells were propagated and used for membrane preparation as described previously [10]. HEK293 cells stably transfected with human NTPDase 2 ECD in pSecTag2B were established by selecting and propagating cells that were resistant to zeocin (200 μg/ml).

Purification of soluble human NTPDase 2 ECD

HEK293 cells stably transfected with the human NTPDase 2 ECD in pSecTag2B were grown in DMEM containing 5% fetal bovine serum and 5% newborn calf serum. Media collected from ten to fifteen 10-cm plates of cells were combined and used for purification of the human NTPDase 2 ECD. Pooled media (100–140 ml) were first subjected to ammonium sulfate fractionation. Approximately 85% of the total activity was recovered in the fraction that was precipitated between 0% and 55% ammonium sulfate saturation. The precipitate was dissolved in 50 mM Tris–HCl, pH 8, and applied to a His.bind column with a bed volume of 3 ml. The column was washed with 30 ml of binding buffer (20 mM Tris–HCl pH 7.9, 0.5 M NaCl, and 5 mM imidazole) followed by 10 ml of washing buffer I (20 mM Tris–HCl pH 7.9, 0.5 M NaCl, and 20 mM imidazole), 10 ml of washing buffer II (20 mM Tris–HCl pH 7.9, 0.5 M NaCl, and 40 mM imidazole), and eluted with 10 ml of elution buffer (20 mM Tris–HCl pH 7.9, 0.5 M NaCl, and 200 mM imidazole). The eluted human NTPDase 2 ECD was concentrated ~10-fold using an Ultrafree-0.5 centrifugal filter device with Biomax-5K NMWL membrane. For further purification, proteins eluted from the His.bind column were applied to a Sephacryl S-200-HR column (1.5 cm × 42 cm) equilibrated with 20 mM Tris–Cl, pH 7.5. The column was eluted with the same buffer and 1-ml

fractions were collected. The fractions containing ATPase activity were combined and concentrated to ~300 μL. The purified ECD was stable for several weeks when stored at 4 °C.

Determination of nucleotide hydrolase activity

For determination of ecto-ATPase activity of transiently transfected HEK293 cells, the cells were harvested by trypsinization, washed and resuspended in isotonic buffer (0.1 M NaCl, 0.01 M KCl, and 25 mM Tris–Cl, pH 7.5), and aliquots (20–60 μg cell protein) were used for ATPase determination with 5 mM MgATP. Enzymatic properties of the human NTPDase 2 and the hu-ck chimeras were characterized using membranes prepared from stably transfected HEK293 cells. Unless indicated otherwise, standard ATPase assays were carried out in a reaction mixture (250 or 500 μL) containing 25 mM Tris–HCl, pH 7.5, 2.5 mM $MgCl_2$ and 2.5 mM ATP for 10 min at 37 °C. Inorganic phosphate released was determined colorimetrically as described previously (*14*). In determining the ATPase activity of the ECD and Km for MgATP and CaATP of the wild-type human NTPDase 2, the hu-ck chimeras, and the human NTPDase 2 ECD, [γ-^{32}P]ATP (~1000 cpm/nmol) was used as the substrate. ^{32}Pi released was separated from radioactive ATP by isobutanol-benzene extraction after complexing with ammonium molybdate [13]. All assays were carried out in duplicates. Values are given as means ± standard deviation of at least three experiments.

Gel electrophoresis and Western blot analysis

SDS–PAGE was performed in 7.5% or 10% polyacrylamide gel in a MINI-PROTEAN II apparatus (Bio-rad). When KW3 antibody was used as the primary antibody for Western blot analysis of wild-type and chimeric human NTPDase 2 proteins, anti-rabbit antibody conjugated to alkaline phosphatase (5000-fold dilution) was used as the secondary antibody. For Western blot analysis of his-tagged ECD, an anti-his antibody conjugated to alkaline phosphatase (2000-fold dilution) was used. The immunoreactive proteins were detected after developing the blot in the alkaline phosphatase substrate (NBT/BCIP) solution. For quantification of protein expression of the chimeras, Western blot analysis was conducted with protein samples prepared from cells transiently transfected with wild-type and chimeric human NTPDase 2 cDNA in the pcDNA3.1-V5/his vector. A linear standard curve was constructed using 0–5 μg cell proteins containing wild-type human NTPDase 2. Anti-V5 antibody (3000-fold dilution) was used as the primary antibody, and anti-mouse antibody conjugated to horseradish peroxidase (3000-fold dilution) was used as the secondary antibody. The immunoreactive proteins were visualized by chemiluminescence and quantified by NIH ImageJ program.

Cibacron blue binding assay

An aliquot of the purified ECD (30 μL containing 2–3 μg of protein) was incubated with 500 μL of Affi-gel Blue gel (Cibacron blue) pre-equilibrated with 20 mM Tris–HCl pH 7.5 and 2 mM $CaCl_2$. The tube was rotated for 30 min at 4 °C to allow binding. After centrifugation, the supernatant was collected and the resin was washed three times with 500 μL equilibrating buffer, then boiled for 5 min with 200 μL of non-reducing SDS sample buffer. Proteins released from the resin as well as the supernatant obtained after the initial binding were subjected to SDS–PAGE and Western blot analysis to determine if nucleotide binding of the ECD is altered.

Results

Expression and purification of the extracellular domain of the human NTPDase 2

To definitively demonstrate that the inhibitory effects of detergents and high temperatures on the human NTPDase 2 are mediated by its transmembrane domains, we first generated a soluble human NTPDase 2 lacking both TMDs. A recombinant plasmid was constructed by inserting the DNA fragment that codes for only the extracellular domain (ECD) of the human NTPDase 2 into the pSecTag2B vector which has an Igκ leader sequence so that the expressed protein, which also contains a myc epitope and poly-his tag at its C-terminus, is secreted. HEK293 cells transfected by the recombinant plasmid did not display any cell surface ATPase activity above that of the mock-transfected cells, whereas ATPase activity was detected in the culture media. The secreted human NTPDase 2 was partially purified by ammonium sulfate fractionation followed by affinity chromatography on a nickel (His.bind) column. The protein fraction eluted from the His.bind column by buffer containing 200 mM imidazole had a specific ATPase activity of 2–4 μmol min^{-1} mg $protein^{-1}$ and contained proteins other than the ECD (Fig. 2, lane 1). The majority of the unrelated proteins could be seperated from the ECD by molecular sieve chromatography on a Sephacryl S-200-HR column. The purified ECD had a specific activity of 10–20 μmol min^{-1} mg $protein^{-1}$ with CaATP as the substrate at pH 7.5. SDS–PAGE showed the presence of a major protein of 62 kDa as well as a protein of higher molecular mass (Fig. 2, lane 2). However, only the 62 kDa protein, the molecular mass expected of the ECD, was immunoreactive with the anti-his antibody (Fig. 2, lane 4).

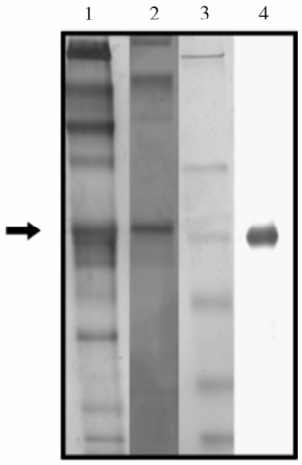

Fig. 2. SDS–PAGE and Western blot analysis of purified human NTPDase 2 ECD. ECD (2.7 μg) eluted from His.bind column (lane 1); and ECD (0.38 μg) eluted from Sephacryl S-200-HR column (lane 2), were subjected to SDS–PAGE analysis followed by silver staining; lane 3, 10 μL of SeeBlue protein marker; lane 4, ECD (0.38 μg) eluted from Sephacryl S-200-HR column, was subjected to Western blot analysis using anti-his antibody conjugated with alkaline phosphatase. The immunoreactive protein has a molecular mass of ~62 kDa.

ATP and ADP hydrolysis by the human NTPDase 2 ECD

The enzymatic properties of the soluble human NTPDase 2 ECD differ significantly from that of the membrane-bound enzyme. While the Ca- and Mg-ATPase activities of the full-length human NTPDase 2 are similar at pH 7.5, the human NTPDase 2 ECD displays a CaATPase activity which is ~4.7-fold higher than its MgATPase activity at the same pH. The Km of the human NTPDase 2 ECD for CaATP is 15.4 ± 3.1 μM, approximately 13-fold lower than that obtained with the membrane-bound enzyme, which is 240 ± 40 μM. In addition to an increased affinity for ATP, human NTPDase 2 ECD also shows an enhanced ability in hydrolyzing ADP. The full-length human NTPDase 2 displays a low CaADPase/CaATPase ratio, i.e., 0.072 ± 0.0031, at pH 7.5, whereas the CaADPase/CaATPase ratio of the ECD is 10-fold higher at 0.81 ± 0.085.

The membrane-bound human NTPDase 2 has not been purified; however, the specific activities of the purified full length chicken [16] and rabbit NTPDase 2 [17] are in the range of ~1000 μmol min^{-1} mg $protein^{-1}$. Assuming that the purified human NTPDase 2 has similarly high activity, the lower activity of the ECD suggests that a conformational change has occurred that affects catalysis. However, the complete binding of the ECD to Cibacron blue (data now shown) indicates that the conformational change does not affect substrate binding.

ATPase activity of the human NTPDase 2 ECD is not inhibited by detergents, high temperatures, and substrate

We previously showed that the activity of the human NTPDase 2 decreases markedly by 0.01% NP-40 [10]. Furthermore, when ATPase assays are carried out for 10 min, the specific activity of the human NTPDase 2 increases in the range of 15–37 °C, but declines at higher temperature. As a result, the activity of the human NTPDase 2 at 55 °C is ~50% of that at 37 °C [10]. We proposed that detergents decrease the activity of the human NTPDase 2 by disrupting inter- and intra-molecular TMD interaction which is important for stabilizing the extracellular domain. TMD interaction can also be disturbed at high temperatures which increase membrane fluidity. However, the effect of temperature on ATPase activity may be partly attributed to the different extent of inactivation of the human NTPDase 2 by substrate at different temperatures. This is illustrated in Fig. 3A which shows that the rate of decline of ATPase activity varies with temperature of the assay. The specific activity of the 10-min ATPase reaction was decreased to ~60%, 70%, and 80% of the 1-min ATPase reaction at 23 °C, 37 °C, and 55 °C, respectively. To minimize the effect of substrate inactivation that may complicate the interpretation of the temperature dependence of ATPase activity, we determined the ATPase activities in 1-min reactions at different temperatures. Fig. 4A shows that the activity of the human NTPDase 2 still declined at higher temperature and the activity at 55 °C was ~50% of that at 37 °C. In contrast, the activity of the human NTPDase 2 ECD lacking both TMDs increased with temperature in the range of 12–55 °C, and its activity at 55 °C was 4-fold higher than that at 37 °C (Fig. 4B). Furthermore, the specific activity of the ECD remains constant during the 10-min reaction at 37 °C (Fig. 3B) indicating that the ECD, unlike the membrane-bound human NTPDase 2, is not susceptible to substrate inactivation.

The resistance of the activity of the ECD to detergent inhibition is shown in Fig. 5. While 0.1% NP-40 inhibited the membrane-bound human NTPDase 2 by more than 90%, it increased the activity of the ECD by ~50%. Thus the removal of the TMDs not only abolished the inhibition of the human NTPDase 2 activity by detergent, which was expected, but also prevented the loss of activity at high temperatures and inactivation by substrate. These results

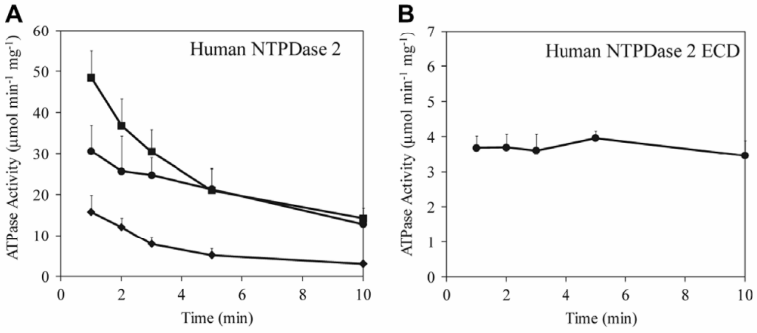

Fig. 3. Time courses of ATP hydrolysis by human NTPDase 2 and its ECD. (A) ATPase assays were carried out in 0.25 ml reaction mixture containing 25 mM Tris–Cl, pH 7.5, 1 mM $MgCl_2$, and 1 mM [γ-^{32}P]ATP with membrane containing wild-type human NTPDase 2 (1 μg) for the indicated times at 23 °C (●), 37 °C (■), or 55 °C (◆). (B) CaATP hydrolysis was carried out in 0.25 ml reaction mixture containing 25 mM Tris–Cl, pH 7.5, 1 mM $CaCl_2$, and 1 mM [γ-^{32}P]ATP with purified human NTPDase 2 ECD (0.5 μg) at 37 °C for the indicated times ($n = 4$). The ATPase activity of human NTPDase 2 declines with time, but not that of the ECD.

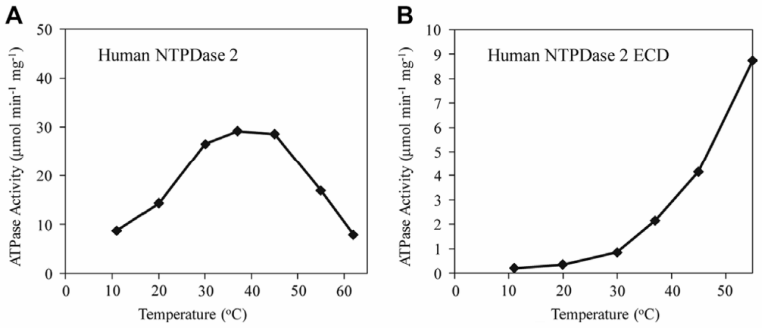

Fig. 4. Effect of temperature on ATPase activity of the human NTPDase 2 and its ECD. ATPase assays were carried out for 1 min in 0.25 ml reaction mixture containing 25 mM Tris–Cl, pH 7.5, 1 mM $CaCl_2$, and 1 mM [γ-^{33}P]ATP with (A) membrane containing wild-type human NTPDase 2 (1 μg) or (B) purified human NTPDase 2 ECD (0.5 μg) at the indicated temperatures. Data presented were from one of three separate experiments. The activity of the human NTPDase 2 decreases at higher temperature, but not the activity of its ECD.

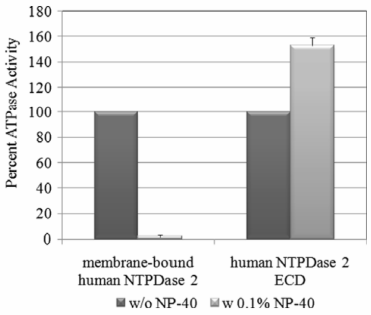

Fig. 5. Effect of NP-40 on ATPase activities of the human NTPDase 2 and its ECD. The ATPase activity of wild-type human NTPDase 2 (0.5 μg) and its the ECD (0.5 μg) was determined in 0.25 ml reaction mixture containing 25 mM Tris–Cl, pH 7.5, 1 mM $CaCl_2$, and 1 mM [γ-^{32}P]ATP with or without 0.1% NP-40 at 37 °C for 10 min. ($n = 3$). The human NTPDase 2 activity is decreased by NP-40, but not the activity of its ECD.

support the conclusion that the TMDs in the full-length human NTPDase 2 are responsible for mediating the inhibitory effects of detergent, high temperature, and substrate.

Expression of the hu-ck chimeras

To further determine if the enzymatic properties of the human NTPDase 2 are affected by specific TMDs attached to the ECD, we constructed chimeras of the human NTPDase 2 in which one or both of its TMDs are exchanged with that of the chicken NTPDase 8 which, unlike human NTPDase 2, is not negatively affected by detergents and high temperatures and does not undergo inactivation by substrates.

The two NTPDases share five apyrase conserved regions (ACRs), which were found in the potato apyrase and several other soluble NTPDases [2,3]. ACR1-4 are located in the N-terminal half of the NTPDases, whereas ACR5 is near the C-terminus. ACR1 of the human NTPDase 2 and the chicken NTPDase 8 differ in only one amino acid, while ACR5 of the two NTPDases differ in two amino acids. We took advantage of the ACR1 and ACR5 sites and used the overlap extension strategy [21] to generate three chimeric constructs. Table 1 shows that cells transiently transfected with the hu-ck ACR1 have similar activity as that of the mock-transfected cells. There was also little protein expression (data not shown). On the other hand, cells transfected with hu-ck ACR5 and ACR1,5 have approximately 10% of the wild-type ATPase activity (Table 1). KW3, a polyclonal antibody against the human NTPDase 2 [10,20] detected the hu-ck ACR5 chimera protein, but not the hu-ck ACR1,5 chimera protein. In order to obtain accurate quantification of the expressed proteins, we generated V5 and his-tagged wild-type human NTPDase 2 and the hu-ck ACR5 and hu-ck ACR1,5 chimeras by inserting their cDNAs into pcDNA3.1 vector. HEK cells transiently transfected with the human NTPDase 2 in pcDNA3.1 showed similar protein expression, as probed by KW3, and ATPase activity as cells transfected by the human NTPDase 2 in pcDNA3.

HEK293 cells transiently transfected with the wild-type and chimeric cDNA in pcDNA3.1 expressed proteins that

Table 1
Ecto-ATPase activities of HEK293 cells transiently transfected with wild-type human NTPDase 2 cDNA, and hu-ck chimeric cDNAs in pcDNA3

	ATPase activity (μmol min^{-1} mg protein^{-1})	% ATPase activity
WT Hu NTPDase 2	2.33 ± 0.40	100
Hu-ck ACR1	0.043 ± 0.0057	1.82 ± 0.54
Hu-ck ACR5	0.20 ± 0.073	8.67 ± 4.7
Hu-ck ACR1,5	0.17 ± 0.056	7.24 ± 3.6
Vector	0.054 ± 0.011	2.36 ± 0.72

HEK293 cells were transfected with pcDNA3 containing the indicated inserts as described in "Experimental procedures". Values reported are averages of four separate transfection experiments ± standard deviations.

could be detected by anti-V5 antibody (Fig. 6). The expression of the hu-ck ACR5 chimera was ~70% of that of the wild-type human NTPDase 2, but the expression of the hu-ck ACR1,5 chimera was reduced to less than 25% as quantified by NIH ImageJ (Table 2). The normalized ATPase activities of the hu-ck ACR5 and hu-ck ACR1,5 chimeras are approximately 9% and 46%, respectively (Table 2). These results indicated that introduction of either the N-terminal or C-terminal TMD of chicken NTPDase 8 into the human NTPDase 2 resulted in either loss or significantly reduced enzyme activity. On the other hand, incorporation of both TMDs of the chicken NTPDase 8 into the human NTPDase 2 affected protein expression, but had less adverse effect on activity.

ATP and ADP hydrolysis by the hu-ck ACR5 and hu-ck ACR1,5 chimeras

HEK293 cells stably transfected by hu-ck ACR5 and hu-ck ACR1,5 chimeric cDNAs were generated by geneticin selection and plasma membrane enriched fractions were used for biochemical characterization. Unlike the ECD which is more active with CaATP than with MgATP, both hu-ck ACR5 and hu-ck ACR1,5 chimeras are more like the wild-type enzyme and display similar CaATPase and MgATPase activities. The MgADPase/MgATPase ratio of the hu-ck ACR1,5 chimera is also the same as the wild-type human NTPDase 2, i.e., 0.054 ± 0.006 at pH 7.4. On the other hand, the hu-ck ACR5 chimera displays a higher MgADPase/MgATPase ratio, i.e., 0.176 ± 0.0118 at pH 7.4, that is intermediate between that of the human NTPDase 2 (0.051 ± 0.010) and chicken NTPDase 8 (0.28 ± 0.031). The Km of the hu-ck ACR5 for MgATP is 0.29 ± 0.021 mM at pII 7.4 and the Km of hu-ck ACR1,5 chimera is 0.32 ± 0.030 mM at pH 7.4, both somewhat higher than that of the wild-type enzyme (0.21 ± 0.022 mM) [10] and significantly higher than that shown by the ECD. These results further demonstrate that kinetic parameters are altered when the soluble ECD is anchored to the membrane by two TMDs.

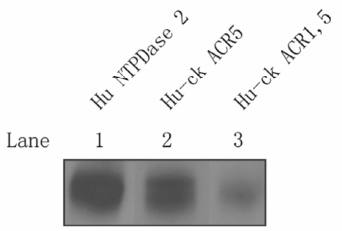

Fig. 6. Protein expression of human NTPDase 2, hu-ck ACR5 chimera, and hu-ck ACR1,5 chimera. Lysate (10 μg) of cells transiently transfected with human NTPDase 2, hu-ck ACR5 chimera, or hu-ck ACR1,5 chimera cDNA in pcDNA3.1 vector were subjected to SDS–PAGE followed by Western blot analysis using anti-V5 antibody as the primary antibody and anti-mouse antibody conjugated to horseradish peroxidase as the secondary antibody. The immunoreactive proteins were visualized by ECL method.

Introduction of C-terminal TMD of the chicken NTPDase 8 into the human NTPDase 2 is insufficient in abolishing inhibition of ATPase activity by NP-40, high temperature, and substrate

The activity of the hu-ck ACR5 chimera is still decreased by low concentration of NP-40, although inhibition by 0.05% NP-40 was ~50% (Fig. 7, lower curve) compared to 95% of the wild-type enzyme (Fig. 5). Fig. 7 further shows that the ATPase activity of the membrane treated with glutaraldehyde is approximately 2.5-fold higher than that of the untreated membrane in the absence of NP-40. Nevertheless, 0.05% NP-40 still caused an ~50% decrease of activity (Fig. 7, upper curve). Thus, unlike the glutaraldehyde-treated wild-type human NTPDase 2 whose activity was not decreased by detergent [10], protein cross-linking does not completely prevent inactivation of the hu-ck ACR5 chimera by detergent.

The response of the hu-ck ACR5 chimera ATPase activity to temperature (Fig. 8, lower curve) is unlike the wild-type enzyme (Fig. 4A). The activity of the hu-ck ACR5 chimera obtained in 1-min reactions does not change significantly in the range of 37–62 °C. Surprisingly, its activity is greater at lower temperatures and decreases in the range of 19–37 °C. The activity at 37 °C is ~58% of the activity at 19 °C.

Similar to the wild-type human NTPDase 2 (Fig. 3A), the specific ATPase activity of membrane containing hu-ck ACR5 chimera decreases to ~28% after 10-min of reaction (Fig. 9A, lower curve). The inactivation by substrate is less severe after protein cross-linking with glutaraldehyde

Table 2
Ecto-ATPase activities and protein expression of HEK293 cells transiently transfected with wild-type human NTPDase 2 cDNA, hu-ck ACR5, and ACR1,5 chimeric cDNAs in pcDNA3.1 vector

	ATPase activity (μmol min^{-1} mg $protein^{-1}$)	ATPase activity (%)	Protein expression (%)	Normalized ATPase activity (%)
Hu NTPDase 2	2.24 ± 0.38	100	100	100
Hu-ck ACR5	0.14 ± 0.052	6.3	68.7 ± 7.01	9.2
Hu-ck ACR1,5	0.25 ± 0.047	11.0	24.1 ± 4.16	45.7
Vector	0.026 ± 0.0071	1.1	N/A	N/A

HEK293 cells were transfected with plasmids containing the indicated inserts as described in "Materials and methods". Values reported are averages of three separate transfection experiments ± standard deviations. Protein expression levels were evaluated by Western blot, as described in "Materials and methods". The immunoreactive proteins were visualized by chemiluminescence and quantified by NIH ImageJ program.

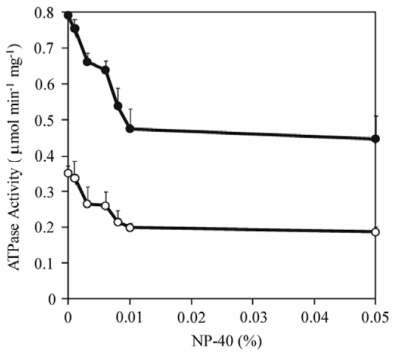

Fig. 7. Effect of NP-40 on the ATPase activities of membranes containing the hu-ck ACR5 chimera protein with or without cross-linking by glutaraldehyde. Membranes containing the hu-ck ACR5 chimera protein (150 μg of membrane proteins) were preincubated in 0.2 ml solution containing 20 mM Mops, pH 7.5, and 5 mM $MgCl_2$, with (●) or without (○) 10 mM glutaraldehyde for 20 min at room temperature, after which 20 μL of 0.25 M lysine was added to stop the cross-linking reaction. Aliquots of membrane proteins were used for ATPase assays in 0.5 ml reaction mixture containing 25 mM Tris–Cl and 2.5 mM MgATP at 37 °C for 10 min. ($n = 3$). The activity of the hu-ck ACR5 chimera is still reduced by NP-40, but inhibition by NP-40 is less after the membrane is cross-linked by glutaraldehyde.

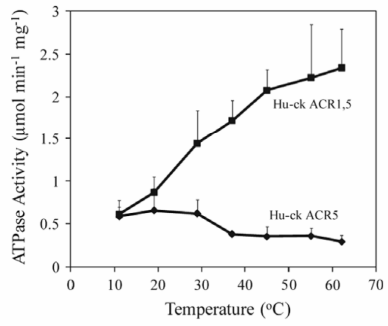

Fig. 8. Effect of temperature on the ATPase activities of the hu-ck ACR5 and hu-ck ACR1,5 chimeras. Membranes containing the hu-ck ACR5 (~15 μg of membrane proteins) (■) or hu-ck ACR1,5 chimera (~10 μg of membrane proteins) (◆) were used for ATPase assays in 0.25 ml reaction mixture containing 25 mM Tris–Cl, pH 7.5, 1 mM $MgCl_2$, and 1 mM [γ-^{32}P]ATP at the indicated temperatures for 1 min ($n = 3$). The activity of the hu-ck ACR5 chimera decreases whereas that of the hu-ck ACR1,5 chimera increases at higher temperatures.

(Fig. 9A, upper curve). Approximately 70% of the activity of the glutaraldehyde-treated membrane remains after 10-min of reaction.

Taken together, these data indicate that introducing the C-terminal TMD of the chicken NTPDase 8 alone into the human NTPDase 2 does not abolish inactivation of the enzyme by detergent, high temperature, and substrate. However, it is important to note that inactivation by NP-40 is reduced and that the chimera exhibits a temperature dependence curve that differs from that of the wild-type enzyme.

Introduction of both TMDs of the chicken NTPDase 8 stabilizes the human NTPDase 2 against inhibition by NP-40, high temperature, and substrate

In marked contrast to the wild-type human NTPDase 2 (Fig. 4A) and the hu-ck ACR5 chimera (Fig. 8 lower curve), the activity of the hu-ck ACR1,5 chimera is resistant to detergent inactivation by NP-40 (Fig. 10). In addition to NP-40, other detergents which inhibit NTPDase 2, i.e., Triton X-100, deoxycholate, Chaps, and alkylglucosides, also have no effect on the hu-ck ACR1,5 chimera (Table 3). The response of the hu-ck ACR1,5 chimera to digitonin is similar to that of the chicken NTPDase 8 [8], and not human NTPDase 2 which is stimulated more than 2-fold by digitonin. Treatment of the membrane containing the hu-ck ACR1,5 chimera with glutaraldehyde decreases, rather than increases, its ATPase activity (Fig. 10 and Fig. 9, lower curves). The remaining activity is also resistant to inhibition by NP-40.

The ATPase activity of the hu-ck ACR1,5 chimera increases with temperature and is undiminished at 62 °C (Fig. 8, upper curve). The specific activity of the hu-ck ACR1,5 chimera at 62 °C is 36% higher than that at 37 °C. The temperature dependence curve of the activity of the hu-ck ACR1,5 chimera is very similar to that of the chicken NTPDase 8 (Fig. 4B in Ref. [10]). The hu-ck ACR1,5 chimera is also no longer susceptible to inactivation by substrate. The activity of the hu-ck ACR1,5 chimera does not decrease with time with or without glutaraldehyde treatment (Fig. 9B).

In summary, substitution of the TMDs of the human NTPDase 2 with that of the chicken NTPDase 8 has converted the enzyme to a more stable protein. Catalysis at the active site in the ECD of the hu-ck ACR1,5 chimera is no longer negatively affected by membrane perturbation in the lipid bilayer by detergent and temperature. Remarkably, it has also become resistant to substrate-induced conformational change to a less active state.

Discussion

Detergents are indispensable in membrane protein research as they are required for membrane protein solubilization and purification [22–24]. In most cases, membrane proteins maintain their native structure and remain in a functional state after solubilization by 1–5% detergent solutions. In contrast, cell surface NTPDases, except for chicken NTPDase 8, lose their activities in the presence of low concentration of several detergents [8,10,11,18, 25,26]. For example, NP-40 at 0.01%, a concentration of the detergent lower than its critical micelle concentration (CMC), decreases the ATPase activity of the human NTPDase 2 by ~90% [10]. This unusual sensitivity to detergents

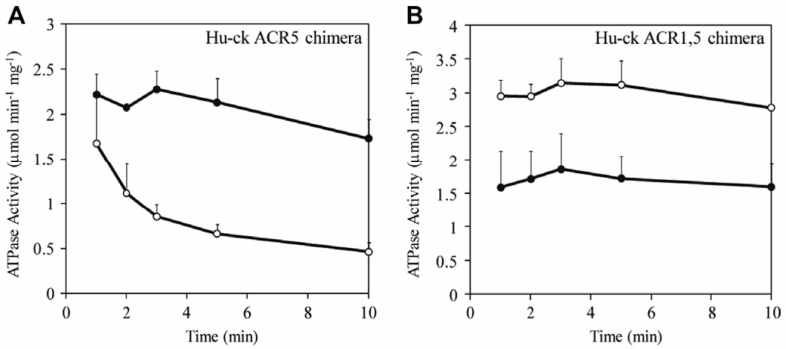

Fig. 9. Time course of ATP hydrolysis by hu-ck ACR5 chimera and hu-ck ACR1,5 chimera with or without glutaraldehyde cross-linking. (A) Membranes containing the hu-ck ACR5 chimera protein (150 μg of membrane proteins) were preincubated in 0.2 ml solution containing 20 mM Mops, pH 7.5, and 5 mM $MgCl_2$, with (●) or without (○) 10 mM glutaraldehyde for 20 min at room temperature, after which 20 μL of 0.25 M lysine was added. Aliquots of membrane proteins were used for enzyme assays at 37 °C for the indicated times. (B). Membranes containing the hu-ck ACR1,5 chimera protein (120 μg of membrane proteins) were preincubated in 0.23 ml solution containing 20 mM Mops, pH 7.5, and 5 mM $MgCl_2$, with (●) or without (○) 10 mM glutaraldehyde for 20 min at room temperature, after which 23 μL of 0.25 M lysine was added. Aliquots of membrane proteins were used for enzyme assays at 37 °C for the indicated times ($n = 3$). The activity of hu-ck ACR5 chimera declines with time, but not that of the glutaraldehyde-treated membranes. The activity of the hu-ck ACR1,5 chimera is decreased after cross-linking with glutaraldehyde, but both remain constant with time.

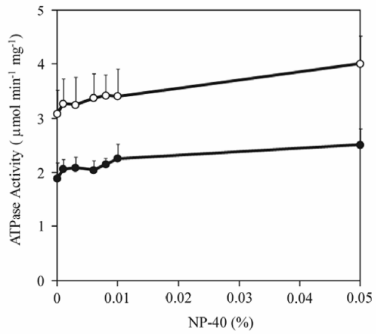

Fig. 10. Effect of NP-40 on the ATPase activities of membranes containing the hu-ck ACR1,5 chimera protein with or without cross-linking by glutaraldehyde. Treatment of the membrane containing hu-ck ACR1,5 chimera by glutaraldehyde was as described in the legend of Fig. 9. Aliquots of membrane proteins were used for ATPase assays in 0.5 ml reaction mixture containing 25 mM Tris–Cl and 2.5 mM MgATP at 37 °C for 10 min. ($n = 3$). The activity of the hu-ck ACR1,5 chimera is not inhibited by NP-40. Cross-linking of the membrane results in reduced activity, but the activity is not inhibited by NP-40.

Table 3
Effect of detergents on the ATPase activities of human NTPDase 2 and hu-ck ACR1,5 chimera

	Human NTPDase 2 Percent activity	Hu-ck ACR1,5 chimera Percent activity
None	100	100
NP-40	3.2 ± 1.6	127.1 ± 1.3
Triton X-100	3.0 ± 1.0	132.6 ± 4.7
Deoxycholate	73.9 ± 6.9	104.6 ± 8.0
Chaps	41.3 ± 1.1	109.2 ± 12
Lauryl maltoside	40.1 ± 3.5	130.7 ± 2.7
Digitonin	238.6 ± 8.8	147.1 ± 17
Octylglucoside	8.6 ± 1.1	99.2 ± 10

Aliquots of membrane containing wild-type human NTPDase 2 or hu-ck ACR1.5 chimera protein were used for ATPase assays in 0.25 ml reaction mixture containing 25 mM Tris–Cl and 5 mM MgATP with or without 0.1% of indicated detergent at 37 °C for 10 min. The 100% value for human NTPDase 2 ranged 45.44 ± 1.43 μmol min^{-1} mg $protein^{-1}$ and the 100% value for hu-ck ACR1.5 chimera is 2.97 ± 0.30 μmol min^{-1} mg $protein^{-1}$ ($n = 3$).

distinguishes the NTPDases from other membrane-bound ATPases such as the ion-motive ATPases, and has made purification of the NTPDases a difficult task.

Since NP-40 inactivates the human NTPDase 2 at a concentration insufficient to solubilize proteins from the membrane, the activity loss of the human NTPDase 2 is most likely due to a disturbance of its TMD interaction by the detergent dissolved in the membrane bilayer. Human NTPDase 2 also undergoes inactivation by the substrate (Ref. [10] and Fig. 3A) and loses its activity at temperatures higher than 37 °C (Ref. [10] and Fig. 4A). However, it is not known if TMD interaction is also involved in these effects. To provide definitive answers to this question, we generated a soluble human NTPDase 2 construct lacking both TMDs. In contrast to the full-length enzyme, the soluble human NTPDase 2 ECD is no longer inactivated by detergents, which is expected. Its activity now increases with temperature up to 55 °C (Fig. 4B). Most surprisingly, the soluble human NTPDase 2 ECD is no longer susceptible to substrate inactivation. We previously hypothesized that substrate inactivation in the full-length enzyme is due to substrate-induced conformational change of the active site to a less active state. However, the lack of substrate inactivation of the ECD activity indicates that the TMDs of the human NTPDase 2 are implicated in this reg-

ulation. In addition to these altered responses to detergent, temperature, and substrate, the soluble human NTPDase 2 ECD has a higher affinity for ATP, and shows a preference for Ca^{2+} instead of Mg^{2+}. This result, as well as those from previous studies on the ECD of human and chicken NTPDase 8 from our laboratory [8,19] and recent study on bacterially expressed soluble rat NTPDase 1, 2, and 3 [27], indicate that changes of the enzymatic properties of the ECD from that of the full-length enzymes can be attributed to the lack of transmembrane domains. It is likely that the environment of the active site is altered when the ECD is severed from the TMDs. It should also be noted that the activities of soluble ECDs of several NTPDases obtained after expression in mammalian cells are generally lower than that of the membrane bound forms [[8,18,19,28] and this study], These results indicate that the soluble forms of these enzymes are less active and that full activity of the NTPDases is only obtained when their ECD are anchored to the membrane by their native TMD [29,30].

Unlike human NTPDase 2, the activity of the chicken NTPDase 8, either in the native tissues [7,11,14] or expressed in human cells [14,15], is maintained in the presence of 5% NP-40. It is not inactivated by its substrate, and its activity increases with temperature up to 55 °C [15]. The TMDs of the chicken NTPDase 8 contain a larger number of small (glycine and alanine) and small polar (serine and threonine) amino acids than the TMDs of the human NTPDase 2 (Fig. 11) We are interested in determining if the interaction of the respective TMDs is responsible for the different stabilities of the human NTPDase 2 and the chicken NTPDase 8 to detergent and temperature. We previously generated ck-hu ACR1, ck-hu ACR5, and ck-hu ACR1,5 chimeras in which the N- or C-terminal region, up to ACR1 and ACR5, respectively, of the chicken NTPDase 8 or both are substituted by the corresponding region of the human NTPDase 2 [15,19]. Incorporation of either the N- or C-terminal TMD of the human NTPDase 2 renders the chicken NTPDase 8 susceptible to inhibition by detergents and high temperatures, although the two chimeras displayed different temperature dependence [15]. Thus the interactions of the human NTPDase 2 N-terminus/chicken NTPDase 8 C-terminus and chicken NTPDase 8 N-terminus/human NTPDase 2 C-terminus are not equivalent. We later showed that chimeras in which the entire chicken NTPDase 8 ECD is ligated to only the N- and C-terminal TMD and the cytoplasmic regions of human NTPDase 2 have similar protein expression and characteristics as the ck-hu ACR chimeras (unpublished data). Thus, the regions between the TMD and the ACR1 and ACR5 of the human NTPDase 2 make no contribution to the protein expression or activity of the ck-hu chimeras.

N-terminal TMD sequence:

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Hu NTPDase 2  SLLPPLLLAAAGLAGLLLLCVP
Ck NTPDase 8  VVAGLLTATCVFSIIALILSAV
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C-terminal TMD sequence:

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Hu NTPDase 2  FSSWVVLLLLFASALLAALVLLL
Ck NTPDase 8  SLWAGAISFIVLAIVAGLVAILL
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Fig. 11. Amino acid sequences of *N*- and C-terminal TMDs of human NTPDase 2 and chicken NTPDase 8. Small amino acid residues (glycine and alanine) are in italic and underlined. Small polar amino acid residues (serine and threonine) are in bold. The TMHMM program (at http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used for TMD prediction.

To determine if the reverse can occur, that is, if a more stable human NTPDase 2 can be generated by TMD exchange, three chimeras containing the human NTPDase 2 ECD but different pairs of TMDs were constructed. In our initial efforts, the N- and C-termini exchanged consisted of only the N- or C-terminal TMD and the short cytoplasmic domains. However, these chimeras were inactive even though they were expressed (data not shown). These negative results indicate that the regions between the TMDs and ACR1 and ACR5 of either NTPDases are likely to be important for proper protein folding of the human NTPDase 2, which will need further investigation. Of the three hu-ck ACR chimeras, which contain sequences between the TMDs and ACR1 and ACR5, two display activity while expression levels vary. The expression level of the hu-ck ACR5 chimera is ~70% of the wild-type human NTPDase 2, whereas that of the hu-ck ACR1,5 chimera is only ~25%. Protein expression of the inactive hu-ck ACR1 is also low, suggesting that the N-terminal TMD of the human NTPDase 2 is necessary for optimal protein expression in HEK293 cells. The normalized activities of the hu-ck ACR5 and hu-ck ACR1,5 are ~10% and ~45% of that of the wild-type human NTPDase 2, respectively. The expression and the activity of the hu-ck chimeras contrast with that of the ck-hu chimeras generated previously. The three ck-hu chimeras, which contain the chicken NTPDase 8 ECD but different pairs of TMDs, have similar protein expression and activity as the wild-type chicken NTPDase 8 when expressed in HEK293 cells [19].

Of the two active hu-ck chimeras, the hu-ck ACR5 chimera retained similar responses to detergent, high temperature, and substrate inactivation as the wild-type human NTPDase 2. On the other hand, we obtained unambiguous evidence that the hu-ck ACR1,5 chimera, in which both TMDs of the human NTPDase 2 were substituted by that of the chicken NTPDase 8, is no longer susceptible to inactivation by detergents and substrate and its activity increases with temperature. It is the only one of the six human NTPDase 2 chicken NTPDase 8 chimeras generated that is not negatively affected by these parameters. These results strongly support the conclusion that the activity of the ECD, whether originating from the chicken NTPDase 8 or the human NTPDase 2, is maintained if they are anchored to the membrane by the chicken NTPDase 8 TMDs, whose interaction is resistant to disruption by detergent and high temperature. The interaction of the chicken NTPDase 8 TMDs is also able to prevent the

conformational change of the active site induced by the substrate, and neither the chicken NTPDase 8 nor hu-ck ACR1,5 displays substrate inactivation.

Previous study showed that glutaraldehyde crosslinking increases the activity of the human NTPDase 2 more than 2-fold and abolishes its inactivation by detergent and substrate [10], suggesting that the resultant quaternary structure is not susceptible to the conformation change induced by the disruption of TMD interaction and substrate. On the other hand, the activity of the chicken NTPDase 8 decreases slightly when it is cross-linked by glutaraldehyde [15]. Interestingly, the activity of all hu-ck and ck-hu chimeras were stimulated after glutaraldehyde crosslinking, except the hu-ck ACR1,5 chimera (ref. 15, and this study). We previously hypothesized that the TMDs of the chicken NTPDase 8 impose a tight and stable structure of the active site of the chicken NTPDase 8. This may also occur in the hu-ck ACR1,5 chimera. Glutaraldehyde crosslinking may cause further restraint in the conformation and result in a decrease of the activity.

In summary, three major findings emerged from this study. Firstly, biogenesis of the human NTPDase 2 is differently affected by the specific TMDs attached to the human NTPDase 2 ECD. Second, catalysis that occurs in the extracellular domain of the human NTPDase 2 is tightly regulated by the TMD attached to its N- and C-termini. Catalytic activity is lost in the hu-ck ACR1 chimera and is reduced in the ECD, the hu-ck ACR5, and the hu-ck ACR1,5 chimeras. The enzymatic properties of the ECD are also significantly altered when compared to the wild-type enzyme. These results indicate that maximal turnover depends on a specific structure of the ECD that is only obtained when it is anchored to the membrane by its native TMDs. Thirdly, resistance to inactivation due to membrane perturbation and substrate of the chicken NTPDase 8 is recapitulated in the hu-ck ACR1,5 chimera which contains both TMDs of the chicken NTPDase 8, providing convincing evidence that strong interaction of the TMDs is determined by their specific amino acid sequences. Future investigations that examine the effects of introduction of amino acids that promote stronger interaction of the TMDs into the TMDs of the human NTPDase 2 should yield greater insight into the mechanism by which TMD interaction regulates catalytic activity of NTPDases.

Acknowledgments

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Chapter 3

Inhibition of the Human NTPDase 2 by Modification of an Intramembrane Cysteine by *p*-Chloromercuriphenylsulfonate and Oxidative Cross-linking of the Transmembrane Domains

Abstract

Human NTPDase 2 is a cell surface integral membrane glycoprotein that is anchored to the membranes by two transmembrane domains while the bulk of the protein containing the active site faces the extracellular milieu. It contains ten conserved cysteine residues in the extracellular domain that are involved in disulfide bond formation and one free cysteine residue, C26, which is located in the N-terminal transmembrane domain. The human NTPDase 2 activity is inactivated by membrane perturbation that disrupts interaction of the transmembrane domain and is inhibited by *p*-chloromercuriphenylsulfonate (pCMPS), a sulfhydryl reagent. In this report, we show that C26 is the target of pCMPS modification, since a mutant in which C26 was replaced by a serine was no longer inhibited by pCMPS. Mutants in which cysteine residues are placed in the C-terminal transmembrane domain near the extracellular surface were still modified by pCMPS, but inhibition of their ATPase activity was less than that of the wild-type enzyme. Thus loss of ATPase activity of the human NTPDase 2 in the presence of pCMPS probably results from disturbance of both transmembrane domain interaction and its active site. Inhibition of human NTPDase 2 activity by pCMPS and membrane perturbation is attenuated when the enzyme is cross-linked by glutaraldehyde. On the contrary, NTPDase 2 dimers formed from oxidative cross-linking of wild-type enzyme and mutants containing single cysteine residue in the C-terminal transmembrane domain displayed reduced ATPase activity. Similar reduction of

activity was also obtained upon intramolecular disulfide formation of mutants that contain a cysteine residue in each of the two transmembrane domains. These results indicate that mobility of the transmembrane helices is necessary for maximal catalysis.

Introduction

Proteins that constitute the NTPDase family are membrane-bound nucleotide hydrolases that are activated by either Mg^{2+} or Ca^{2+} (1). They are located on the cell surface and intracellular membranes and are anchored to the membranes by either one or two transmembrane domains whereas the bulk of the NTPDase protein containing the active site is either in the intracellular lumen or facing the extracellular milieu (2). Different members of the NTPDase family have different substrate preferences. NTPDase 2 is primarily an NTPase (3, 4), whereas NTPDases 4, 5 and 6 are primarily NDPases (5-8), with NTPDase 1, 3, 7 and 8 displaying NDPase/NTPase ratio ranging from 0.25 to 1 (9, 10).

Unlike the membrane-bound ion-motive ATPases, i.e., the P-, V-, and F-type ATPases, the NTPDases have no specific inhibitors. Most of the cell-surface NTPDases, i.e., NTPDase 1, 2, 3, and 8, have reduced activity in the presence of detergents (1). These results are explained by the effects of the detergents on the intra- and inter-molecular interactions of the two transmembrane domains that are located near the N- and C- termini of these proteins, which in turn regulate catalysis in the extracellular domain (11-13). Most membrane-bound NTPDases are inhibited by diethylpyrocarbonate (14-16), which reacts with a conserved histidine in the apyrase conserved region 1 (ACR1) (16-18). 5'-Fluorosulfonyladenosine, an ATP analog, has also been shown to inactivate the human NTPDase 2 (19), the porcine pancreas NTPDase 1 (20) and the Torpedo electric organ ATP

diphospohhydrolase (21). NTPDases that hydrolyze both NTP and NDP, i.e., NTPDase 1, 3, and 8, are inhibited by high concentrations (2-10 mM) of azide (22-27). Azide inhibition of the purified chicken ecto-ATP-diphosphohydrolase (NTPDase 8) is of the mixed and uncompetitive types and is most pronounced with MgADP as the substrate whereas inhibition is markedly diminished with Ca-nucleotides as the substrates (23).

NTPDase 2, which displays an ADPase/ATPase ratio of 0.05-0.1, is not inhibited by high concentrations of azide (15, 28). Chicken smooth muscle and human tumor NTPDase 2 are inhibited by mercurials, e.g., *p*-chloromercuriphenylsulfonate (pCMPS) or *p*-chlomercuribenzoate (pCMB) (19, 28, 29), which react with free cysteine residues. Although cell surface NTPDases contain variable number of cysteine residues, ten of these in the extracellular domain are conserved and have been shown to be involved in disulfide bond formation (30). In the human NTPDase 2, there is only one additional free cysteine residue, C26, located in the N-terminal transmembrane domain (TMD1), and close to the cell surface. This cysteine residue is conserved in the TMD1 of the NTPDase 2 of other species (Fig. 3.1.A), but not those of NTPDase 1, 3, and 8 (Fig. 3.1.B). In this report, we show that C26 in TMD1 of the human NTPDase 2 is the target of pCMPS modification. Mutants in which C26 was replaced by serine (C26S) or alanine (C26A) were no longer inhibited by pCMPS, while other characteristics of the human NTPDase 2 were not affected. To investigate whether the human NTPDase 2 is still inhibited by pCMPS if a free cysteine residue is located in the

C-terminal TMD (TMD2), we also generated mutants containing only one cysteine residue in the TMD2 (Fig. 3.2.). Our results showed that inhibition of these mutants by pCMPS was reduced when compared to the wild-type enzyme.

A

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chicken gizzard            MARRAAAVLLLLALGCLLGILLLCLGSGDAR
rat brain A              MAGKLVSLVPPLLLAAAGLTGLLLLCVPTQDVR
rat brain B              MAGKLVSLVPPLLLAAAGLTGLLLLCVPTQDVR
mouse hepatoma           MAGKLVSLVPPLLLAAVGLAGLLLLCVPTQDVR
human NTPDase 2        1 MAGKVRSLLPPLLLAAAGLAGLLLLCVPTRDVR 33
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B

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NTPDase 1(h)          MEDTKESNVKTFCSKNILAILGFSSIIAVIALLAVGLTQNK
         (r)          MEDIKDSKVKRFCSKNILIILGFSSVLAVIALLAVGLTHNK
NTPDase 3(h)   MFTVLTRQPCEQAGLKALYRTPTIIALVVLLVSIVVLVSITVIQIHK
         (r)   MFTVMTRQPCEQAGFRALSRTPAIVTLVVLLVSIVVLVTLTLIQIHH
NTPDase 8(h)                  MGLSRKEQVFLALLGASGVSGLTALILLLVEAT
         (m)                  MGLSWKERVFMALLGVAAASGLTMLVLILVKAI
         (ck)                   MEYKGKVVAGLLTATCVFSIIALILSAVDVK
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Figure 3.1. N-terminal sequences of cell-surface NTPDases. A. N-terminal sequences of chicken NTPDase 2 (U74467), rat brain NTPDase 2A (Y11835), rat brain NTPDase 2B (AF129103), mouse NTPDase 2 (AF042811) and human NTPDase 2 (EF495152/AF144748). The N-terminal transmembrane domain (TMD1) is underlined. NTPDase 2 from different species contain a conserved cysteine residue (bold), which is C26 in human NTPDase 2. B. N-terminal sequences of human and rat NTPDase1/CD39 (P49961, U81295), human and rat NTPDase 3 (AF034840, AJ437217) and human, mouse and chicken NTPDase 8 (AY903954/AY430414, AY364442, AF426405). The N-terminal transmembrane domain (TMD1) is underlined. TMD1 of these proteins do not contain a conserved cysteine residue.

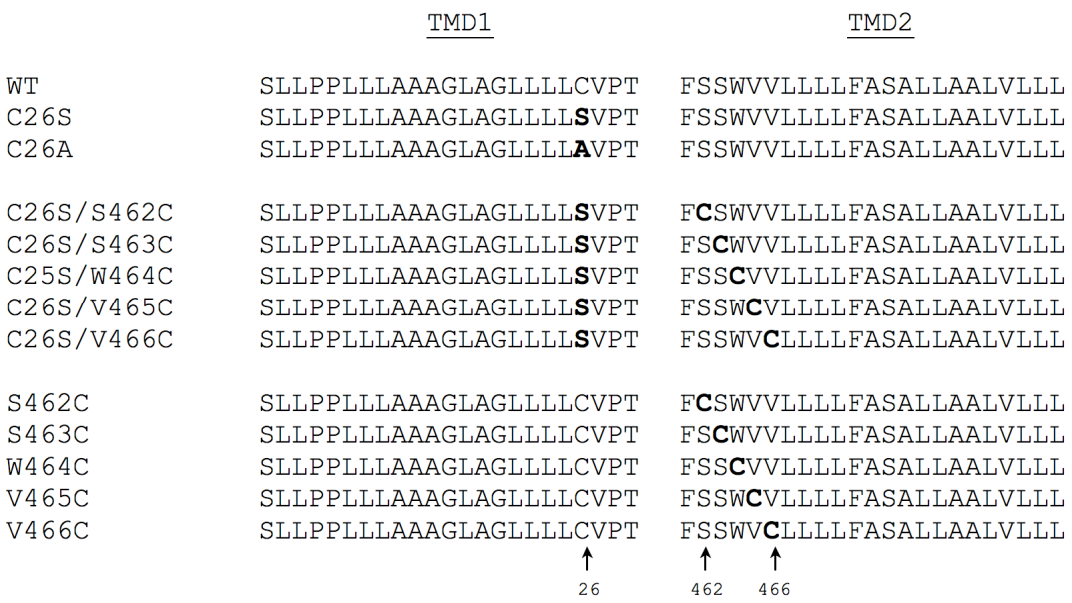

Figure 3.2. Amino acid sequences of TMD1 and TMD2 of the wild-type and mutant NTPDase 2 used in this study. The mutated amino acid residues are shown in bold.

In a previous report (4), we showed that treatment of the wild-type human NTPDase 2 by glutaraldehyde or concanavalin A (ConA), which promotes oligomer formation, abolished the time dependent inactivation of the enzyme by substrate, thus giving rise to apparent stimulation of enzyme activity in a 10 min assay. Glutaraldehyde and ConA cross-linking also attenuated the decrease of activity in the presence of NP-40 and high temperature, indicating that the

oligomerized human NTPDase 2 is less susceptible to the inhibitory effect of NP-40, high temperature, and substrates. Since recent evidence obtained in our laboratory indicated that the strength of the interaction of the TMD is an important determinant in enzyme stability and activity (12, 13), we generated additional mutants containing two cysteine residues, one in each of the two TMDs, and determined the effect of oxidative cross-linking of these cysteine residues on enzyme activity (Fig 3.2.). In contrast to the results obtained by cross-linking by glutaraldehyde and ConA, inter- and intramolecular cross-linking of the cysteine residues in the TMD resulted in significant decrease of the human NTPDase 2 activity. On the other hand, immobilization of the TMD helices due to intramolecular disulfide bond formation abolished inactivation of the residual activity of the human NTPDase 2 by detergents and higher temperatures.

Materials and methods

Materials

Dulbecco's modified Eagle's media (DMEM), OptiMEM, fetal bovine serum, penicillin-streptomycin solution, trypsin-EDTA, subcloning and library efficiency DH5α cells, and Lipofectamine were purchased from Invitrogen. Newborn calf serum was purchased from Gemini Bio-Products Inc. *Pfu* Turbo DNA polymerase was purchased from Stratagene. DpnI was purchased from New England Biolab. DNA miniprep kit was purchased from QIAGEN. SDS-PAGE

reagents and Bio-Rad DC protein assay kit were purchased from Bio-Rad. PVDF membrane and [γ-^{32}P]ATP were purchased from PerkinElmer Life Sciences Inc. Goat anti-rabbit IgG conjugated to alkaline phosphatase was purchased from Promega (Madison, WI). Alkaline phosphatase substrate tablets (NBT/BCIP) were purchased from Roche. Nucleotides, detergents, and all other biochemical reagents were purchased from Sigma Chemical Co. Oligonucleotides used as primers for PCR and sequencing were synthesized at the San Diego State University Microchemical Core Facility or Integrated DNA Technologies. DNA sequencing service was provided by the San Diego State University Microchemical Core Facility. Rabbit polyclonal antibody against the C-terminus (LRQVHSAKLPSTI-COOH) of the human NTPDase 2 was a kind gift from Dr. Terence Kirley of the Department of Pharmacology and Cell Biophysics at the University of Cincinnati.

Site directed mutagenesis

The following human NTPDase 2 mutants were generated for this study. In the C26S and C26A mutants, the single free cysteine, C26, was replaced by serine or alanine. The C26S/S462C, C26S/S463C, C26S/W464C, C26S/V465C and C26S/V466C mutants contain a single free cysteine residue in TMD2 and were generated by replacing S462, S463, W464, V465 and V466 by cysteine. The S462C, S463C, W464C, V465C, and V466C mutants contain a cysteine residue in TMD1 (C26) and a cysteine residue in TMD2 at position 462, 463, 464, 465, and 466, respectively (Fig. 3.2.). The human NTPDase 2 mutant cDNAs were generated by PCR using either the wild-type or the C26S human NTPDase 2 cDNA

in pcDNA3 as the template and appropriate mutagenic primers as previously described (31). The forward mutagenic primers used were respectively, 5'-cctcctactgctgtccgtcccccacccag-3'for C26S, 5'-cctcctactgctggccgtccccacccgc-3' for C26A, 5'-ggcacagacttctgctcctgggtcgtcctc-3' for S462C and C26S/S462C, 5'-cacagacttcagctgc-tgggtcgtcctcctg-3' for S463C and C26S/S463C, 5'-gacttcagctcctgcgtcgtcctcctgctgc-3' for W464C and C26S/W464C, 5'-cttcagctcctggtgcgtcctcctgctgctc-3' for V465C and C26S/V465C and 5'-cagctcctgggtctgcctcctg-ctgctcttc-3' for V466C and C26S/V466C, with the codons that give rise to the specified mutation underlined. After transformation in DH5α cells and propagation of the ampicilin-resistant colony, plasmids containing the mutant cDNAs were isolated and sequenced to verify the desired mutation and absence of unwanted mutation.

Transfection and membrane preparation

Transient transfection of HEK293 cells by wild-type and mutant human NTPDase 2 cDNA in pcDNA3 and selection of stable transfectants were performed as described previously (4, 12, 13, 27). The plasma membrane enriched fraction from stably transfected cells was prepared by differential and sucrose gradient centrifugation as described previously (4, 12, 13, 27).

Determination of ATPase activity

Ecto-ATPase activity of the intact transfected cells was determined in 250 μL reaction mixture containing 25 mM TrisCl, pH 7.5, 5 mM $MgCl_2$ and 5 mM [γ-^{32}P]ATP (~ 1.5 x 10^6 cpm/μmol ATP). ATPase activity of membranes prepared

from the stably transfected cells was determined in 250 μL reaction mixture containing 25 mM HEPES (pH 7.5), 4 mM $MgCl_2$, and 1 mM [γ-^{32}P]ATP. ^{32}Pi released was determined as described previously (19). To determine the ATPase activity in the presence of pCMPS, membranes were preincubated in the reaction mixture with the indicated concentrations of pCMPS for 5 min at room temperature before the reactions were initiated by the addition of ATP. For glutaraldehyde cross-linking, membranes at a protein concentration of 0.3 mg/ml were incubated in 20 mM MOPS and 5 mM $MgCl_2$ with 10 mM glutaraldehyde for 20 min at room temperature. Aliquots of membranes were assayed for ATPase activity in the absence or presence of the indicated concentrations of pCMPS.

Oxidative cross-linking

Oxidative cross-linking of cysteine residues of wild-type and mutant NTPDase 2 was carried out using membranes prepared from stably transfected cells. The oxidizing agent, copper phenanthroline (CuP), was prepared by combining cupric sulfate and 1,10-phenanthroline at a 1:3 molar ratio in water. Membranes at a concentration of 0.5 mg/mL were treated with the indicated CuP concentration in 12 mM HEPES at pH 7.5 at 37 °C for 20 min. For samples to be directly applied to the gel, the oxidation reactions were stopped by adding equal volume of 2X non-reducing SDS loading buffer containing 10 mM N-ethylmaleimide and 10 mM EDTA. For samples to be used for ATPase assays, reactions with CuP were stopped by 10-fold dilution of the mixtures with buffer containing 25 mM HEPES at pH 7.5, and 2 mM EDTA.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed using a 7.5% polyacrylamide gel in a MINI-PROTEAN II apparatus (Bio-Rad). For Western blot analysis, antibody against the C-terminus of the human NTPDase 2 (5000-fold dilution) was used as primary antibody and goat anti-rabbit IgG conjugated to alkaline phosphatase (5000-fold dilution) was used as the secondary antibody. The immunoreactive proteins were detected using alkaline phosphatase substrate (NBT/BCIP) solution.

Results

Inhibition of the expressed human NTPDase 2 by pCMPS

We previously reported that pCMPS inhibited the plasma membrane ATPase of the human small cell lung carcinoma xenograft, an NTPDase 2 (19) and the ecto-ATPase activity of intact lung tumor cells (28). Inhibition of ATPase activity by mercurials was also observed in plasma membranes prepared from chicken gizzard smooth muscle (29), a tissue abundant in ecto-ATPase/NTPDase 2 (32). The sensitivity to pCMPS inhibition was retained in the ATPase in membranes prepared from HEK293 cells stably transfected with the human NTPDase 2 cDNA (Fig. 3.3., lower curve, -◇-). Approximately 40% inhibition was obtained at a pCMPS concentration of 2.5 μM. Maximal inhibition, ~70%, was obtained at a pCMPS concentration of 0.25 mM.

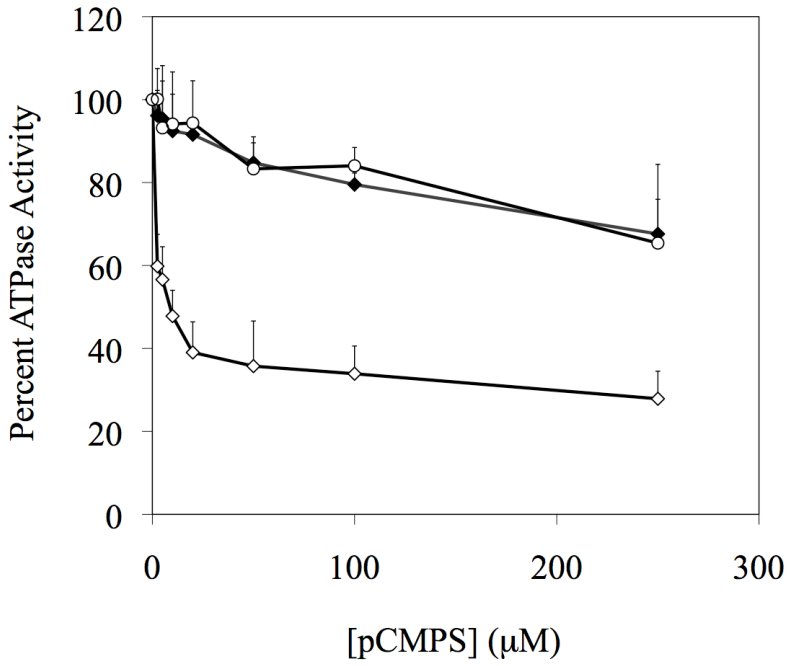

Figure 3.3. Effect of pCMPS on the ATPase activity. Membranes containing wild-type human NTPDase 2 were pretreated with (-◆-) or without (-◇-) 10 mM glutaraldehyde or C26S mutant NTPDase 2 without glutaraldehyde (-○-) as describe in the "Materials and methods". Aliquots of membranes were assayed for ATPase activity in the absence or presence of the indicated concentrations of pCMPS. The 100% activities for wild-type human NTPDase 2 with or without glutaraldehyde treatment and C26S mutant were 18.0 ± 1.3, 23.9 ± 1.6, and 19.9 ± 0.1 μmol min^{-1} mg^{-1} respectively.

The target of pCMPS modification is cysteine residue 26

The wild-type human NTPDase 2 contains the same ten conserved cysteine residues, i.e. C75, C99, C242, C265, C284, C310, C323, C328, C377, and C409, in the extracellular domain as in other cell surface NTPDases. These conserved cysteine residues have been shown to be involved in disulfide bond formation in human NTPDase 3 (30) and most likely serve the same function in human NTPDase 2. Human NTPDase 2 contains an additional free cysteine residue, C26,

located in TMD1 (Fig. 3.1.A), which is the most likely target of pCMPS. If this is the case, a mutant protein in which C26 is replaced by other amino acids should become resistant to pCMPS inhibition. Two mutants, C26A and C26S, which lack the free cysteine, were generated and gave very similar results. Only those obtained with C26S were summarized here. Substitution of C26 by serine had minimal effect on either protein expression (Fig. 3.4., lane 2) or activity (Table 3.1.). However, pCMPS inhibition was markedly reduced (Fig. 3.3., upper curve, -o-). The conclusion that C26 is the target of pCMPS modification is further supported by the results of oxidative cross-linking. The wild-type human NTPDase 2 is a 66 kDa protein (Fig. 3.5., lane 1). Treatment of the membranes by pCMPS did not alter protein mobility in SDS-PAGE (Fig. 3.5., lane 2). Upon oxidative cross-linking with Cu-phenanthroline, the majority of the wild-type 66 kDa monomer was converted to a dimer due to disulfide bond formation between the C26 of two monomers (Fig. 3.5., lane 3). Dimer formation was not detected (Fig. 3.5., lane 4) if the human NTPDase 2 was first treated with pCMPS, indicating that C26 has reacted with pCMPS and oxidative cross-linking by CuP could not occur. In contrast, the C26S mutant protein (Fig. 3.5., lane 5), which lacks a free cysteine residue, did not form dimer upon oxidative cross-linking (Fig. 3.5., lane 6). These results provided conclusive evidence that dimer formation upon oxidative cross-linking requires the presence of free C26. Dimer formation was abolished if C26 was either substituted by serine or modified by pCMPS.

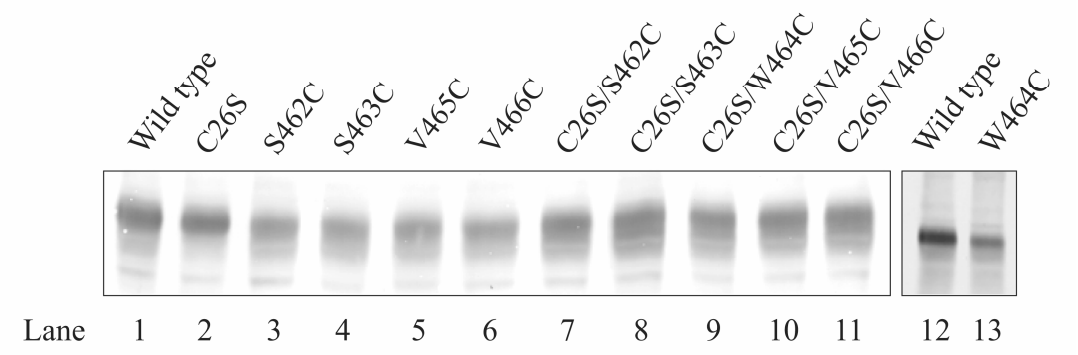

Figure 3.4. Protein expression of wild-type and mutant human NTPDase 2 in HEK cells. Cell lysates (20 μg protein) of HEK293 cells transiently transfected with wild-type and mutant human NTPDase 2 cDNAs in pcDNA3 were subjected to SDS-PAGE and Western blot analysis. Protein expression of the W464C mutant was performed in a separate experiment.

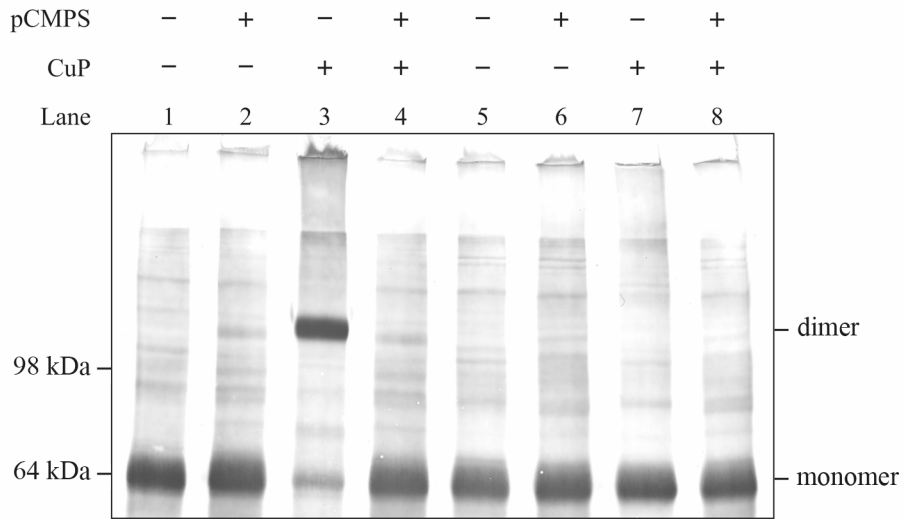

Figure 3.5. Oxidative cross-linking of wild-type and C26S mutant human NTPDase 2 protein without and with covalent modification by pCMPS. Membranes (10 μg) containing wild-type human NTPDase 2 were incubated for 5 min without and with 0.5 mM pCMPS in 18 μL at room temperature, after which 2 μL CuP was added to a concentration of 0.5 mM to the indicated samples and incubated at 37°C for 20 min. Aliquots of the membranes (7.5 μg) were subjected to SDS-PAGE and Western blot analysis. Lanes 1-4: membranes containing wild type human NTPDase 2. Lanes 5 and 6, membranes containing C26S mutant NTPDase 2.

Table 3.1. ATPase activity of HEK cells transfected with wild-type and cysteine mutant human NTPDase 2 cDNAs. HEK293 cells were transfected with wild-type and mutant human NTPDase 2 in pcDNA3 as described in "Materials and methods". ATPase activity of the intact cells was determined 48 hours after transfection. The ATPase activity of the cells transfected with wild-type human NTPDase 2 cDNA ranged from 0.8 to 4.5 μmol min^{-1} mg^{-1}. Values reported are averages of three separate transfection experiments ± standard deviations.

Human NTPDase 2 cDNA	Percent wild-type ATPase activity
Wild-type	100
C26S	88.7 ± 8.4
C26S/S462C	86.6 ± 9.1
C26S/S463C	73.5 ± 9.7
C26S/W464C	4.9 ± 1.1
C26S/V465C	80.0 ± 9.0
C26S/V466C	97.3 ± 3.3
S462C	58.1 ± 2.1
S463C	55.4 ± 8.4
W464C	2.4 ± 0.04
V465C	60.1 ± 8.2
V466C	63.9 ± 9.2

Inhibition by pCMPS is reduced if the free cysteine residue is located in the TMD2

We then set out to determine if pCMPS will inhibit the ATPase activity of the human NTPDase 2 by reacting with a cysteine residue in TMD2 located in a position similar to that occupied by C26 in TMD1 near the exterior of the bilayer. Different programs predicting transmembrane topology were used to define the TMD2 region. Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html)

and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) predict that TMD2 begins with F461, thus placing S462 and S463 within TMD2, whereas TopPred (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) and DAS (http://www.sbc.su.se/~miklos/DAS/) designate S463 as the first amino acid of TMD2 (see Fig. 3.2.). Five mutants, C26S/S462C, C26S/S463C, C26S/W464C, C26S/V465C, C26S/V466C were generated to cover both possibilities. These mutant cDNAs were generated by PCR using the cDNA encoding the C26S mutant as the template. The resultant mutant proteins contain only a single free cysteine residue in positions 462-466 of the human NTPDase 2 polypeptide (Fig. 3.2.).

ATPase activity of HEK cells transiently transfected with these mutant cDNAs ranged from 75-100% of that of the cells transfected with the wild-type human NTPDase 2 cDNA, except for C26S/W464C, which lost ~ 95% of activity (Table 3.1.). Protein expression of all five mutants, including that of C26S/W464C, was similar to that of the wild-type protein (Fig. 3.4., lanes 7-11).

Membranes were prepared from HEK cells stably transfected with the C26S/S462C, C26S/S463C, C26S/V465C, and C26S/V466C mutant cDNAs and used for ATPase assays in the absence and presence of 0.1 mM pCMPS. Although all four mutant proteins contain a free cysteine, the inhibition of the ATPase activities by pCMPS was greatly reduced compared to the wild-type enzyme containing C26 (Table 3.2.). The activity of C26S/S462C and the S26C/S463C mutant proteins were inhibited by 24% and 14%, while the ATPase activity of the C26S/V465C and C26S/V466C mutant proteins were inhibited by ~40%.

Table 3.2. Inhibition of wild-type and mutant human NTPDase 2 ATPase activity by pCMPS. Membranes used for ATPase assays were prepared from the stably transfected cells as described in "Materials and methods". The ATPase activity of the membranes containing wild-type and mutant human NTPDase 2 ranged from 25 to 50 μmol min^{-1} mg^{-1}. Values reported are averages of three separate ATPase assay ± standard deviations. Percent inhibition was calculated using ATPase activity of the wild-type and different mutants obtained in the absence of 0.1 mM pCMPS as 100%.

	Percent inhibition (by 0.1 mM pCMPS)
Wild-type	77.4 ± 2.9
C26S	7.6 ± 5.1
C26S/S462C	24.2 ± 1.4
C26S/S463C	14.4 ± 3.0
C26S/V465C	39.9 ± 3.6
C26S/V466C	37.7 ± 5.6
S462C	80.7 ± 1.1
S463C	90.7 ± 0.7
V465C	91.5 ± 1.4
V466C	82.8 ± 1.2

The significantly reduced inhibition of the ATPase activity of the mutants containing a single cysteine residue in the TMD2 by pCMPS was not due to a failure of reaction of the mercurial with the introduced cysteine residues. Similar to the wild-type enzyme (Fig. 3.5., lanes 3 and 4), the mutant proteins were able to form dimers when subjected to oxidative cross-linking by CuP (Fig. 3.6., lanes 1, 3, 5 and 7), albeit to different extent. However, no dimer formation was obtained with three of the mutant proteins if the membranes were previously incubated with pCMPS (Fig. 3.6., lanes 2, 4, and 8). Dimer formation of C26S/V465C was

markedly reduced if the membranes were pre-treated with pCMPS (Fig. 3.6., lane 6).

It is interesting to note that the largest amount of dimer was obtained with the C26S/S463C mutant protein. The extent of dimer formation of this mutant protein (Fig. 3.6., lane 3) was comparable to that of the wild-type enzyme (Fig. 3.5., lane 3). The facile cross-linking of cysteine residue at position 26 and 463 was further supported by the observation that significant amount of dimer formation was only obtained with the wild-type enzyme and the C26S/S463C mutant protein when the reaction with CuP was carried out at 4°C (data not shown).

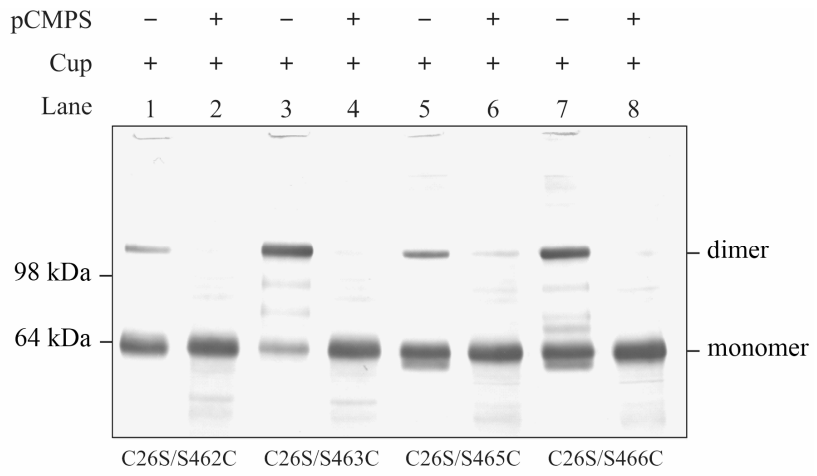

Figure 3.6. Oxidative cross-linking of human NTPDase 2 mutant proteins containing a single cysteine residue in TMD2 without and with covalent modification by pCMPS. Membranes containing C26S/S462C (lanes 1 and 2), C26S/S463C (lanes 3 and 4), C26S/V465C (lanes 5 and 6) and C26S/V466C (lanes 7 and 8) mutant NTPDase 2 were incubated without and with 0.5 mM pCMPS as described in the legend of Figure 3.5., after which 2 μL CuP was added to a concentration of 0.5 mM and incubated for 20 min at 37°C. Aliquots of the membranes (7.5 μg) were subjected to SDS-PAGE and Western blot analysis.

Intramolecular cross-linking of human NTPDase 2 mutants that contains one cysteine residue each in TMD1 and TMD2

We showed previously that treatment of the wild-type human NTPDase 2 with glutaraldehyde, which promotes oligomer formation, abolished the inhibitory effects of NP-40, high temperature, and substrate (4). Fig. 3.3. (upper curve, -♦-) shows that the inhibition of the ATPase activity of glutaraldehyde-treated membranes by 50 μM pCMPS was negligible when ~60% inhibition of the ATPase activity was obtained in untreated membranes. These results suggest that oligomers of the human NTPDase 2 are less susceptible to pCMPS inhibition. However, because of the large number of lysine and other amino acid residues that can react with glutaraldehyde in the human NTPDase 2 protein, intramolecular cross-linking within an NTPDase 2 monomer may also occur and the possibility that some of the effects of glutaraldehyde are due to intramolecular cross-linking cannot be ruled out. This possibility is of particular interest since recent evidence suggests that the strength of inter-helical interaction of TMD1 and TMD2 within the monomer of the NTPDases is more important in regulating catalysis than intermolecular interaction of TMD between two different NTPDase 2 monomers (13). Oxidative cross-linking between two cysteine residues in the TMD1 and TMD2 would provide one means of strengthening intramolecular TMD interaction. Thus, we generated mutants containing one cysteine residue in TMD1 (C26) and a second cysteine in TMD2 in proximity to C26, i.e., S462C, S463C, W464C, V465C, and V466C (Fig. 3.2.).

ATPase activity of HEK293 cells transiently transfected with these mutant cDNAs was approximately 60% of that of cells transfected with wild-type human NTPDase 2 cDNA, except for W464C, which lost ~98% of activity (Table 3.1.). Protein expression of S462C, S463C, W464C, V465C, and V466C was comparable to that of the wild-type enzyme (Fig. 3.4., lanes 3-6, 13). Similar to the wild-type enzyme, the ATPase activity of membranes obtained from HEK cells stably transfected with the four active mutants was inhibited 80-90% by 0.1 mM pCMPS (Table 3.2.). This was expected since these four mutants retain the original free cysteine residue, C26.

Upon CuP treatment, the mutants with a cysteine residue each in the TMD1 and TMD2, S463C, V465C, and V466C, formed fewer dimers than C26S/S463C, C26S/V465C, C26S/V466C, which contain only one cysteine residue in TMD2 (compare lane 6 with lane 14, lane 8 with lane 16, lane 10 with 18 in Fig. 3.7.). This was most noticeable with the C26S/S463C and S463C mutant pair (Fig. 3.7., lane 6 and lane 14).

S462C was an exception in that it formed more dimers than C26S/S462C after CuP treatment (Fig. 3.7., lane 4 and lane 12). It appears that the presence of C26 in the S462C mutant promotes dimer formation between C26 and C462 of different monomers, suggesting that these two residues are at the interface between two different monomers.

Higher order oligomers were consistently obtained in mutant proteins containing two free cysteine residues after oxidative cross-linking although

accounting for only a small amount of the total protein. The majority of the S463C, V465C, V466C mutant proteins remained as monomers after CuP treatment. These results suggest that intramolecular cross-linking was generally favored over intermolecular cross-linking when these mutants are treated with CuP.

Interestingly, there was more dimer formation in the S462C mutant than the corresponding mutant containing a single cysteine residue, C26S/S462C, after oxidative cross-linking (compare lane 12 with lane 4 in Fig. 3.7.). Nevertheless, intramolecular cross-linking in the S462C mutant also occurred as shown by the greater decrease of activity described in the next section.

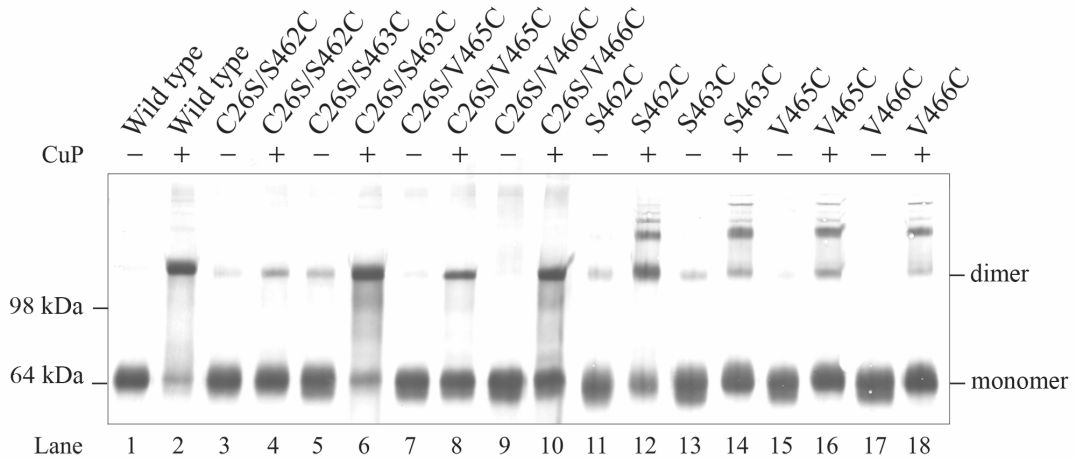

Figure 3.7. Oxidative cross-linking of wild-type human NTPDase 2 and mutant proteins containing one or two cysteine residues. Membranes (10 μg) containing wild-type and mutant NTPDase 2 were incubated without or with 0.5 mM CuP in 20 μL solution containing 12 mM HEPES buffer at pH 7.5 for 20 min at 37°C after which 20 μL of 2X SDS gel sample buffer were added. The entire samples were used for SDS-PAGE and Western blot analysis.

Effect of inter- and intra-molecular oxidative cross-linking on ATPase activity

The ATPase activity of the wild-type human NTPDase 2 and the mutant proteins without and with CuP oxidative cross-linking were compared. Of the proteins containing one cysteine residue, the wild-type enzyme and the C26S/S463C mutant enzyme suffered ~70% loss of activity after cross-linking (Table 3.3.). The reduction of activity correlated with the extent of intermolecular cross-linking since the largest amounts of dimer were obtained in these two proteins after CuP treatment (Fig. 3.7.). The activity loss of the C26S/S462C, C26S/V465C, and C26S/V466C mutants, which form less dimers, was 40-60% after CuP treatment.

ATPase activity of the S462C, S463C, V465C, and V466C mutant proteins that contain a cysteine residue each in the TMD1 and TMD2 was reduced to 20-40% after CuP treatment, with the S463 mutant showing the greatest loss of activity (Table 3.3.). Taken together, these data and the Western blot analysis shown in Fig. 7 indicate that the loss of ATPase activity was a consequence of both intramolecular cross-linking and oligomer formation after CuP treatment. In conclusion, both inter- and intra-molecular cross-linking of the TMDs caused the human NTPDase 2 to suffer marked loss of enzyme activity.

Inhibition of ATPase activity by NP-40 is reduced after intramolecular oxidative cross-linking of the TMDs

Similar to the wild-type human NTPDase 2, the activity of all the mutants generated in this study was inhibited 90% by 0.1% NP-40. As described above, CuP treatment reduced the ATPase activity of mutant NTPDase 2 to a variable

extent. However, the residual activity of the oxidatively cross-linked wild-type human NTPDase 2 and C26S/S463C, which formed the most dimers, was inhibited by NP-40 only by 50-60% (Fig. 3.8.). In contrast, the residual activity of the C26S/S462C, C26S/V465C, and C26S/V466C mutants, which formed less dimers, was still inhibited 90% by NP-40. Thus, the reduced inhibition by NP-40 correlated with the amount of dimer formed.

Table 3.3. Effect of CuP oxidative cross-linking on the ATPase activity of wild-type human NTPDase 2 and mutants. Membranes were preincubated with or without CuP and the ATPase activity was determined as described in "Materials and methods". The ATPase activity of the membranes ranged from 13 to 18 μmol min^{-1} mg^{-1}. Values reported are averages of three separate experiments ± standard deviations. Percent activity was calculated using ATPase activity of the wild-type and mutants obtained without CuP cross-linking as 100%.

	Percent activity
Wild-type	26.2 ± 6.5
C26S	87.7 ± 11.3
C26S/S462C	60.0 ± 3.2
C26S/S463C	28.0 ± 3.8
C26S/V465C	58.2 ± 10.2
C26S/V466C	40.5 ± 2.6
S462C	26.4 ± 9.5
S463C	17.5 ± 7.5
V465C	28.6 ± 2.7
V466C	39.3 ± 4.1

All of the mutants containing one cysteine residue each in the TMD1 and TMD2 showed decreased inhibition by NP-40 after oxidative cross-linking. The S463C mutant was completely resistant to inhibition by NP-40 after intramolecular cross-linking, while the inhibition of the cross-linked S462C, V465C, and V466C mutant enzymes by 0.1% NP-40 was reduced to 40-60%. These results suggested that both inter- and intra-molecular oxidative cross-linking of the TMDs increased TMD interaction and reduced or abolished the inhibitory effect of NP-40.

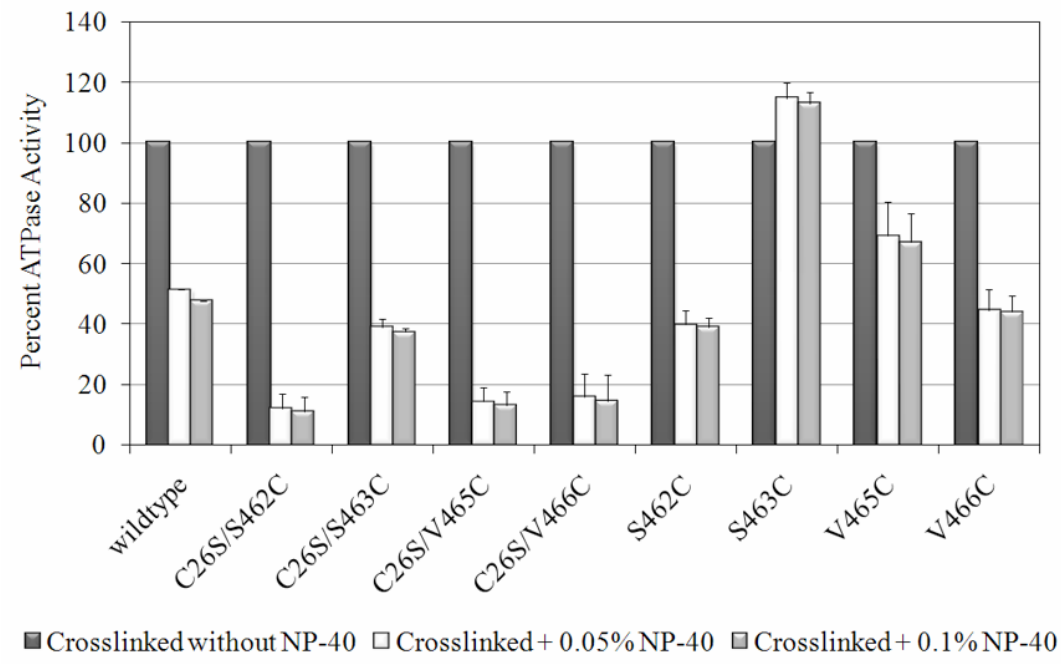

Figure 3.8. Effect of inter- and intra-molecular cross-linking on the NP-40 inactivation of ATPase activity of wild-type and mutant human NTPDase 2. Membranes (10 μg) containing C26S/S462C, C26S/S463C, C26S/V465C, C26S/V466C, S462C, S463C, V465C, or V466C mutant NTPDase 2 were incubated for 20 min without or with 0.5 mM CuP in 20 μL reaction mixture at 37°C for 20 min. Aliquots of the membranes were assayed for ATPase activity in the absence or presence of the indicated concentrations of NP-40. ATPase activity of the cross-linked wild-type and mutants was used as 100% value. Values reported are average of three separate experiments ± standard deviations.

Inter- and intra- molecular oxidative cross-linking of TMDs abolishes inhibition of ATPase activity by higher temperatures

The effect of temperature on the NTPDase 2 mutants containing one cysteine in TMD2, or with a cysteine residue each in the TMD1 and TMD2, was similar to that of the wild-type enzyme in that ATPase activity declines at higher temperature (4). The effect of high temperature was attributed to the disruption of TMD interaction due to increased membrane fluidity, which in turn destabilizes the active site. This is illustrated by the data obtained with the C26S/S463C and S463C mutants. The ATPase activity of these mutant enzymes at 60^{o}C was usually ~30% of the activity obtained at 37^{o}C (Fig. 3.9.A and B). After CuP treatment, the residual ATPase activity of the C26S/S463C mutant increased, rather than diminished, at high temperature up to 61^{o}C (Fig. 3.9.C), so that the ratio of the ATPase activity obtained at 61 ^{o}C to that at 37 ^{o}C was ~1.5. This was also seen with the wild-type human NTPDase 2, the activity of which increased with temperature after CuP treatment (data not shown). In contrast, the activity of the C26S/S462C, C26S/V465C, and C26S/V466C mutants, which remained mostly as monomers after CuP treatment, decreased with temperature with or without CuP treatment (data not shown).

The S462C, S463C, V465C, and V466C mutants, which contain one cysteine residue each in TMD1 and TMD2, were insensitive to high temperature following CuP treatment. Data obtained with S463C are shown in Fig. 3.9.D. After CuP cross-linking, the ATPase activity of the S463C mutant increased with

temperature and the ratio of the ATPase activity obtained at 61 ^{o}C to that at 37 ^{o}C was ~3.5.

In summary, our data indicate that the inhibitory effect of high temperature on ATPase activity can be abolished by CuP treatment, when significant inter- and intra-molecular oxidative cross-linking of the human NTPDase 2 protein occurs.

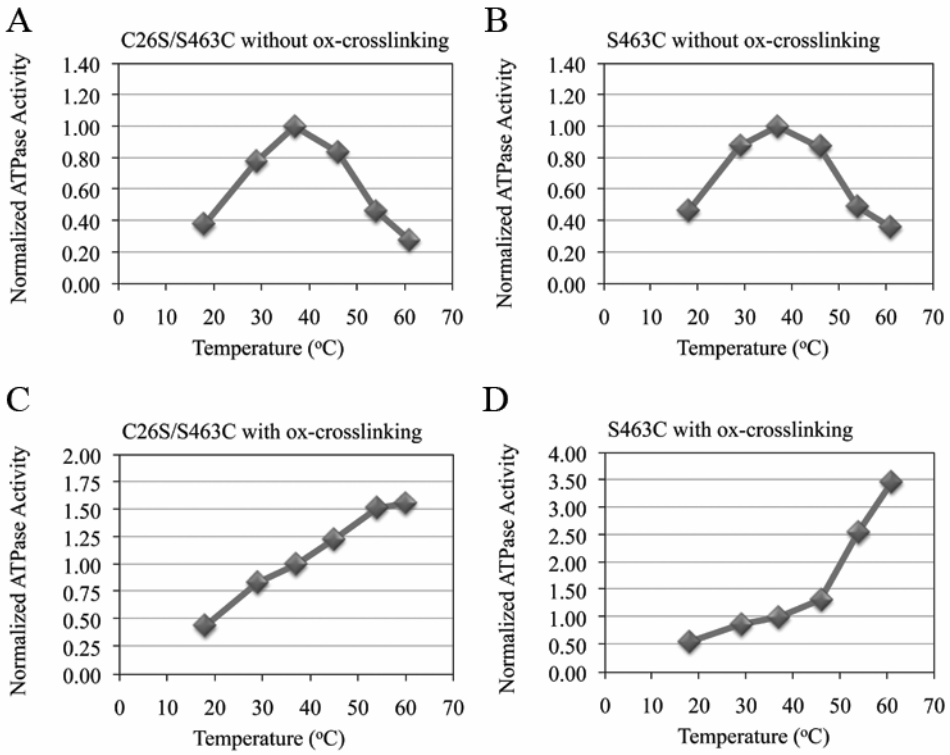

Figure 3.9. Effect of inter- or intra-molecular cross-linking on the effect of temperature on ATPase activity of the C26S/S463C and S463C mutants. Membranes (10 μg) containing S463C or C26S/S463C mutant NTPDase 2 were incubated for 20 min without or with 0.5 mM CuP in 20 μL reaction mixture at 37^{o}C for 20 min. Aliquots of the membranes were assayed for ATPase activity in an 1-minute reaction at the indicated temperatures.

Discussion

Inhibition of a cell surface ATPase by *p*-chlomercuribenzoate (pCMB) was first reported by Karasaki (33). This and subsequent studies showed that (i) cell surface ATPase activity in the normal rat liver was localized at the bile canaliculi, (ii) the cell surface ATPase activity in N,N'-dimethylaminoazobenzene-induced rat hepatoma increased and was distributed over the entire surface of the hepatoma cells, and (iii) cytochemical staining of the cell surface ATPase activity in the rat hepatoma cells was abolished by 10 mM pCMB, whereas ATPase staining in normal rat hepatocyte cells was not affected by pCMB (33, 34). The differential effects of pCMB suggested that the ATPase in the normal rat liver differs from that of the rat hepatoma. Previous studies of ecto-ATPases of a human hepatoma cell line and three small cell lung carcinoma cell lines showed that the major ecto-ATPase in these tumor cells was also inhibited by the mercurial, pCMPS (28, 35, 36). This ecto-ATPase, an NTPDase 2, has been cloned from the small cell lung carcinoma, and was inhibited by pCMPS when expressed in HeLa and HEK293 cells (4). Although mercurials, such as pCMPS and pCMB, are not specific ATPase inhibitors, they are the only reagents known to discriminate between the mercurial-sensitive NTPDase 2 and the other cell surface NTPDases that are not inhibited or only inhibited by high concentration of pCMPS (15, 33, 35, 37, 38).

In this report, we showed that the target of pCMPS modification is C26 in the TMD1 of the human NTPDase 2. A mutant NTPDase 2, in which C26 was

replaced by serine, was no longer inhibited by pCMPS. In addition, the wild-type enzyme, which contains C26, would not form dimers upon oxidative cross-linking if it was first treated with pCMPS. We hypothesize that, upon reacting with C26, the bulky hydrophilic side group of pCMPS causes disruption of the TMD interaction and results in inhibition of catalysis at the active site.

If this hypothesis were correct, the human NTPDase 2 should also be inhibited by pCMPS if the free cysteine is situated in the TMD2. To investigate this possibility, we generated five mutants, C26S/S462C, C26S/S463C, C26S/W464C, C26S/V465C, and C26S/V466C, which contain only a single free cysteine residue in positions 462-466 located in TMD2 and near the exterior of the bilayer. Of the five mutants, the C26S/W464C mutant displayed only 5% of the wild-type human NTPDase 2 activity; however, its activity was partially rescued after cross-linking by ConA (data not shown), indicating that W464 is critical for protein stability and ATPase activity. The other four mutants displayed 75-100% of the wild-type human NTPDase 2 activity.

Interestingly, the C26S/S462C, C26S/S463C, C26S/V465C, and C26S/V466C mutants showed reduced pCMPS inhibition compared to the wild-type human NTPDase 2. This was not due to a failure of reaction of the mercurial with the introduced cysteine residues, since CuP-induced dimer formation was also abolished or reduced if these mutants were pre-treated with pCMPS (Fig. 3.6.). We conclude that while the reactivity of the cysteine residue at these positions with pCMPS is the same as C26, the reduced inhibitory effect of pCMPS of these

mutants may be accounted for by the greater distance of C462-C466 from ACR1, proposed to contain a phosphate 1 motif of the human NTPDase 2 active site (30). The closer proximity of the bulky side group of pCMPS linked to C26 to ACR1 may also alter the conformation of the active site in addition to disrupting the interaction of the TMDs.

While using oxidative cross-linking with CuP to ascertain the reaction of the cysteine residues with pCMPS, we noticed that oxidative cross-linking *per se* caused reduction of ATPase activity of the human NTPDase 2 and its mutants. The level of reduction in ATPase activity correlated with the amount of dimer formed. The greatest reduction of the ATPase activity was observed in the CuP-treated wild-type human NTPDase 2 and the C26S/S463C mutant, which formed dimers most readily. Reduction of ATPase activity after CuP treatment was less for the C26S/S462C, C26S/V465C, and C26S/V466C mutants. These results indicate that the cysteine residues at position 26 and 463 are more susceptible to oxidative cross-linking than the cysteine residues at the other positions.

The effect of oxidative cross-linking of cysteine residues in the TMD on ATPase activity was strikingly different from the effect of cross-linking by DSS, glutaraldehyde or ConA. While these reagents all promote dimer or oligomer formation, the major sites of cross-linking by DSS, glutaraldehyde and ConA are in the extracellular domain of the human NTPDase 2 because of their reactivity with lysine residues (DSS and glutaraldehyde) and binding to glycans (ConA). The resultant dimers and oligomers displayed an apparent increase of ATPase activity,

because they were no longer susceptible to inactivation by substrate, i.e., they did not show a decline of activity with reaction time (4). Cross-linking of the human NTPDase 2 by glutaraldehyde and ConA also attenuated the decrease of ATPase activity in the presence of NP-40 and high temperature (4), suggesting that cross-linking of the extracellular domain of the NTPDase 2 by these reagents also promotes TMD interaction. On the other hand, CuP cross-linking of the wild-type human NTPDase 2 and the mutants containing only one free cysteine residue in the TMD2 is restricted to the TMD and gave rise to mostly dimers. The loss of ATPase activity of such dimers can only be attributed to the negative effect of reduced TMD mobility on catalysis.

Despite the loss of ATPase activity due to oxidative cross-linking, inhibition of residual activity by NP-40 and high temperature was attenuated in these dimers. This was seen most clearly with the wild-type human NTPDase 2 and the C26S/S463C mutant, which formed most dimers. On the other hand, the activity of the C26S/S462C, C26S/V465C, and C26S/V466C mutants, which remained mostly as monomers after CuP treatment, was still decreased by NP-40 and high temperature. These results indicate that while intermolecular TMD cross-linking impairs enzyme activity, such dimers become insensitive to membrane perturbation by detergents and high temperatures.

Since our recent studies indicated that the strength of intramolecular interaction of the TMD of the NTPDases is important in regulating their responses to membrane perturbation (12, 13), we further investigated the effect of

intramolecular covalent disulfide bond formation between TMD1 and TMD2. The mutants utilized were S462C, S463C, W464C, V465C, and V466C, which contain C26 in TMD1 and a cysteine residue in TMD2. Oxidative cross-linking of these mutants by CuP resulted in some oligomer formation; however, there was a general reduction of dimer formation, except for S462C, indicating that intramolecular cross-linking is favored. Compared to the activities of the untreated membranes, the activity of the CuP treated mutants was reduced to 20-40% (Table 3.3.), further supporting the conclusion that reduced TMD mobility limits the dynamics of the active site in achieving maximal activity. However, the extent of activity inhibition of the different mutants after oxidative cross-linking varied, indicating that intramolecular cross-linking of C26 and cysteine residues at different positions in TMD2 may change the conformation of the active site differently.

Similar to the results obtained with cross-linked dimers, the residual activity of the intramolecularly cross-linked monomers was less sensitive to inhibition by NP-40. Inhibitory effect of NP-40 on the activity of the oxidatively cross-linked S462C, V465C, and V466C mutants was reduced to 30-60%, while that of the oxidatively cross-linked S463C mutant was completely abolished (Fig. 3.8.). Furthermore, unlike the wild-type human NTPDase 2 or mutants without CuP treatment, which had decreased ATPase activity at temperature higher than 37^{o}C, the activity of the intramolecularly cross-linked mutants at 61^{o}C was ~ 3.5 fold greater than that at 37 ^{o}C (Fig. 3.9.D). These results suggest that locking the TMDs

causes the mutant human NTPDase 2 to be less sensitive to the inhibitory effect of membrane perturbation by NP-40 and high temperature.

Since our previous studies showed that a weaker TMD interaction of the human NTPDase 2 contributed to its susceptibility to membrane perturbation, the observation that ATPase activity of human NTPDase 2 was reduced upon strengthening TMD interaction by oxidative cross-linking was unexpected. However, our finding is similar to that obtained with the rat NTPDase 1/CD39 (39). Using various engineered single-cysteine and double-cysteine substituted mutant rat NTPDase 1, Grinthal and Guidotti showed that inter- and intra-TMD disulfide bond formation occured most readily when the cysteine residues introduced in the two TMDs were in the region near the cell surface and was associated with reduction of ATPase activity. Furthermore, the TMDs of the rat NTPDase 1 displayed a high degree of rotational mobility. While primary interfaces in the TMD1 and TMD2 could be demonstrated when CuP treatment was conducted at 4^{o}C, cross-linking was complete at 37^{o}C for all the rat NTPDase 1 mutants tested in which the cysteine residue occupied positions as far as 6 amino acid residues away from the cell surface. In spite of the similar effect of TMD cross-linking on ATPase activity, our results show that the rotational mobility of the TMDs of the human NTPDase 2 is more limited than that of the rat NTPDase 1 since dimer formation was most easily demonstrated with the enzyme containing a single cysteine residue at either position 26 (the wild-type enzyme) or position 463 (the C26S/S463C mutant). Additionally, intramolecular cross-linking of the double cysteine mutants

of the rat NTPDase 1 resulted exclusively in monomers, which was only possible if disulfide bond formation occurred regardless of the helix faces of the cysteine residues, further supporting the conclusion that TMDs of the rat NTPDase 1 are highly mobile. On the other hand, intramolecular cross-linking in the human NTPDase 2 with double cysteine residues was most clearly seen with the S463C mutant, whereas dimer formation actually increased in the S462C mutant probably due to the presence of C26. Thus C26 in TMD1 and S462 in TMD2 probably define an interface between the monomers. In spite of the different TMD mobility in the two NTPDases, the human NTPDase 2 is similar to the rat NTPDase 1 in that (i) its monomer is in rapid equilibrium with dimers and oligomers and a defined quaternary structure probably does not exist for the enzyme, and (ii) mobility of the TMD is necessary for maximal catalysis.

In summary, three major findings emerged from this study. First, the cysteine residue at position 26 was the target of pCMPS modification, which resulted in the loss of ATPase activity of the human NTPDase 2 by disturbing the TMD interaction of the human NTPDase 2 as well as affecting the conformation of the active site. This result differs from that obtained with human NTPDase 3, in which pCMPS modification of C501 located in TMD2 near the cytoplasmic side caused inhibition by interfering with monomer-monomer interactions in the native tetrameric quaternary structure of the human NTPDase 3 (38). Second, intra- and inter-molecular oxidative cross-linking was accompanied by reduction of ATPase activity, suggesting that the mobility of the TMDs is essential for enzyme function.

Third, upon cross-linking of the TMDs, the human NTPDase 2 became resistant to inactivation caused by membrane perturbation. Future investigations addressing communication between TMDs and the active site when the TMD is modified by pCMPS or when the TMD interaction is disturbed by membrane perturbation should yield greater insight into the mechanism by which TMD interaction regulates the catalytic activity of NTPDases.

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Concluding Remarks

The presence of membrane-bound ATPases in eukaryotic cells that differ from the transport ATPases has been described in numerous reports (reviewed in 1). These ATPases appeared in the literature under many different names, such as ecto-ATPase, Mg^{2+} (Ca^{2+})-ATPase, ATP pyrophosphohydrolase, ecto-apyrase, ecto-ATPDase, ATP diphosphohydrolase, E-type ATPase, etc. The unified nomenclature of this family, termed ectonucleoside triphosphate diphosphohydrolase family (E-NTPDase family), was coined in 1999 during the "Second International Workshop on Ecto-ATPases and Related Ectonucleotidases", held in Diepenbeek, Belgium (2), and later shortened to NTPDases to include intracellular and soluble NTPDases. NTPDases are (i) ubiquitous, (ii) dependent on Mg^{2+} or Ca^{2+} for activity, (iii) insensitive to the specific inhibitors of the P-, V-, and F-type ATPases, and (iv) have broad substrate preference for NTP and NDP, but not NMP.

Molecular cloning of the NTPDases was not achieved until the 1990s (3, 4) because of the difficulties in obtaining pure proteins. Purification of these enzymes has been challenging, since most NTPDases are inactivated by detergents normally used for solubilizing membrane-bound proteins. So far, only four NTPDases have been purified from their native tissues, i.e., the rabbit and chicken NTPDase 2 (5, 6), and the chicken NTPDase 8 from oviduct and liver (7, 8). The specific activities of these purified enzymes are in the range of 1000-7000 μmol min^{-1} mg $protein^{-1}$, which are at least one order of magnitude higher than that of the transport ATPases.

A human ecto-ATPase, later identified as NTPDase 2, was previously

characterized in intact human hepatoma Li-7A cells and three lines of human small cell lung carcinoma cells (9, 10). The enzyme was only partially purified from the plasma membranes of human oat cell carcinoma xenograft because of the small amount of the enzyme in the plasma membranes and the limited amount of xenograft. The ecto-ATPase activity in the native membranes displayed several unusual characteristics. Its activity was decreased by several detergents, e.g., NP-40, bile salts, and octylglucoside. It was inhibited by pCMPS and dithiothreitol, but was activated by the lectin, ConA (10, 11).

The human NTPDase 2 was later cloned from the human small cell lung carcinoma NCIH69 cells (GenBank Accession No. EF495152) (12) and expressed in HeLa and HEK293 cells. The expressed human NTPDase 2 hydrolyzed ATP and UTP, the physiological ligands of the P2 receptors, in the presence of either Mg^{2+} or Ca^{2+}. It was primarily an NTPase, whose ADPase activity was less than 5% of either the ATPase or UTPase activities. More importantly, similar to the activity in the native membranes, the expressed human NTPDase 2 was inactivated by detergents and inhibited by pCMPS, but was activated by cross-linking reagents. Its activity also decreased at high temperature and it was susceptible to substrate inactivation (12). The data reported in this dissertation show that most of the effects are mediated by the transmembrane domains of the enzyme.

Decrease of human NTPDase 2 activity due to membrane perturbation.

Detergents are indispensable in membrane protein research as they are required for membrane protein solubilization and purification. In most cases,

membrane proteins maintain their native structures and remain in a functional state after solubilization by 1-5% detergent solutions. In contrast, cell surface NTPDases, except for chicken NTPDase 8 (7, 8, 13), lose their activities in the presence of low concentration of several detergents (11, 14-19). For example, the ATPase activity of the human NTPDase 2 was decreased by ~95% by NP-40 at 0.01% (Fig. 1.3.), a concentration of the detergent lower than its critical micelle concentration. This unusual sensitivity to detergents distinguishes the NTPDases from other membrane-bound ATPases.

Since NP-40 inactivates the human NTPDase 2 at a concentration insufficient to solubilize proteins from the membrane, the activity loss of the human NTPDase 2 was most likely due to a disturbance of its TMD interaction by the detergent dissolved in the membrane bilayer. Furthermore, human NTPDase 2 also lost its activity at temperatures higher than 37ºC (Fig. 1.1.). Since high temperature can increase membrane fluidity, its effect on the human NTPDase 2 was likely to be also mediated by the TMDs of the enzyme (Fig. C.1.).

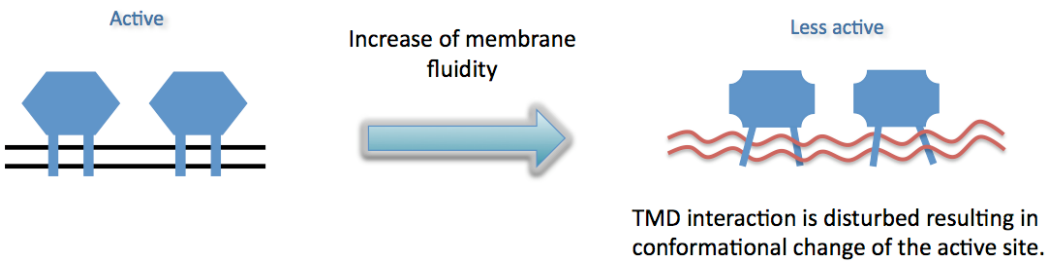

Figure C.1. The human NTPDase 2 activity is decreased upon increase of membrane fluidity.

To determine if the TMDs are involved in these responses, a soluble human NTPDase 2 construct lacking both TMDs was generated. In contrast to the full-length enzyme, the soluble human NTPDase 2 ECD was no longer inactivated by detergents and its activity increased with temperature up to 55ºC (Fig. 2.4.B). We conclude that the TMDs in the full-length human NTPDase 2 mediate the inhibitory effects of detergents and high temperature. However, it should be noted that the ATP hydrolysis rate of the soluble human NTPDase 2 wss less than 5% of the activity of the purified membrane-bound enzyme, assuming a value of ~1000 μmol min^{-1} mg $protein^{-1}$. Thus anchoring of the human NTPDase 2 ECD by its TMD to the membrane appears to be necessary for its full activity. Other changes in the soluble human NTPDase 2 included a higher affinity for ATP and a preference for Ca^{2+} over Mg^{2+}. These results, as well as those from previous studies on the soluble ECD of human and chicken NTPDase 8 from our laboratory (19, 20) and a recent study on bacterially expressed soluble rat NTPDase 1, 2, and 3 (21), suggest that alteration of the enzymatic properties of the ECD from that of the full-length enzymes can be attributed to the lack of transmembrane domains. It is likely that the environment of the active site in the full-length enzyme and that of the ECD are different.

Unlike human NTPDase 2, the activity of the chicken NTPDase 8, either in the native tissues or expressed in human cells, was maintained in the presence of 5% NP-40 and its activity increases with temperature up to 55ºC (13). The TMDs of the chicken NTPDase 8 contain a larger number of small (glycine and alanine)

and small polar (serine and threonine) amino acids than the TMDs of the human NTPDase 2 (Fig. 2.11.). The different responses of the human NTPDase 2 and the chicken NTPDase 8 to membrane perturbation (Fig. C.2.A) make them excellent models in determining if the interaction of the respective TMDs is responsible for the different stabilities of these two NTPDases under these conditions.

Previously, Mukasa et al. generated ck-hu ACR1, ck-hu ACR5, and ck-hu ACR1,5 chimeras in which the N- or C- terminal region, up to ACR1 and ACR5 respectively, of the chicken NTPDase 8 or both were substituted by the corresponding regions of the human NTPDase 2. Incorporation of either the N- or C-terminal TMD of the human NTPDase 2 was sufficient to render the chicken NTPDase 8 susceptible to inactivation by detergents and high temperatures (13, 20). To determine if the reverse can occur, that is, if a more stable human NTPDase 2 can be generated by TMD exchange, three chimeras containing the human NTPDase 2 ECD but different pairs of TMDs were constructed. In our initial efforts, the N- and C-termini exchanged consisted of only the N- or C- terminal TMD and the short cytoplasmic domains. However, these chimeras were inactive even though they were expressed (See Appendix C), suggesting that the regions between the TMDs and ACR1 and ACR5 of either NTPDase are important for proper protein folding of the human NTPDase 2, which will require further investigation. Of the three hu-ck ACR chimeras, which contain sequences between the TMDs and ACR1 and ACR5, two displayed activity while expression levels

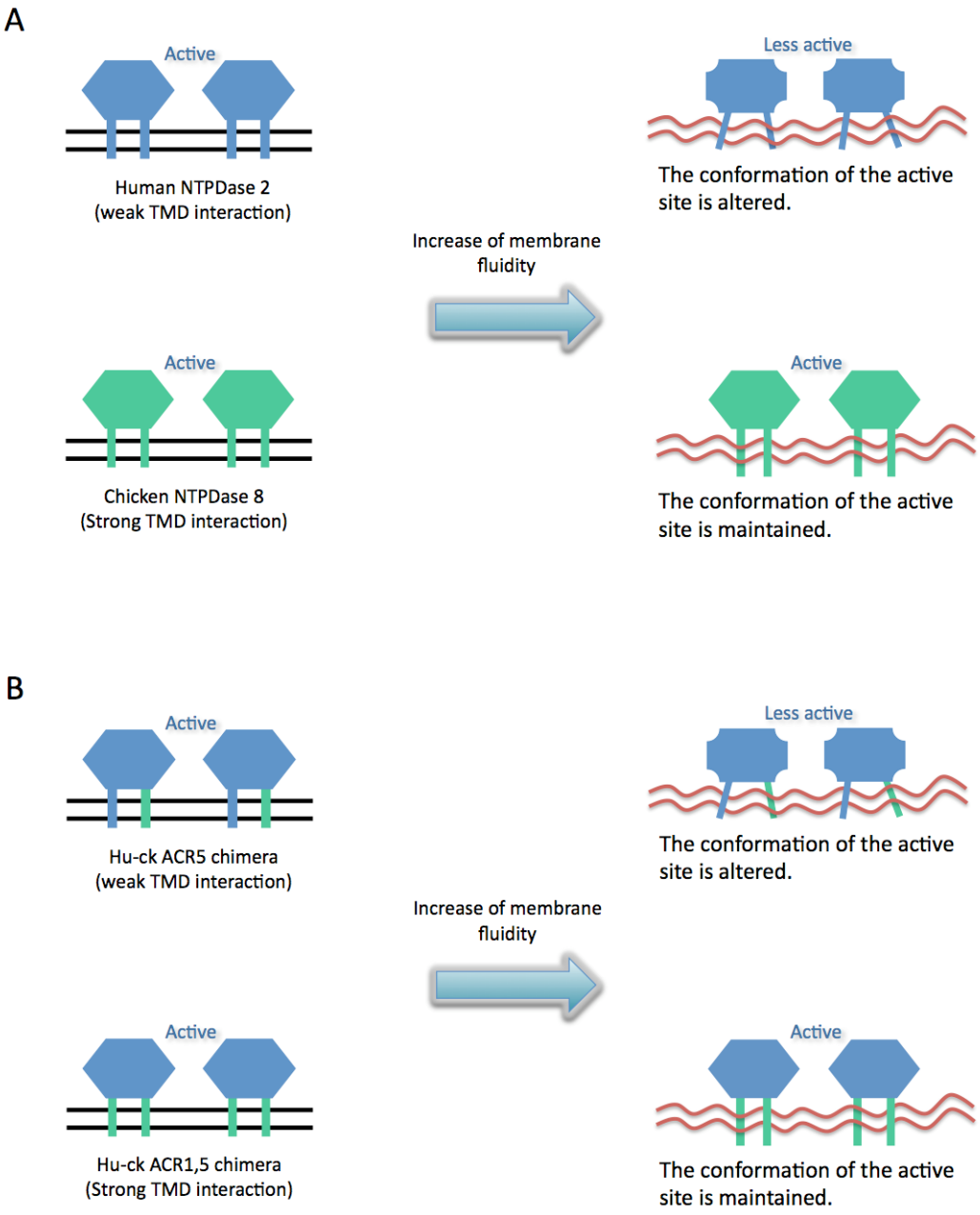

Figure C.2. A. The effect of membrane perturbation on the human NTPDase 2 and the chicken NTPDase 8. B. The effect of membrane perturbation on the hu-ck ACR5 and ACR1,5 chimeras.

varied. Protein expression of the hu-ck ACR5 chimera was ~ 70% of the wild- type human NTPDase 2, whereas that of the hu-ck ACR1,5 chimera was ~25%. Protein expression of the inactive hu-ck ACR1 was negligible, suggesting that the N-terminal TMD of the human NTPDase 2 is necessary for optimal protein expression in HEK293 cells. The normalized activities of the hu-ck ACR5 and hu-ck ACR1,5 were ~10% and ~40% of that of the wild-type human NTPDase 2, respectively.

The hu-ck ACR5 chimera, which contains the N-terminus of the human NTPDase 2, retained similar responses to detergent and high temperature as the wild-type enzyme. On the other hand, the hu-ck ACR1,5 chimera, in which both TMDs of the human NTPDase 2 were substituted by that of the chicken NTPDase 8, was no longer susceptible to inactivation by detergents and its activity increased with temperature (Fig. 2.9. and 2.10.B). It is the only one of the six human NTPDase 2–chicken NTPDase 8 chimeras generated that was not negatively affected by these parameters. These and the previous results of Mukasa et al. strongly support the conclusion that the activity of the ECD, whether originating from the chicken NTPDase 8 or the human NTPDase 2, is maintained in the presence of detergents and at high temperature if they are anchored to the membrane by the chicken NTPDase 8 TMDs (Fig. C.2.B). Future investigations that examine the effects of incorporation of amino acids that promote oligomerization of transmembrane helices into the human NTPDase 2 TMD should offer a better understanding of the relationship of amino acid sequence and TMD

interaction. I have attempted to substitute the seven amino acid residues of the two TMDs of the human NTPDase 2 near the extracellular surface with the corresponding amino acid sequences of the chicken NTPDase 8 TMDs. Unfortunately, the resultant chimera was not expressed (Appendix D).

Since the interaction of the TMDs of the human NTPDase 2 is easily disrupted by membrane perturbation, it was of interest to determine if increasing the TMD interaction, for example, by linking the two TMDs of the human NTPDase 2 with a disulfide bond, can attenuate the inhibitory effect of membrane perturbation. Interhelical disulfide bond was formed by oxidative cross-linking of the cysteine residue at position 26 in TMD1 and a cysteine residue introduced individually at positions 462-466 in TMD2. Despite the loss of ATPase activity due to oxidative cross-linking (discussed in the later section), inhibition of the residual activity of the cross-linked double-cysteine human NTPDase 2 mutants by NP-40 was greatly reduced, and their activities increased with temperature. Unexpectedly, the wild-type human NTPDase 2 and the C26S/S463C mutant, which contain only one free cysteine but readily form dimer after CuP treatment, also became less sensitive or insensitive to inactivation by NP-40 and high temperature. These results suggest that upon intra- and inter-molecular cross-linking, the TMD interaction of the human NTPDase 2 becomes more resistant to membrane perturbation (Fig. C.3.).

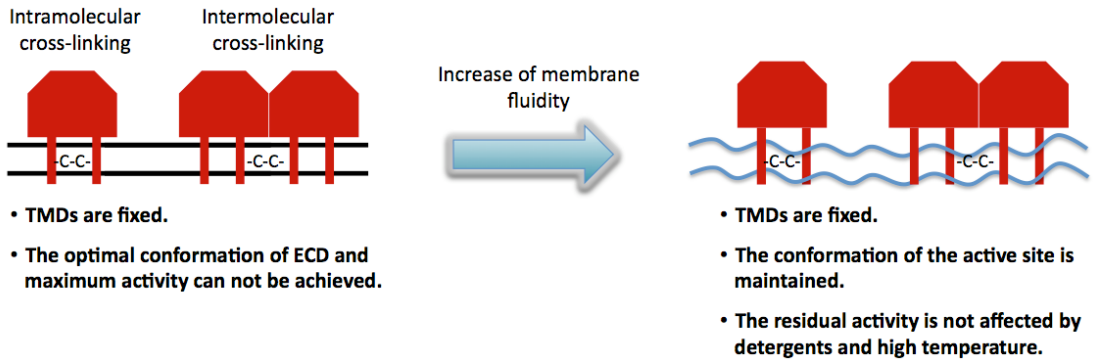

Figure C.3. Intra- and inter-molecular cross-linking between TMDs render the human NTPDase 2 resistant to membrane perturbation.

Inhibition of human NTPDase 2 activity by *p*-chloromercuriphenylsulfonate.

Previous studies showed that the plasma membrane ATPase of tissues abundant in NTPDase 2 was inhibited by *p*-chloromercuriphenylsulfonate (pCMPS) (11, 22), which reacts with free cysteine residues. The sensitivity to pCMPS inhibition was retained in the ATPase in membranes prepared from HEK293 cells stably transfected with the human NTPDase 2 cDNA (Fig. 3.3., lower curve).

Although cell surface NTPDases contain variable number of cysteine residues, ten of these in the extracellular domain are conserved and have been shown to be involved in disulfide bond formation (23). In the human NTPDase 2, there is only one additional free cysteine residue, C26, located in the TMD1 and close to the cell surface. This cysteine residue is conserved in the TMD1 of the NTPDase 2 of other species (Fig. 3.1.A), but not in those of NTPDase 1, 3, and 8 (Fig. 3.1.B).

The study reported in Chapter 3 showed that the target of pCMPS modification is C26 in the TMD1 of the human NTPDase 2, since (i) a mutant NTPDase 2, in which C26 was replaced by serine, was no longer inhibited by pCMPS, and (ii) pCMPS modified human NTPDase 2 did not form dimer by oxidative cross-linking. We hypothesize that, upon reacting with C26, the bulky hydrophilic side group of pCMPS causes disruption of the TMD interaction which results in inhibition of catalysis at the active site. If this hypothesis were correct, the human NTPDase 2 should also be inhibited by pCMPS if the free cysteine were situated in the TMD2. To investigate this possibility, five mutants, C26S/S462C, C26S/S463C, C26S/W464C, C26S/V465C, and C26S/V466C were generated. These mutants contain only a single free cysteine residue in positions 462-466 located in TMD2 and near the exterior of the bilayer. Except for the C26S/W464C mutant, which was inactive, the other four mutants displayed 75-100% of the wild-type human NTPDase 2 activity. Interestingly, pCMPS inhibition of these four mutants was significantly less than that of the wild-type enzyme (Table 3.2.). The significantly reduced inhibition of the ATPase activities of these mutants by pCMPS was not due to a failure of reaction of the mercurial with the introduced cysteine residues, but may be accounted for by the greater distance of C462-C466 from ACR1, proposed to contain a phosphate 1 motif in the human NTPDase 2 active site (4, 24, 25). Since C26 is only about 17 amino acid residues away from ACR1, the bulky side group of pCMPS linked to C26 may also alter the conformation of the active site in addition to disrupting the interaction of the TMDs.

Decrease of human NTPDase 2 activity by oxidative cross-linking of the transmembrane domains.

While using oxidative cross-linking with CuP to ascertain the reaction of the cysteine residues with pCMPS, I noticed that oxidative cross-linking *per se* caused reduction of ATPase activity of the human NTPDase 2 and its mutants (Table 3.3.). For the wild-type human NTPDase 2 and the mutants containing a single cysteine in the TMD2, the extent of reduction in ATPase activity correlated with the amount of dimer formed. The greatest reduction of the ATPase activity was observed in the CuP-treated wild-type human NTPDase 2 and the C26S/S463C mutant, which formed dimers most readily. Reduction of ATPase activity after CuP treatment was less for the C26S/S462C, C26S/V465C, and C26S/V466C mutants. These results indicate that the cysteine residues at position 26 and 463 are more susceptible to oxidative cross-linking than the cysteine residues at the other positions. The loss of ATPase activity of such dimers can only be attributed to the negative effect of limited TMD mobility on catalysis.

As for the mutants containing C26 in TMD1 and a cysteine residue in TMD2, oxidative cross-linking of these mutants by CuP resulted in some oligomer formation; however, the majority of the proteins remained as monomers, indicating that intramolecular cross-linking is favored over intermolecular cross-linking. Compared to the activity of the untreated membranes, the activity of the CuP treated mutants was reduced to 20-40% (Table 3.3.), further supporting the conclusion that reduced TMD mobility limits the dynamics of the active site in

achieving maximal activity (Fig. C.4.). Similar finding has been reported for rat NTPDase 1/CD39 (26), although the rotational mobility of the TMDs of the two NTPDases are different as assessed by the ease of oxidative cross-linking.

In summary, the strengths of TMD interaction appear to be the key in determining the responses of the human NTPDase 2 to membrane perturbation. Although the human NTPDase 2 became resistant to inactivation by membrane perturbation upon oxidative cross-linking, the accompanying reduction of ATPase activity suggests that the mobility of the TMDs is essential for maximal catalysis.

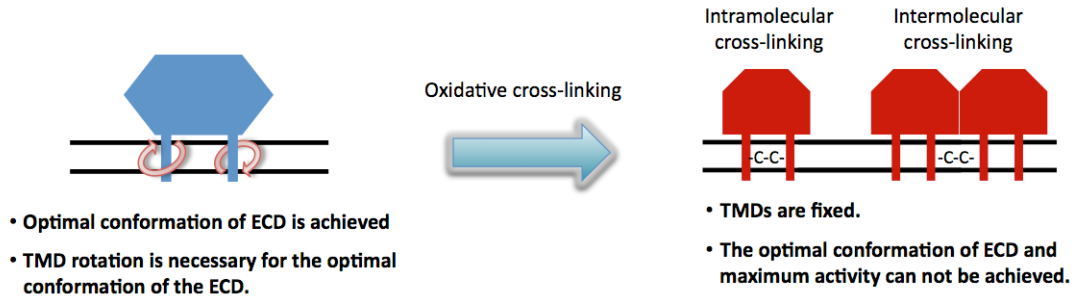

Figure C.4. Oxidative cross-linking of the TMDs limits their rotational mobility resulting in reduction of the ATPase activity.

Inactivation of human NTPDase 2 by its substrate.

One of the most unusual characteristics of the human NTPDase 2 is a decline of ATP hydrolysis activity after the reaction begins, so that the specific activity obtained at 10 min is only ~30% of that obtained at 1 min (Fig. 1.6. and Table 1.2.). The loss of activity with time could not be attributed to product

inhibition nor inactivation of the enzyme at 37^{o}C. The only explanation is that ATP has a negative regulatory effect on the enzyme.

At present, the mechanism of ATP inactivation of human and other NTPDases is not known. It has been proposed that ATP-induced inactivation of the rat brain ecto-ATPase is mediated partially by phosphorylation of membrane proteins (27). This seems unlikely in the case of the human NTPDase 2 since inactivation of the enzyme was also obtained with UTP (data not shown), which does not serve as a substrate for most protein kinases. Our hypothesis is that conformational change in the protein after the onset of ATP hydrolysis shifts the enzyme into a less active state (Fig. C.5.), and such a shift is accelerated at high temperatures (Fig. 2.3.A). Results obtained with the soluble human NTPDase 2, in which substrate inactivation is abolished, suggest that TMD interaction is also involved in the negative regulatory effect of substrate on the human NTPDase 2. Further support for the conclusion was obtained with the hu-ck chimeras. In contrast to the human NTPDase 2, the activity of the chicken NTPDase 8 was not inactivated by its substrate. The results described in Chapter 2 showed that while hu-ck ACR5 chimera retained substrate inactivation as the wild-type human NTPDase 2, the hu-ck ACR1,5 chimera, in which both TMDs of the human NTPDase 2 were substituted by that of the chicken NTPDase 8, was resistant to substrate inactivation. It appears that the different interactions of the TMDs of the two NTPDases correlate with their different susceptibility to substrate inactivation.

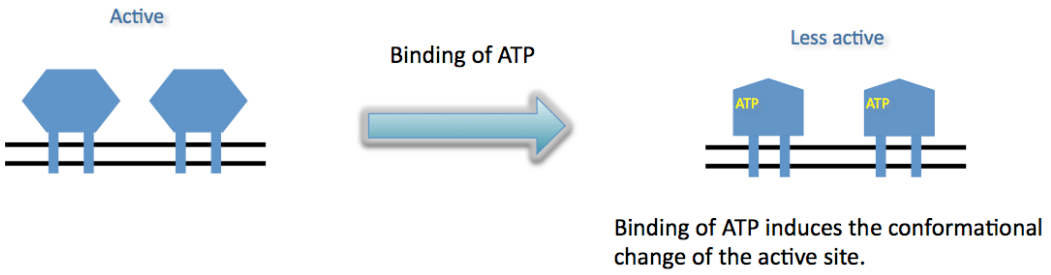

Figure C.5. The proposed mechanism of ATP induced inactivation of the human NTPDase 2.

Modulation of human NTPDase 2 activity by cross-linking by concanavalin A and glutaraldehyde.

Cross-linking reagents, e.g., DSS and glutaraldehyde, which react with lysine residues, and ConA, which binds to glycans of glycoproteins, promoted dimerization or oligomerization of the human NTPDase 2 (12) and increased its ATPase activity. Initially, it was proposed that the oligomeric human NTPDase 2 possesses higher activity than the monomers. However, the results described in Chapter 1 showed that the human NTPDase 2 cross-linked by glutaraldehyde was no longer susceptible to inactivation by substrate, i.e., it did not show a decline of activity with reaction time (Fig. 1.6.). Therefore, the apparent increase of ATPase activity of the oligomeric human NTPDase 2 by cross-linking reagents was related to their ability to prevent substrate inactivation (Fig. C.6.).

Cross-linking of the human NTPDase 2 by glutaraldehyde and ConA also attenuated the decrease of ATPase activity in the presence of NP-40 and high

temperature (Fig. 1.1., 1.3., and C.6.), suggesting that cross-linking of the extracellular domain of the human NTPDase 2 by these reagents also promotes TMD interaction. Furthermore, cross-linking of the human NTPDase 2 by glutaraldehyde also prevents inhibition by pCMPS (Fig. 3.3). This is probably because (i) C26 in the oligomeric human NTPDase 2 does not react with pCMPS, or (ii) the active site in the oligomeric human NTPDase 2 is not affected by the disturbance of TMD interaction caused by pCMPS modification.

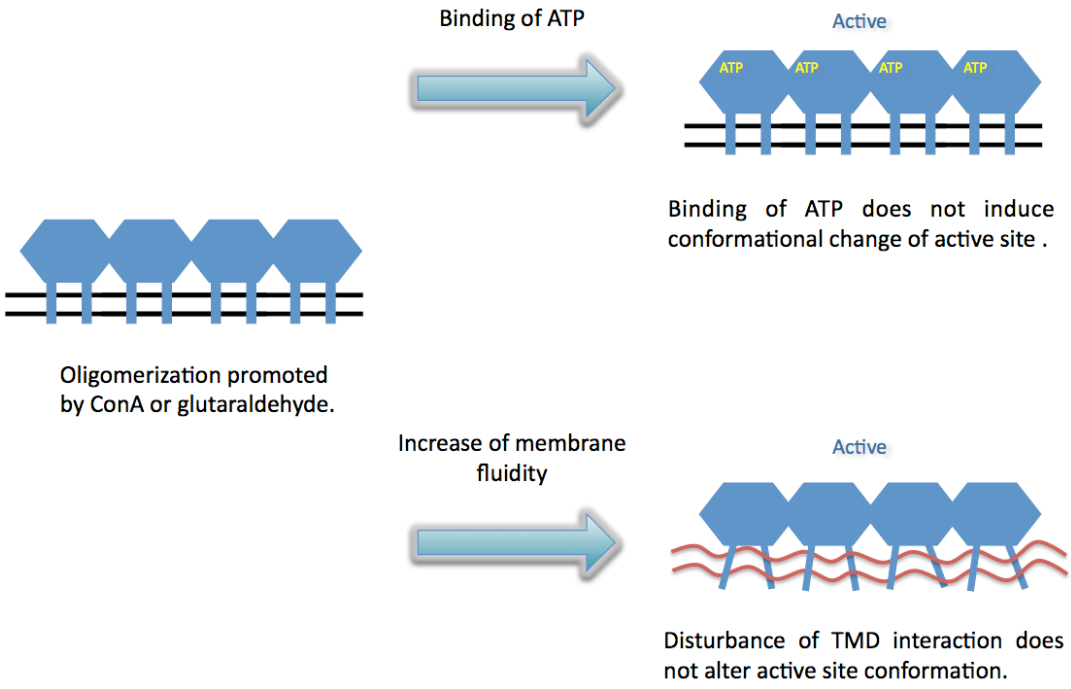

Figure C.6. Inactivation by substrate and membrane perturbation is abolished by cross-linking of the human NTPDase 2.

Future direction

Interaction of TMDs is critical for the functions of all membrane proteins. It is especially important for proteins whose activity is regulated by oligomerization. A common motif that promotes TMD association is the GXXXG motif, first identified in glycophorin A (28). Later reports showed that the glycine residues could be substituted with serine or alanine residues (29, 30). Non-charged amino acid residues, such as asparagine, can also stabilize TMD interaction (31), whereas proline, which induces a helix kink, reduces TMD interaction (32). The human NTPDase 2 contains one GXXXG-like motif in each of the two TMDs; however, these two motifs are not proximal to each other. In addition, the N-terminal TMD contains two proline residues, which may reduce the TMD interaction of the human NTPDase 2 (Fig. C.7.). On the other hand, the chicken NTPDase 8 contains two pairs of GXXXG-like motifs in the TMDs that may strengthen the TMD interaction (Fig. C.7.). These sequence differences may contribute to the observations reported in this dissertation in that the activity of the human NTPDase 2 is readily decreased by membrane perturbation, while that of the chicken NTPDase 8 is not. Further investigation of the strength of interactions of the respective TMD pairs of the human NTPDase 2 and chicken NTPDase 8 using TOXCAT, a system developed for the study of transmembrane helix-helix oligomerization (33), may provide experimental evidence to support this proposal.

In spite of the progress made in human NTPDase 2 research in recent years, there are still many unsolved questions. Future experiments with human NTPDase

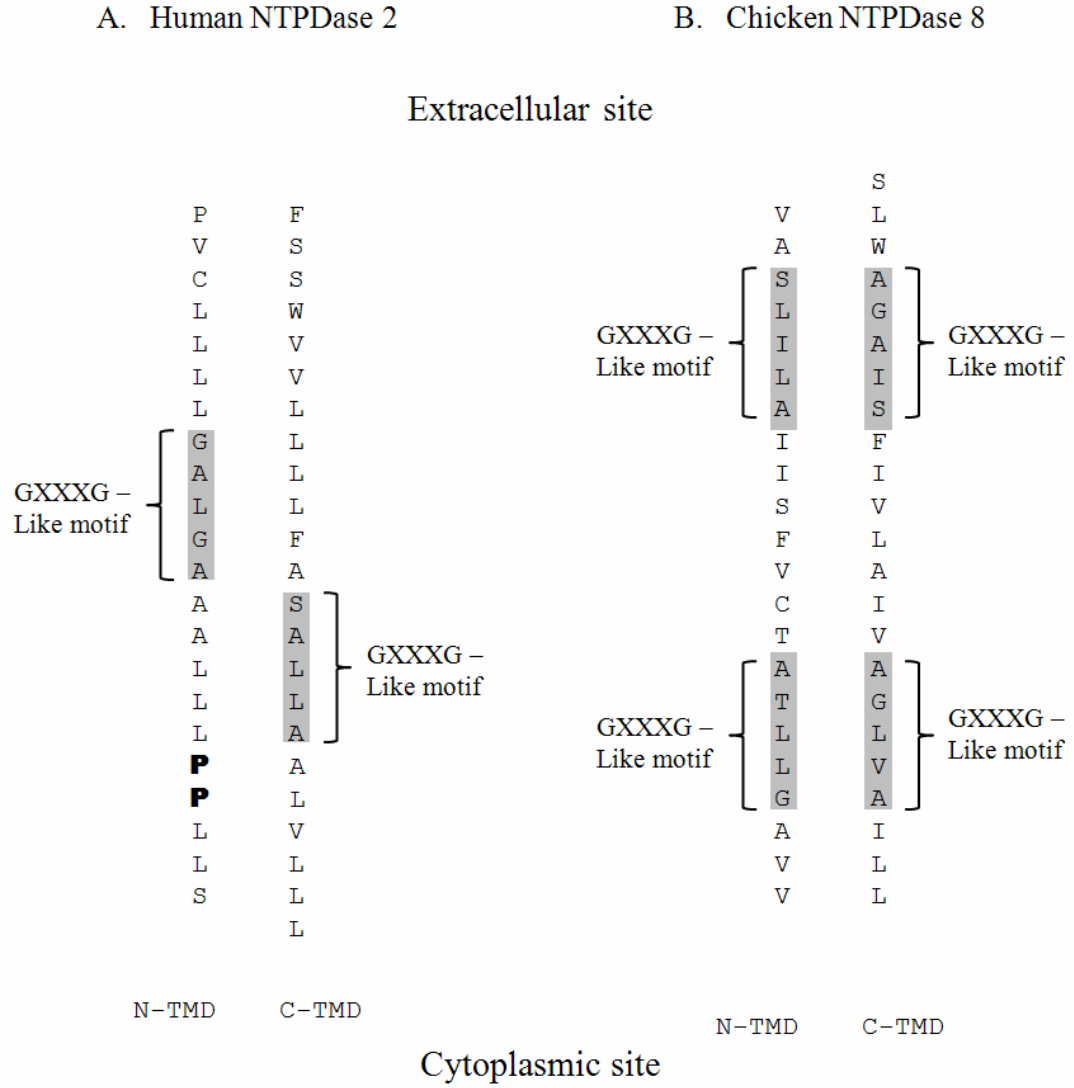

Figure C.7. Amino acid sequences of the TMDs of the human NTPDase 2 and chicken NTPDase 8. A. The human NTPDase 2 contains one GXXXG-like motif each in both TMDs. The N-terminal TMD contains two proline residues (in bold), which may disrupt TMD interaction. B. The chicken NTPDase 8 contains two pair of GXXXG-like motifs that may increase TMD interaction.

2 purified from stably transfected HEK293 cells using digitonin, the only detergent that does not decrease the activity of the human NTPDase 2, will be necessary to determine if the full-length human NTPDase 2 is still inactivated by substrate and pCMPS when it is solubilized from the membrane. In addition, to completely understand the mechanism of substrate-induced inactivation and inhibition of the human NTPDase 2 by pCMPS, as well as the conformational change of the active site due to the disturbance of TMD interaction by membrane perturbation, the three-dimensional structures of the full-length human NTPDase 2 and the ECD are necessary. To date, the crystal structure of the human NTPDase 2 has not been solved. The bacterially expressed ECD of the rat NTPDase 2 has been crystallized by Zebisch and Sträter (21). However, its biochemical properties differ significantly from the full length rat NTPDase 2 expressed in mammalian cells. Furthermore, the protein is not anchored to the membrane nor glycosylated. Therefore, the structure derived from such crystal may not represent the native conformation. Obtaining crystal structure of the purified full-length human NTPDase 2 from over-expression system will be necessary for elucidating the active site of the human NTPDase 2 and its relationship to the TMDs.

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Appendix

A. List of DNA vectors used in this dissertation

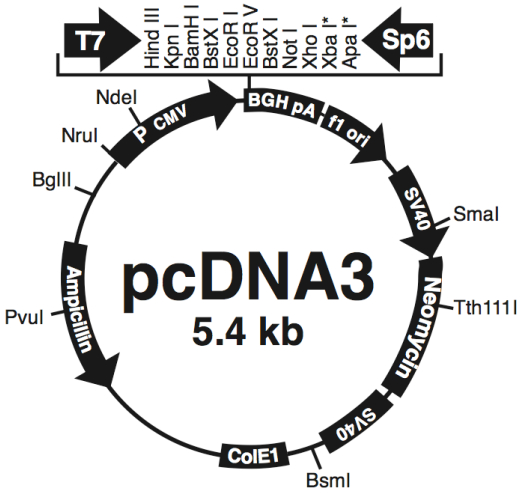
B. List of primers used in this dissertation

C. Molecular cloning of the hu-ck TMD chimeras

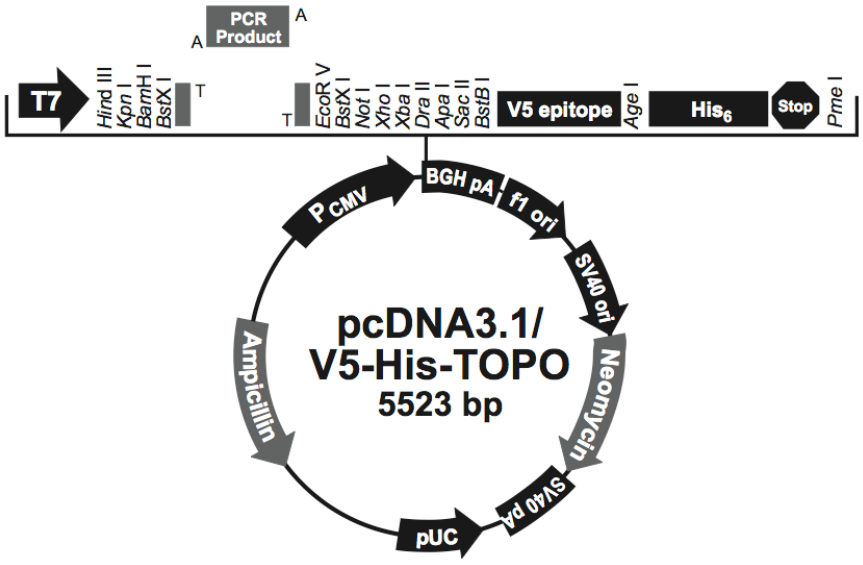
D. Molecular cloning of the hu-ck 7-amino acid TMD1,2 chimera

Appendix A: List of DNA vectors used in this dissertation

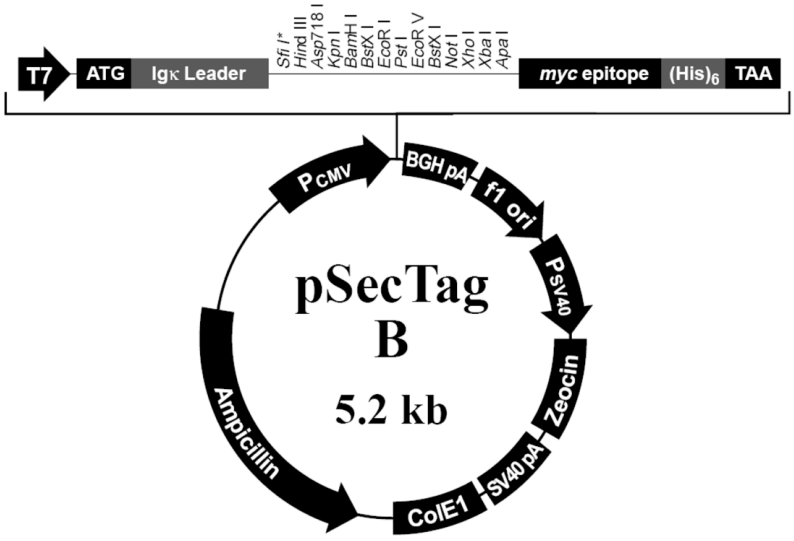
1. **pcDNA3:** cDNAs encoding for wild-type human NTPDase 2, wild-type chicken NTPDase 8, hu-ck ACR1, ACR5, and ACR1,5 chimeras, and all cysteine-substituted mutants were placed in the mammalian expression vector, pcDNA3.



2. **pcDNA3.1/V5-His-TOPO:** cDNAs encoding for wild-type human NTPDase 2, and hu-ck ACR1, ACR5, and ACR1,5 chimeras were placed in the mammalian expression vector, pcDNA3.1, through TA cloning.



3. **pSecTag2B:** The cDNA encoding for the soluble human NTPDase 2 ECD is placed in the mammalian expression vector, pSecTag2B. The secretion signal, mouse Ig kappa-chain, targets the recombinant protein for secretion.



Appendix B: List of primers used in this dissertation

Primers used for constructing hu-ck ACR chimeras

```
Hu-ck ACR1 F: GCCGGCTCCACGCACACGTCCATGTTTATC
Hu-ck ACR1 R: GATAAACATGGACGTGTGCGTGGAGCCGGC
Hu-ck ACR5 F: CGGCTACATGCTGAACCTCACCAACATG
Hu-ck ACR5 R: CATGTTGGTGAGGTTCAGCATGTAGCCG
Ck-XhoI    R: GCTCGAATTCCTCGAGCTATTTGGATTTCCAGAAACAC
pcDNA3     F: GGAGACCCAAGCTTGGTACC
pcDNA3     R: GTCGAGGCTGATCAGCGAGC
```

Primers used for constructing the his-tagged human NTPDase 2

```
His-H69    R: GCTCGAATTCCTCGAGCGGTCAATGATGATGATGATGA
              TGAATGGTGCTTGGCAGCTTGGC
```

Primers used for constructing the soluble ECD of the human NTPDase 2

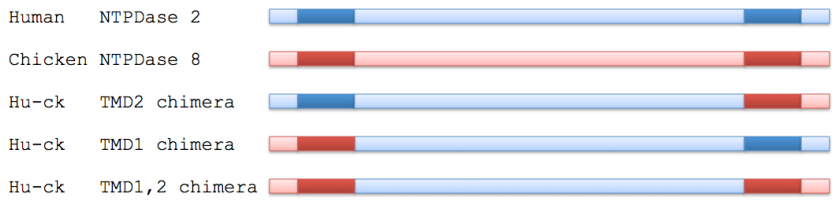
```
Sol ECD    F: GCATACTGCAAGCTTCCCACCCGCGACGTCCG
Sol ECD    R: GCTCGAATTCGCGGCCGCGGAGCTGAAGTCTGTGCC
```

Primers used for constructing cysteine-free and cysteine-substituted mutants

```
C26S       F: CCTCCTACTGCTGTCCGTCCCCACCCAG
C26A       F: CCTCCTACTGCTGGCCGTCCCCACCCGC
S462C      F: GGCACAGACTTCTGCTCCTGGGTCGTCCTC
S463C      F: CACAGACTTCAGCTGCTGGGTCGTCCTCCTG
W464C      F: GACTTCAGCTCCTGCGTCGTCCTCCTGCTGC
V465C      F: CTTCAGCTCCTGGTGCGTCCTCCTGCTGCTC
V466C      F: CAGCTCCTGGGTCTGCCTCCTGCTGCTCTTC
```

Appendix C: Molecular cloning of the hu-ck TMD chimeras

Schematic representation of the cDNAs of the hu-ck TMD chimeras



Molecular cloning of the hu-ck TMD chimeras

(i) Hu-ck TMD2 chimera

The hu-ck TMD2 chimera was constructed using two-step PCR amplification. Two overlapping DNA fragments were generated separately in the first step. The DNA fragment containing the N-terminal portion of the human NTPDase 2 was produced by PCR using the human NTPDase 2 cDNA (in pcDNA3) as the template, a pcDNA3 forward primer annealing to the vector upstream of the start codon and including a BamHI site, and a hu-ck TMD2 reverse chimeric primer annealing to the sequences upstream of the TMD2 of the human NTPDase 2. The sequence of the hu-ck TMD2 reverse primer is 5'-CACCTGCCCACAGACTGGAGCTG AAGTCTGTG -3' in which the nucleotides underlined are from the chicken NTPDase 8 sequence, and the nucleotides not underlined are from the human NTPDase 2 sequence. The DNA fragment containing the C-terminal portion of the chicken NTPDase 8 was obtained by PCR using chicken NTPDase 8 cDNA (in pcDNA3) as the template, a hu-ck TMD2 forward chimeric primer, 5'- CACAGACTTCAGCTCCAGTCTGTGGGCAGGTG -3', annealing to the sequence of the TMD2 of the chicken NTPDase 8, and a ck-XhoI reverse primer, annealing to the C-terminal end of chicken NTPDase 8 cDNA and including an XhoI site. The entire hu-ck TMD1 chimeric cDNA was obtained in the second PCR using the two overlapping DNA fragments generated above as templates, the pcDNA3 forward primer, and the ck-XhoI reverse primer. PCR conditions for generating the overlapping DNA fragment and the chimera were similar to that described in Chapter 2. The PCR product was purified, double digested by BamHI and XhoI, and ligated with the pcDNA3 vector digested by BamHI and XhoI.

(ii) Hu-ck TMD1 chimera

Similar strategy was used to generate the hu-ck TMD1 chimera. The DNA fragment containing the N-terminal portion of the chicken NTPDase 8 was produced by PCR using the chicken NTPDase 8 cDNA (in pcDNA3) as the template, the same pcDNA3 forward primer containing a BamHI site as described above, and a hu-ck TMD1 reverse chimeric primer, 5- GACGTCGCGGG

TGGGTACAGCACTTAGAATG -3', annealing to the sequence of the TMD1 of the chicken NTPDase 8. The DNA fragment containing the C-terminal portion of the human NTPDase 2 was obtained by PCR using the human NTPDase 2 cDNA (in pcDNA3) as the template, a hu-ck TMD2 forward chimeric primer, 5'-CATTCTAAGTGCTGTACCCACCCGCGACGTC -3', annealing to the sequence downstream of the TMD1 of the human NTPDase 2, and the same pcDNA3 reverse primer containing a XhoI site as described above. The entire hu-ck TMD1 chimeric cDNA was obtained by PCR using the two overlapping DNA fragments as templates and the pcDNA3 forward and reverse primers. The PCR product was purified, double digested by BamHI and XhoI, and ligated with the pcDNA3 vector digested by BamHI and XhoI.

(iii) Hu-ck TMD1,2 chimera

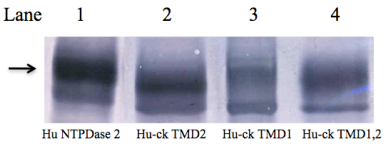
The DNA fragment coding for the N-terminus of the chicken NTPDase 8 extending to TMD1 followed by the extracellular domain of human NTPDase 2 was produced by PCR using the hu-ck TMD1 chimeric cDNA (in pcDNA3) as the template and the pcDNA3 forward primer and the hu-ck TMD2 reverse chimeric primer as described above. The DNA fragment coding for the C-terminal portion of the chicken NTPDase 8 was obtained by PCR as described above. The entire hu-ck TMD1,2 chimeric cDNA was obtained by PCR using the two overlapping DNA fragments obtained above as templates, the pcDNA3 forward primer, and the ck-XhoI reverse primer. The PCR product was purified, double digested by BamHI and XhoI, and ligated with the pcDNA3 vector digested by BamHI and XhoI. DNA sequencing showed that there was no unintended mutation in these recombinant DNA constructs.

Primers used for constructing hu-ck TMD chimeras

```
Hu-Cl TMD1 F: CATTCTAAGTGCTGTACCCACCCGCGACGTC
Hu-Cl TMD1 R: GACGTCGCGGGTGGGTACAGCACTTAGAATG
Hu-Cl TMD2 F: CACAGACTTCAGCTCCAGTCTGTGGGCAGGTG
Hu-Cl TMD2 R: CACCTGCCCACAGACTGGAGCTGAAGTCTGTG
```

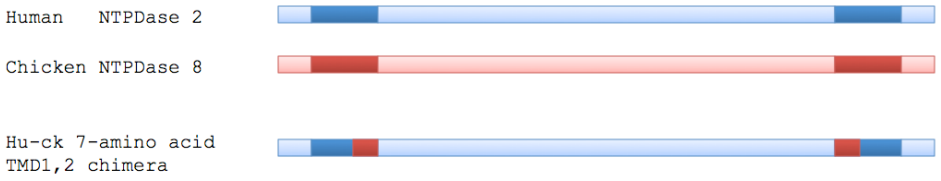
Expressions and ATPase activities of the hu-ck TMD chimeras

The procedures of transfection of human embryonic kidney 293 (HEK293) cells with the hu-ck TMD chimeric cDNAs were similar to that described in Chapter 2. The transfected cells were harvested after 48 hours and used for ATPase assay and western blotting. These chimeras were expressed in the plasma membrane. However, the activities of the HEK293 cells transfected with these chimeric cDNAs are similar to that of the HEK293 cells transfected with pcDNA3 vector.



Appendix D: Molecular cloning of the hu-ck 7-amino acid TMD1,2 chimera

Schematic representation of the cDNAs of the hu-ck 7-amino acid TMD1,2 chimera



Amino acid sequences of the TMDs of the hu-ck 7-amino acid TM1,2 chimera

N-terminal TMD: SLLPPLLLAAAGLAGALILSAV
C-terminal TMD: SLWAGAILLLFASALLAALVLLL

The sequence underlined is from the chicken NTPDase 8 and the sequence not underlined is from the human NTPDase 2.

Molecular cloning of the hu-ck 7-amino acid TMD1,2 chimera

The hu-ck 7-amino acid TMD1,2 chimera was constructed using three-step PCR amplification. Three overlapping DNA fragments were generated separately in the first step. (i) A 0.1 kb DNA fragment encoding the N-terminal cytoplasmic domain and the 15 amino acids from the TMD1 of the human NTPDase 2 close to the cytoplasmic domain and the seven amino acids from the TMD1 of chicken NTPDase 8 close to the extracellular domain was produced by PCR using the human NTPDase 2 cDNA (in pcDNA3) as the template, a human NTPDase 2 forward primer annealing to the N-terminus of the human NTPDase 2, and a hu TMD1 (7ck) reverse chimeric primer annealing to the sequences of the TMD1 of the human NTPDase 2. The sequence of the hu TMD1 (7ck) reverse primer is 5'-TACAGCACTTAGAATGAGGGCGCCGGCGAGGCCCGC-3' in which the nucleotides underlined encode the seven amino acids from the TMD1 of chicken NTPDase 8 close to the extracellular domain, and the nucleotides not underlined are from the human NTPDase 2 sequence. (ii) An 1.3 kb DNA fragment encoding the extracellular domain of the human NTPDase 2 was produced by PCR using the human NTPDase 2 cDNA (in pcDNA3) as the template, a hu TMD1 (7ck) forward chimeric primer annealing to the sequences downstream of the TMD1 of the human NTPDase 2, and a hu TMD2 (7ck) reverse chimeric primer annealing to the upstream of the TMD2 of the human NTPDase 2. The sequence of the hu TMD1 (7ck) forward primer is 5'- GCCCTCATTCTAAGTGCTGTAACCCGCGACG TCCGG-3' in which the nucleotides underlined encode the seven amino acids from

the TMD1 of chicken NTPDase 8 close to the extracellular domain, and the nucleotides not underlined are from the human NTPDase 2 sequence. The sequence of the hu TMD2 (7ck) reverse primer is 5'- GATGGCACCTGCCCA CAGACTGTCTGTGCCCTTGCG-3' in which the nucleotides underlined encode the seven amino acids from the TMD2 of chicken NTPDase 8 close to the extracellular domain, and the nucleotides not underlined are from the human NTPDase 2 sequence. (iii) A 0.1 kb DNA fragment encoding the C-terminal cytoplasmic domain and the 15 amino acids from the TMD2 of the human NTPDase 2 close to the cytoplasmic domain and the seven amino acids from the TMD2 of chicken NTPDase 8 close to the extracellular domain was produced by PCR using the human NTPDase 2 cDNA (in pcDNA3) as the template, a human NTPDase 2 reverse primer annealing to the C-terminus of the human NTPDase 2 without a stop codon, and a hu TMD2 (7ck) forward chimeric primer annealing to the sequences of the TMD2 of the human NTPDase 2. The sequence of the hu TMD2 (7ck) forward primer is 5'- AGTCTGTGGGCAGGTGCCATCC TGCTGCTCTTCGCC-3' in which the nucleotides underlined encode the seven amino acids from the TMD2 of chicken NTPDase 8 close to the extracellular domain, and the nucleotides not underlined are from the human NTPDase 2 sequence.

The second PCR step generates an 1.4 kb DNA fragment using the two overlapping DNA fragments, the 1.3 kb fragment and the first 0.1 kb fragment encoding the N-terminus of the human NTPDase 2, as templates, and the human NTPDase 2 forward primer and the hu TMD2 (7ck) reverse primer. The entire hu-ck 7-amino acid TMD1,2 chimeric cDNA was obtained in the third PCR using the two overlapping DNA fragments, the 1.4 kb fragment and the remaining 0.1 kb fragment, as templates, and the hu NTPDase 2 forward and reverse primers. *Taq* polymerase is used in the third PCR. The hu-ck 7-amino acid TMD1,2 chimeric cDNA is inserted into pcDNA3.1 vector through TA cloning. DNA sequencing showed that there was no unintended mutation in this recombinant DNA construct.

Primers used for constructing hu-ck 7-amino acid TMD1,2 chimera

Hu TMD1 (7CK) F GCCCTCATTCTAAGTGCTGTAACCCGCGACGTCCGG
Hu TMD1 (7CK) R TACAGCACTTAGAATGAGGGCGCCGGCGAGGCCCGC
Hu TMD2 (7CK) F AGTCTGTGGGCAGGTGCCATCCTGCTGCTCTTCGCC
Hu TMD2 (7CK) R GATGGCACCTGCCCACAGACTGTCTGTGCCCTTGCG

Expression and ATPase activity of the hu-ck 7-amino acid TMD1,2 chimera

The procedures of transfection of human embryonic kidney 293 (HEK293) cells with the the hu-ck 7-amino acid TMD1,2 chimeric cDNA were similar to that described in Chapter 2. The transfected cells were harvested after 48 hours and used for ATPase assay and western blotting. The chimera was not expressed and the activity of the HEK293 cells transfected with the chimeric cDNA is similar to that of the HEK293 cells transfected with pcDNA3 vector.