Either the Carboxyl- or the Amino-Terminal Region of the Human Ecto-ATPase (E-NTPDase 2) Confers Detergent and Temperature Sensitivity to the Chicken Ecto-ATP-diphosphohydrolase (E-NTPDase 8)†

Takashi Mukasa, Yonghee Lee, and Aileen F. Knowles*

Department of Chemistry and Biochemistry, San Diego State University, San Diego, California 92182-1030

Received January 4, 2005; Revised Manuscript Received June 23, 2005

ABSTRACT: Human ecto-ATPase (E-NTPDase 2) and chicken ecto-ATP-diphosphohydrolase (E-NTPDase 8) are cell surface nucleotidases with two transmembranous domains, one each at the N- and C-termini. Hydrolysis of substrates occurs in active sites residing in their extracellular domains. Human ecto-ATPase activity is decreased by NP-40 and at temperatures higher than 37 °C. Reduction of activity is abolished by prior cross-linking of the ecto-ATPase by lectin and chemical cross-linking agents [Knowles, A. F., and Chiang, W.-C. (2003) *Arch. Biochem. Biophys. 418*, 217−227]. In contrast, the chicken ecto-ATP-diphosphohydrolase is not inhibited by NP-40, and activity is approximately 2-fold higher at 55 °C. To determine if the transmembranous domains of the two E-NTPDases mediate their respective responses to detergents and high temperature, we first constructed a chimera (ck-hu ACR5) in which the C-terminus of the chicken ecto-ATP-diphosphohydrolase is substituted by the corresponding region of the human ecto-ATPase. While this chimera displays many similar enzymatic characteristics as the parental chicken ecto-ATP-diphosphohydrolase, its inhibition by NP-40, high temperature, and substrate resemble that of the human ecto-ATPase, which donates the C-terminus including the C-terminal transmembranous domain. Additionally, comparison of the effects of ConA, disuccinimidyl suberate, and glutaraldehyde on the parental enzymes and the chimera indicated that catalysis which occurs in the extracellular domains of the two E-NTPDases responds differently to conformational constraints. Enzyme activity of a second chimera (ck-hu ACR1) in which the N-terminus of the chicken ecto-ATP-diphosphohydrolase is substituted by the corresponding region of the human ecto-ATPase is also inhibited by NP-40 and is less active at 55 °C; however, its temperature dependence differs from that of ck-hu ACR5. These results indicate that (1) the C- and N-termini of the two E-NTPDases encompassing the two transmembranous domains are important elements in determining the sensitivity of the human ecto-ATPase to NP-40 and high temperatures; (2) incorporation of either the C- or N-terminus of the human ecto-ATPase alone in the chicken ecto-ATP-diphosphohydrolase is sufficient to impart negative regulation on ATP hydrolysis due to membrane perturbation; and (3) interactions of the two sets of heterologous transmembranous domains are not equivalent, which are most likely related to their different amino acid sequences.

Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases)[1] are a family of nucleotidases that have broad substrate specificities. More than 30 E-NTPDases have been cloned to date. Eight subfamilies of these enzymes (E-NTPDase 1−8) can be distinguished by the extent of their sequence homologies, substrate specificity, and tissue and subcellular distribution. Most of these enzymes are integral membrane proteins. Except for E-NTPDase 5 and 6, all have two transmembranous domains (TMD) at their N- and C-termini with short cytoplasmic domains. E-NTPDases 1, 2, 3, and 8 are cell surface nucleotidases with large extracellular domains containing the active sites, i.e., they are true ecto-nucleotidases. E-NTPDase 1 (also called CD39), 3, and 8 hydrolyze both nucleoside triphosphates and diphosphates in the presence of Mg^{2+} and Ca^{2+} with ADPase/ATPase ratios ranging from 0.25 to 1 and are sometimes referred to as ecto-apyrases or ecto-ATP-diphosphohydrolases (ecto-ATPDases). In contrast, E-NTPDase 2 preferentially hydrolyzes NTP with an ADPase/ATPase ratio of ∼0.1 despite the presence of the same five apyrase conserved regions (ACR) as the ecto-ATPDases in their sequences. The cell surface E-NTPDases also contain 10 conserved cysteine residues in the extracellular domain, but glycosylation is variable. Thus, the molecular masses of these enzymes range from 66 to 85 kDa even though the polypeptide chains deduced from cDNA sequences have a similar size, i.e., 54 kDa.

Our laboratory has been investigating two of the E-NTPDases, a human E-NTPDase 2 (*1*−*3*) and a chicken E-NTPDase 8 (*4*, *5*). Biochemical determination (*6*−*8*) and

† Supported by the California Metabolic Research Foundation.

* Corresponding author phone: 619-594-2065; fax: 619-594-4624; e-mail: aknowles@chemistry.sdsu.edu.

[1] Abbreviations: E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; TMD, transmembranous domain; ecto-ATPDase, ecto-ATP-diphosphohydrolase; ACR, apyrase conserved region; ConA, concanavalin A; DSS, disuccinimidyl suberate; NP-40, nonidet P-40; pCMPS, *p*-chloromercuri-phenylsulfonate; PFO, pentafluorodecaoctanoic acid; ECD, extracellular domain.

10.1021/bi050019k CCC: $30.25
Published on Web 07/30/2005

Northern blot analysis (*9*) indicated that E-NTPDase 2 is abundant in muscle and brain and is also highly expressed in some tumors. Mouse and human E-NTPDase 2 were cloned from mouse hepatoma (*10*), human bladder tumor (*11*), and human hepatoma and small cell lung carcinoma (*3*). Biochemical characteristics of heterologously expressed human E-NTPDase 2, a glycoprotein of 66 kDa (*3*), are similar to those in the native tissues (*1*, *2*). The chicken E-NTPDase 8, the first of this subfamily to be cloned, is present in the liver (*5*), oviduct (*4*, *12*), and stomach (*13*). The protein is more extensively glycosylated, and the mature proteins have molecular masses of 80−85 kDa.

Previous studies of the human ecto-ATPase and chicken ecto-ATPDase in both native tissues and heterologous expression systems indicated that besides their different substrate preference, they also differ in their responses to various activity modulators (*3*, *5*, *7*, *15*), the most striking being their different responses to detergents, such as NP-40 and Triton X-100, and high temperatures. The human ecto-ATPase is decreased by low concentrations (0.01%) of NP-40 and temperatures above 37 °C (*3*), whereas the chicken ecto-ATPDase can be solubilized from membranes by 5% NP-40 (*4*, *5*) and suffers little loss of activity even after 1 h at 60 °C (*7*). Furthermore, noncovalent and covalent cross-linking agents stimulate the activity of the human ecto-ATPase (*3*) but have little or even inhibitory effects on the chicken ecto-ATPDase (*7*, *15*), suggesting that the activities of the two enzymes are regulated differently by their quaternary structures.

Because detergents partition in the lipid bilayer and temperature affects membrane fluidity, which likely affect intramolecular or intermolecular interaction of TMDs that span the lipid bilayer, we are interested in determining the involvement of the TMDs of the two E-NTPDases in their activity regulation. Previous studies of rat E-NTPDase 1 (CD39/ecto-apyrase) using constructs lacking the N-terminal or C-terminal TMD or both indicated that anchoring of the extracellular domain by the two TMDs is required for full activity (*16*). The same study also showed that CD39 constructs lacking the TMDs are no longer inhibited by detergent (*16*). In this study, we wish to determine if substitution of the C- and N-terminal TMD of chicken ecto-ATPDase by the corresponding regions of the human ecto-ATPase will alter its responses to temperature and detergent. Our results indicate that both TMDs of the chicken ecto-ATPDase are required for its stability. Exchange of either of its TMDs with that from the less stable human ecto-ATPase reduces enzyme stability, and the chimeras demonstrate responses to detergent and high temperature that are more characteristic of the latter.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's media (DMEM), OptiMEM, fetal bovine serum, Lipofectamine, trypsin-EDTA, subcloning efficiency DH5α cells, and antibiotics were purchased from Invitrogen. PCR Master Mix was purchased from ABgene (Rochester, NY). DNA miniprep kit and QIA gel extraction kit were purchased from QIAGEN. Restriction enzymes were purchased from New England Biolabs. SDS−PAGE reagents and Bio-Rad DC protein assay kit were purchased from Bio-Rad (Hercules, CA). Prestained protein ladder was purchased from Fermentas (Hanover, MD). PVDF membrane was purchased from PerkinElmer Life Sciences Inc. Goat anti-mouse IgG conjugated to alkaline phosphatase was purchased from Promega (Madison, WI). Alkaline phosphatase substrate tablets (NBT/BCIP) were purchased from Roche. Detergents, ATP, ADP, and all other biochemical reagents were purchased from Sigma Chemical Co. Disuccinimidyl suberate (DSS) was purchased from Pierce (Rockford, IL). Pentadecafluorooctanoic acid (PFO) was purchased from Oakwood Products (West Columbia, SC). HeLa cells were obtained from Dr. Jacques Perrault of the San Diego State University Biology Department. The mouse anti-chicken ecto-ATPDase monoclonal antibody, MC18, was the generous gift of Dr. Randy Strobel of Metropolitan State University, St. Paul, Minnesota. 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.

Chimera Construction. Chicken ecto-ATPDase cDNA cloned from chicken liver (*5*) and human ecto-ATPase cDNA cloned from small cell lung carcinoma cells NCIH69 (*3*) were inserted in the expression vector pcDNA3 (Invitrogen). The recombinant plasmids were used as templates in the construction of the ck-hu ACR5 and ck-hu ACR1 chimeras.

In the ck-huACR5 chimera, the portion of chicken ecto-ATPDase from ACR5 to the C-terminus and containing the C-terminal transmembranous domain (TMD2) was exchanged for the corresponding region of the human ecto-ATPase. Amino acid sequences in ACR5 of the chicken ecto-ATPDase (aa437−aa451) and human ecto-ATPase (aa435−aa449) differ only in two amino acid residues (see Figure 3A). The chimera consists of the N-terminal 455 amino acids of chicken ecto-ATPDase and the C-terminal 46 amino acids of human ecto-ATPase. The ck-hu ACR5 chimera was constructed using the strategy of overlap extension (*17*). A 1.4 kb DNA fragment containing the 5′-end of chicken ecto-ATPDase cDNA was generated by PCR using chicken ecto-ATPDase cDNA (in pcDNA3) as the template, a forward primer (pcDNA3F: 5′-GGAGACCCAAGCTTGGTACC-3′) annealing to the vector upstream of a BamHI site, and a reverse chimeric primer annealing to ACR5 (ck-hu ACR5R: 5′-ggatcAGGTTGGTCAGGTTCAGCATG*aagccc*-3′ in which nucleotides in lower case are from the chicken ecto-ATPDase sequence, nucleotides in italicized lower case are from the human ecto-ATPase sequence, and underlined nucleotides in capital letters are those that are identical in both sequences). A 200-bp DNA fragment containing the 3′-end of human ecto-ATPase cDNA was obtained by PCR using the forward chimeric primer annealing to ACR5 (ck-hu ACR5F: 5′-*gggctt*CATGCTGAACCTGACCAACCT-gatcc-3′) and reverse primer annealing to the vector downstream of a XhoI site (pcDNA3R: 5′-GTCGAGGCTGAT-CAGCGAGC-3′) using human ecto-ATPase cDNA (in pcDNA3) as the template. PCR was carried out in a 50 μL reaction mixture (ABgene) containing 0.5 μg of cDNA template, 0.5 μM of primers, 0.1 mM dNTP, and 1.25 units of Taq DNA polymerase. Thermal cycling on a PTC-200 Peltier thermal cycler began with 4.5 min at 94 °C followed by 39 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 3 min and ending with 10 min at 72 °C. The chimera was then obtained in a two-step PCR reaction. In the first

step, the 1.4-kb and 200-bp DNA fragments that overlap at ACR5 were extended by DNA polymerase. Cycling of the first step began with four cycles of 94 °C for 30 s, 65 °C for 30 s, and decreasing to 30 °C at 0.5 °C /s, 30 °C for 30 s and ending with 3 min at 65 °C, after which primers that anneal to the vector, pcDNA3F and pcDNA3R, were added for the second step of PCR. The second step began with 1 min at 94 °C followed by 37 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 3 min and ending with 10 min at 72 °C. The ~1.5 kb PCR product was gel purified, digested with BamHI and XhoI, and ligated to the pcDNA3 vector digested by the same restriction enzymes. Recombinant plasmid isolated from transformed DH5α *Escherichia coli* cells was sequenced in both orientations to verify that the desired chimera was obtained.

A similar strategy was used for the construction of ck-hu ACR1 in which the N-terminus of the chicken ecto-ATPDase extending to ACR1 and containing the N-terminal transmembranous domain (TMD1) is exchanged for the N-terminus of the human ecto-ATPase. Amino acid sequences in ACR1 of the chicken ecto-ATPDase (aa45-aa51) and human ecto-ATPase (aa48-aa54) differ in only one amino acid (see Figure 3B). The ck-hu ACR1 chimera consists of the N-terminal 49 amino acids of the human ecto-ATPase and the C-terminal 443 amino acids of the chicken ecto-ATPDase. A 200-bp DNA fragment containing the 5′ end of the human ecto-ATPase cDNA was generated by PCR using human ecto-ATPase cDNA as the template, the pcDNA3F primer (see above), and a reverse chimeric primer annealing to ACR1 (ck-hu ACR1R: 5′-gacatagagagccgtgtgtgaagaaccagc-3′, the underlined and italicized sequence is complementary to that coding for amino acids in the human ecto-ATPase ACR1, whereas the remaining sequence is complementary to that coding for amino acids immediately following ACR1 in the chicken ecto-ATPDase). A 1.4-kb DNA fragment containing the 3′-end of the chicken ecto-ATPDase was obtained by PCR using the chicken ecto-ATPDase cDNA as the template, a forward chimeric primer containing nucleotides (underlined) annealing to its ACR1 (ck-hu ACR1F: 5′-gctggttcttcacacacggctctctatgtc-3′), and the pcDNA3R primer (see above). Other experimental conditions are the same as that described for the construction of hu-ck-ACR5.

Preparation of Plasma Membranes from Transfected HeLa or HEK293 Cells. Transient and stable transfection of HeLa or HEK293 were carried out as described previously (*3*). Although enzyme activities obtained from transfected HEK293 cells were usually higher than that from HeLa cells, the expressed enzymes displayed the same characteristics. Plasma membranes were prepared from five to ten 10-cm plates of stably transfected HeLa or HEK293 cells by differential and sucrose gradient centrifugation as described previously (*3*). Specific ATPase activities varied with different membrane preparations. There was little loss of activity after storage of the membranes at −20 °C for 6−12 months.

ATPase and ADPase Assays. ATPase and ADPase assays were carried out in duplicates in a 0.5 mL reaction mixture with 5−15 μg of membrane proteins as described previously (*3*). Substrates were either 5 mM MgATP or MgADP. The amount of phosphate released was determined colorimetrically using 0.1 to 0.4 mL of deproteinized supernatant solutions with 2 mL of AAM reagent (*4*). Protein concentrations of the plasma membranes were determined using the Bio-Rad DC (detergent compatible, Lowry-based) protein assay kit with bovine serum albumin as standard. Each of the experiments was performed at least twice. In experiments in which specific activities are reported, data from one experiment are shown.

Gel Electrophoresis and Western Blot Analysis. SDS−PAGE (*18*) and PFO−PAGE (*19*) were performed in a MINI−PROTEAN II apparatus (Biorad) as described previously (*3*). For Western blot analysis, monoclonal antibody MC18 (200-fold dilution) was used as the primary antibody, and goat anti-mouse IgG conjugated to alkaline phosphatase (5000-fold dilution) was used as the secondary antibody. Immunoreactive proteins were detected using alkaline phosphatase substrate (NBT/BCIP) solution.

RESULTS

Biochemical Characteristics of the Expressed Chicken ecto-ATPDase (NTPDase8) and Human ecto-ATPase (NTPDase2) and Their Different Responses to Modulators. The chicken ecto-ATPDase and human ecto-ATPase were respectively cloned from chicken liver and human small cell lung carcinoma cells, NCIH69, by RT-PCR (*3*, *5*). Both cDNA were inserted in the expression vector, pcDNA3, and the recombinant plasmids were used to transfect HeLa cells. Stably transfected HeLa cells were obtained by geniticin selection. Membrane fractions enriched in plasma membranes were used for biochemical characterization of the expressed E-NTPDases.

The two E-NTPDases have different abilities in hydrolyzing ADP. The human ecto-ATPase displays a low MgADPase/MgATPase ratio, i.e., 0.051 ± 0.010 at pH 7.4 and 0.12 ± 0.013 at pH 6.4, whereas the chicken ecto-ATPDase displays a higher MgADPase/MgATPase ratio, i.e., 0.28 ± 0.031 at pH 7.4 and 0.80 ± 0.032 at pH 6.4. Ca^{2+} can substitute for Mg^{2+} in the divalent ion−nucleotide substrate complex for most E-NTPDases. However, CaATPase activity of the chicken ecto-ATPDase is only ~25% of its MgATPase activity, whereas human ecto-ATPase displays similar CaATPase and MgATPase activities at saturating substrate concentrations.

In addition to differences in their preferences for substrates and divalent ions, the two E-NTPDases also differ in their responses to several modulators that have different mechanisms of action. At 37 °C, the chicken ecto-ATPDase is not affected by the sulfhydryl reagent, pCMPS, nor by NP-40 and ConA, whereas it is inhibited by 10 mM azide, an inhibitor of cell surface ecto-ATPDases including E-NTPDase 1, 3, and 8 (*20*−*22*). Human ecto-ATPase is insensitive to azide inhibition but is inhibited ~70% by 0.25 mM pCMPS and >90% by 0.1% NP-40. It is markedly stimulated by ConA (Table 1).

Both chicken ecto-ATPDase and human ecto-ATPDase have 10 conserved cysteine residues in their extracellular domains, which are most likely involved in disulfide bond formation in the stabilization of protein structures. However, ecto-ATPases from different species have an additional conserved cysteine residue located in the TMD near the N-terminus (TMD1) but close to the extracellular surface (*23*). Site-directed mutagenesis of the human ecto-ATPase has shown that this cysteine, cys26 in human ecto-ATPase, is the target of pCMPS modification (unpublished results).

Table 1: Effects of Different Modulators on the ATP Hydrolysis Activities of Chicken ecto-ATPDase and Human ecto-ATPase[a]

	chicken ecto-ATPDase		human ecto-ATPase	
	μmol of Pi min^{-1} mg^{-1}		μmol of Pi min^{-1} mg^{-1}	
modulators	37 °C	55 °C	37 °C	55 °C
none	4.72	9.45	11.1	6.5
NP-40 (0.1%)	5.38	3.26	0.93	1.3
pCMPS (0.25 mM)	4.71	8.48	3.72	2.77
azide (10 mM)	3.15	7.75	10.9	6.49
ConA (50 μg/mL)	3.98	8.6	28.6	35.7

[a] ATPase reactions were carried out with 5−15 μg of membrane proteins prepared from HeLa cells stably transfected with chicken ecto-ATPDase and human ecto-ATPase 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 ATP. After 10 min at 37 or 55 °C, the reaction was terminated by the addition of 0.1 mL of 10% trichloroacetic acid. The amount of Pi released was determined as described in Materials and Methods. The chicken eto-ATPDase is inhibited by 10 mM azide but is not affected by the other modulators (except inhibition by 0.1% NP-40 at 55 °C). The human ecto-ATPase is inhibited by pCMPS and NP-40 and is stimulated by ConA but is not affected by azide.

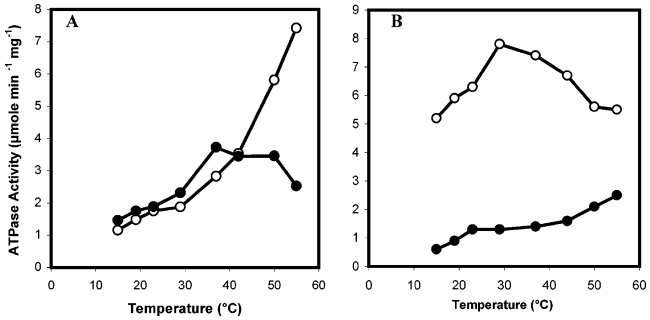

FIGURE 1: Effect of NP-40 on ATPase activities of chicken ecto-ATPDase and human ecto-ATPase at different temperatures. ATPase reaction was carried out for 10 min at the indicated temperatures without (○) or with (●) 0.1% NP-40 with 5 μg of membrane protein. (A) Membranes were prepared from HeLa cells stably transfected with chicken ecto-ATPDase cDNA; (B) Membranes were prepared from HeLa cells stably transfected with human ecto-ATPase cDNA. ATPase activity of chicken eco-ATPDase increased with temperature up to 55 °C and was inhibited by 0.1% NP-40 only at temperatures higher than 40 °C. ATPase activity of human ecto-ATPase decreased at temperatures higher than 30 °C and was inhibited by 0.1% NP-40 at all temperatures.

Of greater interest are the different effects of NP-40 and ConA on the two E-NTPDases. The inhibitory effect of NP-40 and stimulatory effect of ConA on human ecto-ATPase were previously attributed to alteration of quaternary structure of the enzyme by these reagents (*3*). It was further shown that human ecto-ATPase cross-linked by ConA becomes resistant to inactivation by substrate, resulting in apparent stimulation (*3*). In contrast, the chicken ecto-ATPDase activity is not inhibited by NP-40 nor is stimulated by Con A at 37 °C (Table 1) and is not susceptible to inactivation by substrate.

Besides ConA, the human ecto-ATPase activity is stimulated by two chemical cross-linking agents, DSS and glutaraldehyde, both reacting with lysine residues. Table 2 shows that the stimulatory effects of the three cross-linking agents on the human ecto-ATPase are greater at 55 °C (6−10-fold) than at 37 °C (∼2-fold). In contrast, glutaraldehyde and ConA have little or no effect on the chicken ecto-ATPDase, while DSS is clearly inhibitory.

Different Effects of Temperature and Detergent on Chicken ecto-ATPDase and Human ecto-ATPase. The data in Table 1 also show that the effects of modulators on the two E-NTPDases are similar whether the ATPase assays were conducted at 37 or 55 °C. However, it is noticeable that (1) chicken ecto-ATPDase activity is 2-fold higher at 55 °C than at 37 °C, whereas human ecto-ATPase activity is decreased by ∼50% at 55 °C, and (2) while chicken ecto-ATPDase is slightly stimulated by 0.1% NP-40 at 37 °C, it is inhibited ∼60% at 55 °C

The different effects of NP-40 and high temperature on chicken ecto-ATPDase and human ecto-ATPase are further illustrated in Figure 1. Figure 1A shows that chicken ecto-ATPDase activity increases markedly with temperature between 37 and 55 °C. It further shows that NP-40 has a slight stimulatory effect up to 40 °C but becomes inhibitory at higher temperature, so that 0.1% NP-40 inhibits the ATPase activity by ∼70% at 55 °C. In contrast, the human ecto-ATPase activity increases from 15 to 37 °C but declines at higher temperatures. It is inhibited by 0.1% NP-40 at all temperatures (Figure 1B).

We previously showed that glutaraldehyde treatment of membranes containing the human ecto-ATPase prevented inactivation of the enzyme by NP-40 (*3*). Treatment of membranes containing the chicken ecto-ATPDase by glutaraldehyde causes a slight inhibition of ATPase activity at both 37 and 55 °C in the absence of NP-40 (Table 2 and Figure 2) or in the presence of NP-40 at 37 °C (Figure 2A). However, it stabilizes against inactivation of the enzyme by NP-40 at 55 °C (Figure 2B). The protective effect of glutaraldehyde against NP-40 inactivation of the chicken ecto-ATPDase at 55 °C is similar to that previously observed

Table 2: Effects of Cross-Linking Agents on ATPase Activities of Chicken ecto-ATPDase, Human ecto-ATPase, the ck-hu ACR5 Chimera, and the ck-hu ACR1 Chimera[a]

	chicken ecto-ATPDase		human ecto-ATPase		ck-hu ACR5		ck-hu ACR1	
cross-linking agents	37 °C	55 °C	37 °C	55 °C	37 °C	55 °C	37 °C	55 °C
none	100	100	100	100	100	100	100	100
DSS (1 mM)	40.7 ± 4.7	43.3 ± 3.0	195 ± 31.4	583 ± 66	50.0 ± 6.6	97.3 ± 25	84 ± 5.0	107 ± 13.7
glutaraldehyde (10 mM)	78.0 ± 13.3	75.0 ± 1.3	178 ± 23.5	825 ± 112	108 ± 8.0	363 ± 51	204 ± 22.3	258 ± 24.6
ConA (1.5 mg/mL)	91.1 ± 23.4	80.6 ± 12.4	250 ± 51.4	1074 ± 47	93.0 ± 10.2	115 ± 39.6	100 ± 10.6	98 ± 13.9

[a] Membranes (50−150 μg) were preincubated in a 0.1 mL solution containing 20 mM Mops, pH 7.4, and 5 mM $MgCl_2$ without or with 1 mM DSS, 10 mM glutaraldehyde, or 1.5 mg/mL ConA for 20 min at room temperature, after which 10 μL of 0.1 M lysine was added. Aliquots were used for enzyme assays at 37 or 55 °C for 10 min. Values are given as percent activity ± SD (n = 3). The chicken ecto-ATPDase activity is moderately inhibited by DSS, whereas ConA and glutaraldehyde have little effect, while human ecto-ATPase activity is stimulated by all three reagents. In contrast to the chicken ecto-ATPDase, DSS does not inhibit the activities of ck-hu ACR5 or ck-hu ACR1, whereas glutaraldehyde stimulates the ck-hu ACR5 activity at 55 °C and the ck-hu ACR1 activity at both temperatures.

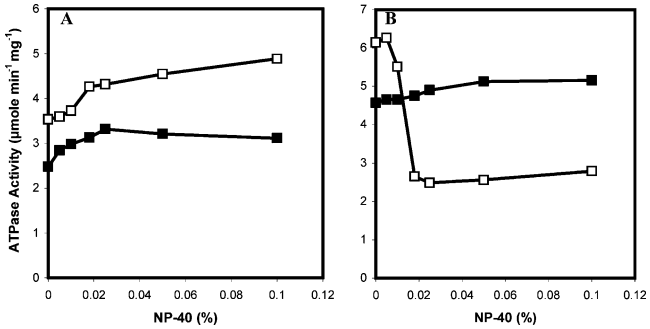

FIGURE 2: Effect of NP-40 on ATPase activities of chicken ecto-ATPDase without and with prior glutaraldehyde cross-linking and assayed at 37 and 55 °C. Membranes containing chicken ecto-ATPDase were preincubated in a 0.1-mL solution containing 20 mM Mops, pH 7.4, 5 mM $MgCl_2$, without (□) or with (■) 10 mM glutaraldehyde. After 20 min at room temperature, 10 μL of 0.1 M lysine was added to stop the reaction. Aliquots of the solution containing 5−15 μg of membrane proteins were used for ATPase assays in the absence or presence of the indicated concentrations of NP-40 for 10 min. (A) ATPase assayed at 37 °C; (B) ATPase assayed at 55 °C. At 37 °C, glutaraldehyde cross-linking decreased activity slightly. At 55 °C, activity of the untreated membranes was decreased to ~40% by 0.02% NP-40, whereas activity of the glutaraldehyde-treated membranes remained constant up to 0.1% NP-40.

for human ecto-ATPase at 37 °C (*3*), although to a lesser degree.

Construction and Expression of ck-hu ACR5 Chimera. The inactivation of the human ecto-ATPase by high temperatures and detergents and the lack of effect of both on the chicken ecto-ATPDase prompted us to determine the structural elements responsible for their different behaviors. Since detergents and increased membrane fluidity at higher temperatures (*24*) are likely to affect interaction of membrane-spanning helices, their different effects on the two E-NTPDases may be attributed to the different manners in which their TMDs respond to membrane perturbation. The question is if introduction of either one of the TMDs of the human ecto-ATPase into the chicken ecto-ATPDase will impart to the latter the properties of inhibition by NP-40 and reduction of activity at high temperatures or if both are required. The two E-NTPDases share nine conserved regions, five of them named ACRs because homologous sequences were found in potato apyrase and several other soluble NTPDases (*25, 26*). Eight of the conserved regions are located in the N-terminal half of the E-NTPDases, whereas only ACR5 is near the C-terminus. The amino acid sequences of human ecto-ATPase and chicken ecto-ATPDase extending from ACR5 to the C-termini are shown in Figure 3A. ACR5 (underlined) of the two E-NTPDases differ in only two amino acids (bold). The 15-residue peptides consist mostly of hydrophobic amino acids and contain the same obligatory glycosylation site, NLT (*27, 28*). Figure 3A also shows that there is little similarity in the sequences of the predicted TMD2 of the two enzymes (shaded). We took advantage of the ACR5 site and used the overlap extension strategy (*17*) to construct the ck-hu ACR5 chimera where the C-terminus (46 amino acids) of human ecto-ATPase that includes the C-terminal intracytoplasmic region, TMD2, and intervening peptide between TMD2 and ACR5 was ligated to the N-terminal polypeptide of chicken ecto-ATPDase (455 amino acids).

Table 3: Effects of Modulators on ATPase and ADPase Activities of ck-hu ACR5and ck-hu ACR1 Chimeras[a]

	ck-hu ACR5		ck-hu ACR1	
addition	ATPase	ADPase	ATPase	ADPase
none	100	100	100	100
NP-40 (0.1%)	20.6 ± 5.2	39.1 ± 4.8	40.5 ± 1.7	43 ± 4.5
pCMPS (0.25 mM)	102 ± 10.9	96.2 ± 7.2	139 ± 15.6	109 ± 2.8
azide (10 mM)	80.4 ± 12.3	39.3 ± 12.5	70.0 ± 8.5	47 ± 2.1
ConA (50 μg/mL)	90.8 ± 11.3	95.1 ± 7.1	100.0 ± 10.6	85 ± 2.1

[a] Reactions were carried out with membranes prepared from HeLa cells stably transfected with the ck-hu ACR5 chimera cDNA and HEK293 cells stably transfected with ck-hu ACR1. Experimental conditions were similar to that described in the legend of Table 1. ATPase and ADPase activities were determined at 37 °C. Values are given as percent activity ± SD (n = 3). Both ATPase and ADPase activities of the chimera were inhibited by NP-40 and azide. They were not affected by pCMPS or ConA.

Introduction of C-Terminus of the Human ecto-ATPase Alters the Properties of Chicken ecto-ATPDase. The ck-hu ACR5 chimera was expressed in HeLa cells. Specific activity obtained from transiently transfected cells was typically ~20% of that of the wild-type chicken ecto-ATPDase probably due to less efficient synthesis of the chimera in HeLa cells since protein expression was also reduced (not shown). The enzymatic properties of the ck-hu ACR5 chimera were determined using cell fraction enriched in plasma membranes. As expected, the chimera retains similar responses to some modulators as the parental chicken ecto-ATPDase. At 37 °C, the chimera is inhibited by azide with greater inhibition of ADPase activity than ATPase activity and is not inhibited by pCMPS nor by ConA. However, in marked contrast to the parental chicken ecto-ATPDase, the ATPase and ADPase activities are inhibited by 0.1% NP-40 at 37 °C (Table 3). Furthermore, ATPase activity of the chimera increases with temperature from 15 to 37 °C but declines at higher temperatures (Figure 4A) so that activity at 55 °C is ~50% of that at 37 °C. The temperature

A

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Human ecto-ATPase      435   GWALGYMLNLTNLIPADPPGLRKGTDFSSWVVLLLLFASALLAALVLLLRQVHSAKLPSTI   495
Chicken ecto-ATPDase   437   GWTLGFMLNLTNMIPTEALEHVKGQQPSLWAGAISFIVLAIVAGLVAILLQCFWKSK       493
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B

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Human ecto-ATPase        1    MAGKVRSLLPPLLLAAAGLAGLLLLCVPTRDVREPPALKYGIVLDAGSSHT    51
Chicken ecto-ATPDase     1    MEYKGKVVAGLLTATCVFSIIALILSAVDVKDVFLPPGTKYGLVFDAGSTHT    52
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FIGURE 3: Amino acid sequences of C- and N-termini of human ecto-ATPase and chicken ecto-ATPDase. (A) Amino acid sequences extending from ACR5 (underlined) to the C-termini of the two E-NTPDases are shown. The predicted TMD2 are shaded. (B) Amino acid sequences from the N-termini to ACR1 (underlined) of the two E-NTPDases are shown. The predicted TMD1 are shaded. The TMHMM program (at http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used for TMD prediction.

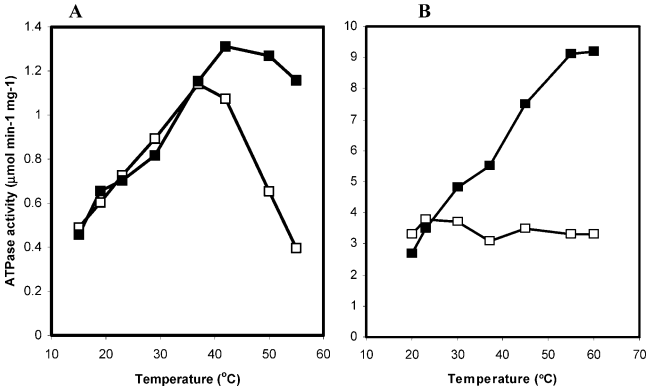

FIGURE 4: Effect of temperature on the ATPase activities of ck-hu ACR5 chimera and ck-hu ACR1 chimera with or without prior cross-linking by glutaraldehyde. Membranes were treated without (□) or with (■) 10 mM glutaraldehyde as described in the legend of Figure 2. Aliquots were used for ATPase assays at the indicated temperatures for 10 min. (A) Membranes were prepared from HeLa cells stably transfected with ck-hu ACR5 chimera cDNA. ATPase activity of untreated ck-hu ACR5 chimera decreases at temperatures higher than 37 °C but not that of glutaraldehyde-treated membranes. (B) Membranes were prepared from HEK293 cells stably transfected with ck-hu ACR1 chimera cDNA. ATPase activity of untreated ck-hu ACR1 chimera is relatively constant at all temperatures, but ATPase activity of glutaraldehyde-treated ck-hu ACR1 chimera increases from 20 to 60 °C.

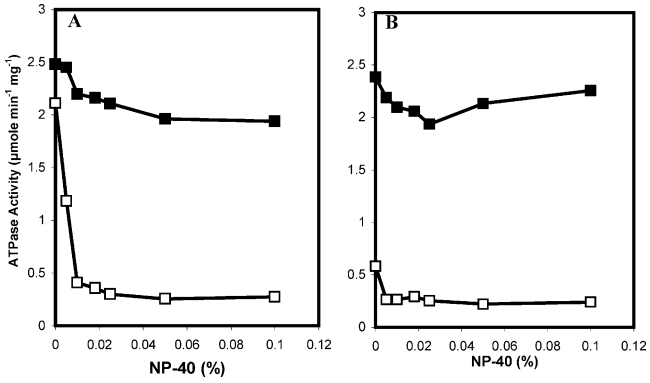

FIGURE 5: Effect of NP-40 on the ATPase activity of ck-hu ACR5 chimera with or without prior cross-linking by glutaraldehyde and assayed at 37 and 55 °C. Membranes were untreated (□) or treated with 10 mM glutaraldehyde (■) as described in the legend of Figure 2. Aliquots containing 10 μg of membrane proteins were used for ATPase assays in the absence or presence of the indicated concentrations of NP-40 for 10 min. (A) Reaction temperature was 37 °C; (B) reaction temperature was 55 °C. Glutaraldehyde cross-linking stabilized the ATPase activity against inactivation by NP-40.

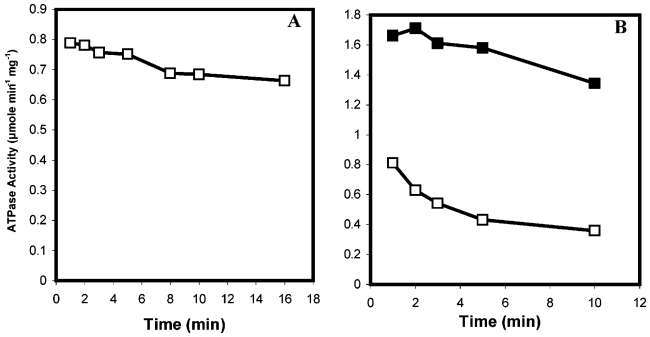

FIGURE 6: Time course of ATP hydrolysis by ck-hu ACR5 chimera. ATPase activities of the membranes prepared from HeLa cells stably transfected with ck-hu ACR5 chimera were determined at 37 or 55 °C at the indicated times. In the experiment shown in (B), the membranes were untreated (□) or treated with 10 mM glutaraldehyde (■) as described in the legend of Figure 2. Aliquots containing 2.5 μg of membrane proteins were added to reaction mixtures preequilibrated to 37 or 55 °C to initiate the reaction. Reactions were terminated at the indicated times by the addition of trichloroacetic acid. (A) Reaction temperature was 37 °C; B, reaction temperature was 55 °C. ATPase activity decreased continuously during the 10-min reaction at 55 °C. Treatment by glutaraldehyde increased the activity at 1 min and also prevented loss of activity during the reaction.

dependence of the chimera ATPase activity and its inhibition by NP-40 clearly differ from that of the wild-type chicken ecto-ATPDase (Figure 1A) but are similar to that of the human ecto-ATPase (Figure 1B). Thus, introduction of the C-terminus of the human ecto-ATPase has conferred on chicken ecto-ATPDase sensitivity to both detergent and high temperature.

Figure 4A further shows that a decrease of activity at high temperature was prevented by prior treatment of the membranes with glutaraldehyde. This behavior is also similar to that of the human ecto-ATPase (*3*) but with some difference. While cross-linking by DSS, ConA, and glutaraldehyde resulted in an ~6−10-fold greater activity of the human ecto-ATPase at 55 °C (Table 2), only treatment by glutaraldehyde resulted in an increase (~3.5-fold) of the chimera ATPase activity at 55 °C, whereas DSS and ConA had little effect (Table 2). Similar to the wild-type chicken ecto-ATPDase (Table 2), activity of the chimera in the DSS-treated membrane is inhibited ~60% at 37 °C. It is concluded that the inhibitory effect of DSS cross-linking results from reaction of the reagent with lysine residues that are important for catalysis.

Glutaraldehyde treatment of the membrane also prevented inactivation of the ck-hu ACR5 chimera ATPase activity by NP-40. Figure 5 shows that the chimera ATPase activity is markedly inhibited by 0.01% NP-40 at both 37 and 55 °C. However, ATPase activity of the chimera in glutarladehyde-treated membranes suffers little loss in the presence of NP-40. Furthermore, the activity is not decreased by high temperature (Figure 5B).

A third marked change that occurs with the introduction of the human ecto-ATPase C-terminus into the chicken ecto-ATPDase is the decline of specific ATPase activity with time at elevated temperature. Similar to the parental chicken ecto-ATPDase (not shown), the time course of ATP hydrolysis by the ck-hu ACR5 chimera is linear at 37 °C, so that a straight line is obtained when specific activities obtained at different time points are plotted against reaction time (Figure 6A). However, specific ATPase activity of the ck-hu ACR5 chimera decreases with time when assays are performed at 55 °C (Figure 6B, lower curve). We have determined that this is not due to instability of the enzyme at 55 °C within the 10-min reaction period (not shown) but rather an inactivation of the enzyme after substrate addition. Glutaraldehyde treatment of membranes results in stimulation of the ck-hu ACR5 chimera ATPase activity by 2-fold at the 1-min time point and also attenuates the decline of activity so that apparent stimulation is increased to 3-fold at the 10-min time point (Figure 6B, upper curve). Thus, the ck-hu ACR5 chimera, which contains the human ecto-ATPase C-terminus, is less stable than the parental chicken ecto-ATPDase at high temperature and becomes susceptible to

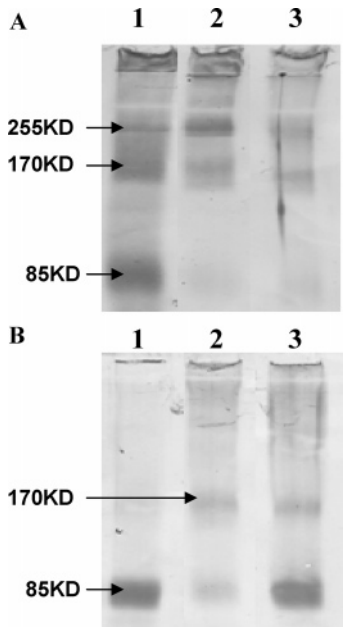

FIGURE 7: Determination of oligomeric structures of chicken ecto-ATPDase and ck-hu ACR5 chimera by PFO gel electrophoresis. Membranes were untreated (lane 1) or treated by 10 mM glutaraldehyde (lane 2) or 1 mM DSS (lane 3) as described in the legend of Table 2. Membranes (25 μg of protein) were mixed with 2× PFO-gel sample buffer so that the final PFO concentration in the samples was 0.2%. The samples were incubated at room temperature for 30 min and then subjected to nondenaturing gel electrophoresis at 4 °C together with molecular weight markers [bovine serum albumin monomer (70 kDa) and its oligomers (140 kDa, 210 kDa, 280 kDa), and myosin (210 kDa)] in gel buffer containing 25 mM Tris, 192 mM glycine, and 0.5% PFO, pH 8.5. Separated proteins were electrophoretically transferred to PVDF membrane. Portion of the blot containing molecular weight markers was stained with Amido black. The remaining blot was probed with monoclonal antibody MC18. (A) Samples containing chicken ecto-ATPDase; (B) samples containing ck-hu ACR5 chimera.

inactivation by substrate, a characteristic displayed by the human ecto-ATPase (*3*).

Effects of Cross-Linking Agents on Quaternary Structures of the Chicken ecto-ATPDase and the ck-hu ACR5 Chimera. Our previous study on human ecto-ATPase (*3*), as well as data presented in this report (Table 2), showed that treatment of membranes with the three cross-linking agents, ConA, DSS, and glutaraldehyde, prevented inactivation of the human ecto-ATPase by high temperature, NP-40, and substrate to a variable extent. Since these reagents brought about oligomerization of the human ecto-ATPase (*3*), we concluded that oligomeric ecto-ATPase is less affected by these factors. On the other hand, this report shows that ConA has no effect on the activities of the chicken ecto-ATPDase or the ck-hu ACR5 chimera at high temperatures, whereas glutaraldehyde and DSS, both lysine cross-linking agents, have very different effects on the two enzymes. DSS is inhibitory to the chicken ecto-ATPDase at both 37 and 55 °C, whereas glutaraldehyde is not. On the contrary, glutaraldehyde cross-linking prevents loss of activity of the ck-hu ACR5 chimera due to high temperature (Table 2, Figure 4A), NP-40 (Figure 5), and substrate (Figure 6B). PFO-gel electrophoresis shows that both DSS and glutaraldehyde promote oligomer formation of the chicken ecto-ATPDase and the chimera (Figure 7), although to a different extent. The wild-type chicken ecto-ATPDase is an 85-kDa protein, but a significant amount of dimer is usually seen in the membranes (Figure 7A, lane 1). The trimer is the dominant species in glutaraldehyde-treated membranes (Figure 7A, lane 2), while both dimer and trimer are obtained in DSS-treated membranes (Figure 7A, lane 3). Interestingly, PFO-gel analysis of the ck-hu ACR5 chimera shows that it is present mostly as a monomer with similar molecular mass as the parental chicken ecto-ATPDase in the untreated membrane (Figure 7B, lane 1). Monomers of the chimera are still present in glutaraldehyde- and DSS-treated membranes in addition to dimers (Figure 7B, lanes 2 and 3). ConA cross-linking causes the complete disappearance of the monomers of both chicken ecto-ATPDase and ck-hu ACR5 chimera (data not shown). These results indicate that the chicken ecto-ATPDase and the ck-hu ACR5 chimera have different propensities for forming oligomers. Nevertheless, all three cross-linking agents promote oligomer formation of both enzymes. Thus, their different effects on the enzymatic activities of the chicken ecto-ATPDase, the ck-hu ACR5 chimera, and the human ecto-ATPase are related to their different mechanisms of action aside from their general effects on facilitating oligomer formation.

Introduction of the N-Terminus of the Human ecto-ATPase also Alters the Responses of the Chicken ecto-ATPDase to NP-40 and High Temperature. The results obtained with the ck-hu ACR5 chimera indicated that the simultaneous introduction of both TMDs of the human ecto-ATPase is not necessary to alter the properties of the chicken ecto-ATPDase. To further demonstrate that this could be accomplished by replacement of a single TMD, we also generated a chimera, ck-hu ACR1, in which the N-terminus of the chicken ecto-ATPDase that includes the N-terminal intracytoplasmic domain, TMD1, and intervening peptide between TMD1 and ACR1 is substituted by the corresponding region of human ecto-ATPase. Interestingly, eight of the fifteen amino acid residues in the intervening sequences between TMD1 and ACR1 are identical between the two E-NTPDases (Figure 3B). Expression of the ck-hu ACR1 in HEK 293 cells was higher than that of ck-hu ACR5, and the specific activity was ~50% of that of the wild-type chicken ecto-ATPDase. The ck-hu ACR1 and ck-hu ACR5 chimeras are similar in many aspects: (1) the ck-hu ACR1 chimera activity retains most of the properties of the parental chicken ecto-ATPDase but is also inhibited by 0.1% NP-40 (Table 3); (2) its activity is not affected by cross-linking by DSS or ConA but is increased by cross-linking by glutaraldehyde in the absence of NP-40 (Table 2 and Figure 4B) and in its presence (not shown); (3) it is susceptible to inactivation by ATP at 55 °C (not shown); and (4) it exists mostly as monomers (not shown). Interestingly, the effect of temperature on ck-hu ACR1 chimera ATPase activity differs from that of ck-hu ACR5. The ck-hu ACR1 chimera activity remains relatively constant in the temperature range of 20−60 °C in the absence of glutaraldehyde (Figure 4B). In addition, its activity is stimulated by glutaraldehyde at both 37 and 55 °C (Table 2). These results suggest that the two different pairs of heterologous TMD in the two chimeras contribute differently to these properties. Nevertheless, the responses of the ck-hu ACR1 chimera to NP-40 and high temperature are distinctly different from the parental chicken ecto-ATPDase. Table 4 summarizes the effects of high

Table 4: Ratios of ATPase Activities with and without 0.1% NP-40 and ATPase Activities at 55 and 37 °C of WT Chicken ecto-ATPDase, WT Human ecto-ATPase, ck-hu ACR5, and ck-hu ACR1 Chimera[a]

constructs	ATPase (+ NP-40)/ ATPase (−NP-40) at 37 °C	ATPase at 55 °C/ ATPase at 37 °C
WT chicken ecto-ATPDase	1.20 ± 0.09	2.08 ± 0.52
ck-hu ACR5	0.27 ± 0.09	0.52 ± 0.14
ck-hu ACR1	0.40 ± 0.02	1.03 ± 0.16
WT human ecto-ATPase	0.025 ± 0.024	0.46 ± 0.09

[a] ATPase assays were carried out as described in the absence or presence of 0.1% NP-40 at 37 °C or at 37 and 55 °C using membranes prepared from cells stably transfected with the various constructs. Reaction time was 10 min. Values are given as ratios ± SD ($n = 4$). Both ratios decreased for the two chimeras when compared to the WT chicken ecto-ATPDase.

temperature and 0.1% NP-40 on the ATPase activities of the parental enzymes and the two chimeras. It is clear that introduction of either the N- or C-terminus of the human ecto-ATPase into the chicken ecto-ATPDase renders it sensitive to NP-40 inhibition and decreases its activity at high temperature, characteristics displayed by the human ecto-ATPase.

DISCUSSION

The cell surface E-NTPDases have unusual membrane topology in that a large extracellular domain is anchored to the membranes by two transmembranous domains near the N- and C-termini flanked by short intracytoplasmic peptides. Only a few other membrane proteins have a similar membrane anchorage, e.g., the P_2X receptors (*29*) that are ATP-gated ion channels; CD36, a multifunctional scavenger receptor (*30*); and the bacterial mechanosensitive channel MscL (*31*). TMD2 of P_2X receptors have been shown to be required for the formation of a trimer that functions as an ion channel (*32*). In MscL, TM1 of the five subunits form the closed pore (*31*). While there is no evidence to indicate that E-NTPDases form channels, Guidotti and co-workers have shown that TMD interaction of rat ecto-apyrase (E-NTPDase 1 or CD39) is involved in quaternary structure formation (*16*), and more importantly, in activity regulation (*16*, *33*, *34*). Engineered soluble rat ecto-apyrase lacking both TMDs and ecto-apyrases with only the N- or C-terminus TMD have lower activities than the native enzyme and are not inhibited by Triton X-100. It was concluded that heterologous interaction of TMDs between monomers is required for oligomerization of the rat ectoapyrase and its full enzymatic activity (*16*). Studies of ecto-ATPase (E-NTPDase 2) from different species also showed that their activities are affected by their oligomeric structures since lectin or chemically cross-linked enzymes display higher activity (*3*, *23*, *24*, *35*−*37*). While these results suggest that oligomeric E-NTPDase 2 is more active than the monomer, our recent study showed that the effects of cross-linking of human ecto-ATPase arise partially from obviation of inactivation of the enzyme by substrate (*3*).

Aside from preventing inactivation by substrate, protein cross-linking also alleviates inhibition of human ecto-ATPase by NP-40 and maintains its activity at high temperatures (*3*). The human ecto-ATPase and many other E-NTPDases are inhibited by detergents (see ref *1* and also review in *38*). Since detergents preferentially partition in the lipid bilayers, their effects on these E-NTPDases are most likely mediated by their TMD that are embedded in the bilayer. The results obtained by Wang et al. suggested that inactivation of rat ecto-apyrase by Triton X-100 is a consequence of disruption of intermolecular interaction of the heterlogous TMDs of two different monomers (*16*). Reduction of human ecto-ATPase activity at high temperatures (Figure 1B) also suggests that it may be related to increased mobility of TMDs since their lipid environment is expected to be more fluid at higher temperatures (*24*). In contrast, the chicken ecto-ATPDase (E-NTPDase 8) activity increases markedly at high temperatures (Figure 1A), and activity is maintained even in the presence of NP-40 at temperatures up to 40 °C (Figure 2A). It is also stimulated by the membrane fluidization agent, benzoyl alcohol (*39*, *40*), at concentrations that are inhibitory to the human ecto-ATPase (data not shown). The stability of the chicken ecto-ATPDase in the presence of agents that increase membrane fluidity may be unique among all the E-NTPDases since the human homologue of chicken ecto-ATPDase is inhibited by 0.1% NP-40 (*41*). The opposite responses of chicken ecto-ATPDase and human ecto-ATPase to detergents and high temperatures make these enzymes excellent model systems for investigating the roles of TMDs in activity regulation. The logical strategy is to generate chimeras of the two enzymes in which the TMDs are exchanged.

Table 5: Summary of Biochemical Characteristics of Human ecto-ATPase, Chicken ecto-ATPDase, ck-hu ACR5, and ck-hu ACR1 Chimeras

	human ecto-ATPase	chicken ecto-ATPDase	ck-hu ACR5 & ck-hu ACR1 chimeras
NP-40 inactivation at 37 °C	yes	no	yes
NP-40 inactivation at 55 °C	yes	yes	yes
reduced activity at high temp	yes	no	yes
inactivation by substrate	yes	no	at high temps
ConA stimulation	yes	no	no
glutaraldehyde stimulation at 37 °C	yes	no	no (ck-hu ACR5) yes (ck-hu ACR1)
glutaraldehyde stimulation at 55 °C	yes	no	yes
DSS effect at 37 °C	stimulation	inhibition	inhibition
DSS effect at 55 °C	stimulation	inhibition	no effect
molecular species	monomer	monomer and dimer	monomer

A comparison of the properties of the two chimeras with the parental enzymes is summarized in Table 5. While the responses of both chimeras to detergent, high temperature, and glutaraldehyde are similar to that of the human ecto-ATPase, their responses to ConA and DSS are similar to that of the chicken ecto-ATPDase. Figure 8 depicts the general topology of a cell surface E-NTPDase and the sites in the protein affected by NP-40 (Figure 8A) and the cross-linking agents (Figure 8B). These models will be used as the basis for the following discussion on the effects of the different modulators on the two E-NTPDases.

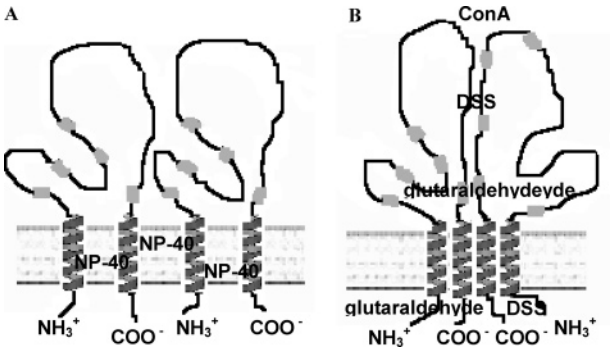

FIGURE 8: Proposed sites of action of NP-40 and cross-linking agents in E-NTPDases with two transmembranous domains. The gray blocks indicate the locations of the five ACR with ACR5 being near the C-terminus and ACR1 near the N-terminus. (A) NP-40 partitions in the membrane and perturbs intra- and intermolecular helical interactions. (B) ConA cross-linking occurs on the protein surface by binding to covalently linked oligosaccharides. Both glutaraldehyde and DSS react with lysine residues. However, cross-linking by glutaraldehyde is usually more extensive than by DSS probably because DSS only reacts with lysine residues separated by a suitable distance that is similar to the length of the spacer.

PFO-gel analysis showed that the human ecto-ATPase exists mostly as monomers in the membrane (*3*). This observation and the fact that human ecto-ATPase is inhibited by NP-40 and high temperature suggests that inter- and intramolecular TMD interaction of human ecto-ATPase is weak. Dissociation of the TMDs perturbs the conformation of ECD which contains the catalytic site and leads to activity inhibition. Cross-linking of the human ecto-ATPase by ConA, DSS, and glutaraldehyde stabilizes the ECD, which becomes resistant to the inactivating effect due to altered TMD interaction. Glutaraldehyde, because of its smaller size, gives rise to greater cross-linking than DSS and is more efficient in preventing inactivation due to NP-40, high temperature, and substrate (*3*).

In contrast to the human ecto-ATPase, TMD interaction in chicken ecto-ATPDase appears to be stronger since the enzyme is not inhibited by NP-40 at 37 °C or by higher temperature. In fact, cross-linking of the enzyme has little effect on the activity or is even inhibitory (Table 2 and Figure 2A). PFO-gel electrophoresis also indicated the presence of dimers and higher oligomers in the membrane (Figure 7A, lane 1). While neither NP-40 nor high-temperature alone has a deleterious effect on the chicken ecto-ATPDase, combination of the two weakens intra- and intermolecular TMD interaction, and a decrease in activity is observed (Figure 1A). However, disruption of these interactions can be prevented and activity is maintained in the presence of NP-40 at 55 °C when the enzyme is cross-linked by glutaraldehyde (Figure 2B).

We hypothesize that the responses of the human ecto-ATPase to NP-40 and high temperature will be manifested by the chicken ecto-ATPDase if the TMDs of the former are grafted onto the ECD of the latter. This hypothesis is supported by results obtained with the ck-hu ACR5 and ck-hu ACR1 chimeras. Incorporation of the C- or N-terminus of the human ecto-ATPase into the chicken ecto-ATPDase molecule apparently results in weakened interaction with the remaining native TMD so that the chicken ecto-ATPDase ECD becomes destabilized and the chimeras have reduced activity at high temperatures (Figure 4) and are inhibited by NP-40 (Tables 3, 4, and Figure 5).

While incorporation of either N- or C-terminus of human ecto-ATPase confers detergent sensitivity to the chicken ecto-ATPDase and abolishes its increase of activity at high temperatures, the interactions of hu-N-terminus/ck-C-terminus (in ck-hu ACR1) and ck-N-terminus/hu-C-terminus (in ck-hu ACR5) are not equivalent. This is seen in the different temperature dependence of the two chimeras (Figure 4). Unlike detergents, the effect of temperature on membrane protein enzyme activity results from its combined effects on membrane fluidity and catalysis (*42*). It is possible that there are some differences in the conformations of ECD of the two chimeras or that the two different sets of heterologous TMD transmit the membrane fluidity effect differently. This issue will need to be resolved in the future with truncated proteins that comprise only the extracellular domain as well as other chimeric constructs containing either naturally occurring TMD or model peptides.

Previous studies on chimeras of rat NTPDase1 and NTPDase2 (*43*) and rat NTPDase1 and human NTPDase2 (*33*) focused on altered substrate preferences. Since all three NTPDases are inhibited by detergents, the chimeras generated are unsuitable for examining the effects of membrane perturbing agents. The unusual property of the chicken ecto-ATPDase provides a unique opportunity to make inquiry into the regulation of enzyme activity by TMDs. We demonstrate for the first time that exchange of part of an E-NTPDase containing a single TMD is sufficient in altering the properties of the recipient enzyme with respect to responses to detergent and high temperature. While one cannot rule out the possibility that the intervening sequences between ACR1 and TMD1 or ACR5 and TMD2 as well as the cytoplasmic domains of the human ecto-ATPase that are also introduced into the chicken ecto-ATPDase contribute to the property of the chimeras, it seems unlikely that these hydrophilic domains are involved in mediating the adverse effects of detergents and membrane fluidizing compounds. An examination of the amino acid sequences of the predicted TMD1 and TMD2 of the human ecto-ATPase (Figure 3) shows that they are more abundant in nonpolar amino acids with bulky side chains, especially leucine, whereas the TMDs of the chicken ecto-ATPDase have a larger number of small and polar amino acids, e.g., glycine, alanine, serine, and threonine, which have been shown to have a higher helix packing values (*44*). Thus, substitution of either of the chicken ecto-ATPDase TMDs by the corresponding TMD of the human ecto-ATPDase is expected to result in a weaker interhelical interaction in the chimeras. The effect of amino acid composition of transmembranous peptides on their propensity to oligomerize has been studied with model peptides of defined amino acid sequences (*45*, *46*). However, "allosteric" regulation of membrane enzyme activity by interaction of TMDs with different sequences has received little attention, even though examples of such regulations are abundant (*47*−*49*). The two E-NTPDases are expected to continue to provide insight into this important issue.

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BI050019K