The Transmembrane Domains of Ectoapyrase (CD39) Affect Its Enzymatic Activity and Quaternary Structure

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Mammalian ectoapyrase (CD39) is an integral membrane protein with two transmembrane domains and a large extracellular region. The enzymatic activity of ectoapyrase is inhibited by most detergents used for membrane protein solubilization. In contrast, the enzymatic activities of soluble E-type ATPases, including potato tuber (Solanum tuberosum) apyrase and parasite ecto-ATPase, are not affected by detergents. Here we show that ectoapyrase is a tetramer and that detergents that reduce the activity of the enzyme promote dissociation of the tetramer to monomers. We expressed a secreted form of the ectoapyrase in COS-7 cells by fusing the signal peptide of murine CD4 with the extracellular domain of the ectoapyrase. The soluble ectoapyrase is catalytically active and its activity is not affected by detergents. Mutants of the ectoapyrase with only the NH2- or the COOH-terminal transmembrane domain are membrane-bound, and their activity is no longer affected by detergents. The enzymatic activity of all of the mutant proteins is less than that of the native enzyme. These results suggest that the proper contacts between the transmembrane domains of the monomers in the tetramer are necessary for full enzymatic activity.

Ectoapyrases, or ATP diphosphohydrolases, hydrolyze extracellular nucleotide tri- and diphosphates in the presence of Ca\(^{2+}\) or Mg\(^{2+}\) (1). The rate of hydrolysis of nucleotide diphosphates is \(50\%\) of that of the triphosphates. Mammalian ectoapyrases play important roles in many biological processes, including the modulation of neural cell activities (for a review, see Ref. 2), prevention of intravascular thrombosis (3, 4), and regulation of immune responses (5). The enzymatic activity of ectoapyrases suggests that their functions might be to regulate purinergic signaling systems (6).

The amino acid sequence of potato apyrase (7) revealed regions of similarity between the potato apyrase and several other proteins: garden pea nucleotide triphosphatase (8), Toxoplasma gondii nucleotide triphosphatase (9), yeast guanosine diphosphatase (10), and the lymphocyte cell activating antigen CD39 (11). We showed that CD39 has ectoapyrase activity (5) and that it is likely that the gene for CD39 is the only ectoapyrase gene in the mammalian genome (12). Subsequently, it was shown that CD39 also has ecto-ADPase activity in endothelial cells and is responsible for inhibition of ADP-induced platelet aggregation (3, 4). The ecto-ATPases in chicken gizzard (13), humans (CD39L1 (14)), and rats (15) are also encoded by genes similar to that for CD39. These latter enzymes hydrolyze nucleoside diphosphates at less than 10\% of the rate of ATP hydrolysis (15).

Mammalian ectoapyrases (CD39) are integral membrane proteins with two transmembrane domains (one at each end of the protein), small cytoplasmic NH2- and COOH-terminal segments, and a large extracellular domain (11) with enzymatic activity (5). This arrangement is unusual for ecto-enzymes, since these are usually attached to the membrane by a single protein or lipid link (16, 17); however, it has been found in a class of channels and pores (for a review, see Ref. 18).

In this paper, we consider whether the transmembrane domains of the ectoapyrase are required for or have an effect on the enzymatic activity of the ectoapyrase. We describe the construction of three mutant cDNAs whose expression produces a secreted soluble protein and membrane proteins lacking either the NH2- or the COOH-terminal transmembrane domains. All of the mutant proteins are enzymatically active, and their activities are not affected by nonionic detergents. However, the specific activities of the mutant proteins are substantially less than that of the native protein. We show that ectoapyrase (CD39) is a noncovalent tetrameric protein and that detergent inhibition of enzymatic activity is caused by dissociation of ectoapyrase tetramers into monomers. We conclude that the interactions between the transmembrane segments of the ectoapyrase monomers in the tetramer regulate the enzymatic activity of the protein.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Nucleotides, bovine thryoglobulin, Escherichia coli β-galactosidase, bovine catalase, bovine heart lactate dehydrogenase, bovine serum albumin, sodium cholate, and potato tuber (Solanum tuberosum) apyrase were purchased from Sigma. Fetal bovine serum, penicillin/streptomycin, Dulbecco’s modified Eagle’s medium, and L-glutamine were purchased from Life Technologies, Inc. Flavobacterium meningosepticum glycopeptidase F, high purity digitonin, and Triton X-100 were purchased from Calbiochem. Murine CD4 cDNA clone was kindly provided by Dr. Leslie J. Berg (Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA).

**DNA Construction**—Rat ectoapyrase (CD39) cDNA had been previously described (12). To construct the hexahistidine-tagged soluble CD39 (sCD39his) cDNA, a DNA fragment encoding the murine CD4 signal peptide and cleavage site sequence (McRAISLRRRLLLLLLLSQQLAVTQGKTLVGLKGEKES (19)) was generated by polymerase chain reaction (PCR),† fused with the extracellular part of rat CD39 cDNA (amino acid residues 29–470). Two more amino acids, NS, were introduced by an EcoRI cloning site between the CD4 signal peptide and CD39 protein. Six histidine residues were added at the COOH terminus for protein purification with HisBind resin (Novagen, Madison, WI). The PCR product was cloned into pcDNA3 mammalian expression

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vector (Invitrogen, San Diego, CA).

To construct the recombinant baculovirus transfer vector, the Max-Bac 2.0 Baculovirus expression vector system from Invitrogen was used. The scDH3his DNA insert was excised from pDNA3 and sub-cloned into pBlueBac4.5 vector downstream of the strong polyhedrin promoter. The recombinant transfer vector was transformed into Spodoptera frugiperda Sf9 cells in a polyvinylidene difluoride membrane (Bio-Rad). The soluble ec-

[14C]FSBA to block labeling. The reactions were stopped by adding 50 mM Na phosphate (pH 8.2), 20 mM EDTA (pH 8.0), 10 mM β-mercapto-

Lysis buffer (10 mM HEPES, pH 7.4, 50 mM sucrose) to prevent inter-

detergent (0.2% digitonin, 0.2% Triton X-100, or 1% cholate), incubated on ice for 30 min, and used immediately for velocity sedimentation.

Velocity Sedimentation—The procedure was performed as described by Lu and Guidotti (27). Marker proteins (50 μg each) were added to detergent solubilized COS-7 crude membrane and then layered onto 10.0 ml of a linear 5–25% (w/v) sucrose density gradient containing detergent (0.2% digitonin, 0.2% Triton X-100, or 1% cholate), 50 mM HEPES-Tris (pH 7.2). The samples were centrifuged for 15 h at 34,000 rpm at 4 °C in a Beckman SW40Ti rotor (Fullerton, CA). In some experiments, the samples were applied to 4.8 ml of a linear 5–20% (w/v) sucrose density gradient and then centrifuged for 15 h at 40,000 rpm at 4 °C in a Beckman SW50.1 rotor. Fractions of 300 μl were collected from the bottom of the tubes. A portion of each fraction (25–50 μl) was used to determine Ca2+-dependent assay activity in 200 μl of reaction buffer containing 1 mM ADP (Tris-buffered, pH 7.4), 4 mM HEPES-Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 0.5 mM NaVO3, and 1 mM EDTA. The Ca2+-stimulated ATPase activity was determined by subtracting values obtained with EGTA alone from those with 1.5 mM CaCl2 plus chelator and by measuring the inorganic phosphate released as described by Mornon et al. (30). The positions of marker proteins were determined by 7.5% SDS-PAGE and Coomassie Blue staining.

The sedimentation coefficients of the detergent-solubilized ectoapryases were determined by comparison with those of the marker proteins, which were thyroglobulin (19.4 S), β-galactosidase (15.9 S), catalase (11.3 S), lactate dehydrogenase (7.3 S), and bovine serum albumin (4.4 S) (29, 30). Sedimentation coefficients were then used to establish the molecular weights of the marker proteins.

Reconstitution of Ectoapryase Monomers into Tetrarmers—Triton X-100-solubilized ectoapryase monomers (100 μl of fraction 13 in Fig. 6B) were layered on 4.8 ml of a linear 5–20% (w/v) sucrose density gradient containing 0.2% digitonin and 50 mM HEPES-Tris (pH 7.2) and centrifuged for 15 h at 40,000 rpm at 4 °C in a Beckman SW50.1 rotor. Fractions of 300 μl were collected from the bottom of the tubes. The Ca2+-stimulated ATPase activity was determined as described above. The total Ca2+-stimulated ATPase activity of tetrameric ectoapryase was integrated and compared with that of the monomeric ectoapryase added to the gradient tube.

Glutaraldehyde Cross-linking Experiments—Glutaraldehyde cross-linking was performed as described by Liu and Guidotti (31) with some modification. Glutaraldehyde (0–8 mM final concentration) (Poly-

Science, Warrington, PA) was incubated with crude membrane (25 μl), soluble ectoapryase (3 μg), or fractions from the sedimentation experiments (50 μl) in phosphate-buffered saline (final volume, 100 μl). Following a 2–10 min incubation at room temperature, the reactions were quenched by the addition of 10 μl of 0.6 M glycine (pH 9.0). The cross-linked recombinant products were separated by 5.5% nonreducing SDS-PAGE and then analyzed by immunoblotting with anti-CD39 antibody.

Ectoapryase Assay—To measure ectoapryase activity, COS-7 cells (105 cells) or crude membranes (12 μg) were resuspended in 180 μl of buffer A (20 mM HEPES-Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, and 1 mM EDTA) with or without 1% detergent (Triton X-100 or digitonin) for 1 h at 4 °C and then incubated for 5 min at 37 °C. The nucleoside
phosphatase reactions were initiated by the addition of 20 μl of the same buffer containing 10 mM ATP. The divalent cation stimulated apyrase activity was determined by measuring the inorganic phosphate released (28) and by subtracting values obtained with EGTA alone from those with 1.5 mM CaCl₂ plus chelator (5).

RESULTS

A characteristic feature of vertebrate ectoapyrases is the sensitivity of their enzymatic activities to most detergents. In contrast, the enzymatic activities of soluble E-type ATPases, including potato tuber (S. tuberosum) apyrase² and parasite ecto-ATPases (9, 32), are not affected by detergents. Since the principal difference between the structures of the mammalian ectoapyrases (detergent-sensitive) and the soluble apyrases (detergent-resistant) is the presence of transmembrane segments in the former proteins, we considered that the interaction of the detergents with the transmembrane domains was central for detergent sensitivity. One possible effect of detergents is the dissociation of protein oligomers. The proposal that the oligomeric state of the ecto-ATPase affects its enzymatic activity was made previously (33, 34). To answer the question about the relationship of the transmembrane domains to the oligomeric state of the protein and its sensitivity to detergents, we made variants of the ectoapyrase with one or no transmembrane domains.

Mutant Forms of the Ectoapyrase—The isolation of a cDNA for rat brain ectoapyrase and its expression in COS-7 cells has been described in a previous paper (12).

We constructed a soluble ectoapyrase, sCD39his (Fig. 1), by fusion of the murine CD4 signal peptide and cleavage sequence (MCRAISLRRLLLLLQLSDDLAVTQGKTLVLGKGEGES) (19) with the extracellular part of rat ectoapyrase (amino acid residues 39–470) (12). The cleavage site of the CD4 signal peptide is between glycine and lysine (underlined above). S. frugiperda Sf9 cells were infected with recombinant sCD39his cDNA baculovirus, and the culture medium was collected after 72–90 h. The soluble ectoapyrase was purified by elution from columns of concanavalin A-Sepharose 4B and HisBind resin (see "Experimental Procedures"). The purified protein had a Ca²⁺-dependent ATPase activity of 68 μmol of P/i/min/mg of protein. [¹⁴C]FSBA, a competitive inhibitor of ectoapyrase (23), labeled a protein with M₉ ~56,000 (Fig. 2A). Anti-CD39 antibody also recognized a protein with the same molecular weight by immunoblotting (Fig. 2B). The NH₂-terminal peptide sequence of this 56-kDa protein is XTLVL. The first amino acid residue (X) was not identified, but the next four amino acid residues are consistent with the sequence expected for cleavage of the CD4 signal peptide between Gly and Lys. Moreover, the amino acid sequence of four tryptic peptides also revealed that this 56-kDa protein has the same amino acid sequences as the wild type ectoapyrase protein (data not shown). Together, these results indicate that the soluble ectoapyrase is secreted and catalytically active. Finally, Coomassie Blue staining of a gel after SDS-PAGE indicated that the 56-kDa protein was the major component (>50% of the total protein) of the purified product (Fig. 2C). Accordingly, the apyrase activity of this 60-kDa protein was estimated to be ~100–200 μmol of P/i/min/mg of protein.

The enzymatic properties of the soluble ectoapyrase were generally similar to those of the wild type enzyme. They both hydrolyze nucleotide di- and triphosphates but not nucleotide monophosphates. The relative hydrolysis rates of the soluble ectoapyrase for various nucleotide phosphates were 100 ± 2% (ATP), 57 ± 4% (ADP), 85 ± 5% (dATP), and 0 ± 1% (AMP), similar to those of the ectoapyrase in Epstein-Barr virus-transformed LGL2 cells (5) (Table I). The soluble ectoapyrase had a Kₘ of 220 μM for ATP (at 0.5 mM Ca²⁺) compared with a Kₘ of 75 μM for the wild type enzyme. The enzymatic activity of soluble ectoapyrase was dependent on either Ca²⁺ or Mg²⁺ and was insensitive to vanadate (inhibitor of P-type ATPases) or azide (inhibitor of F-type ATPases). We conclude that neither transmembrane domains nor cytoplasmic domains are required for apyrase activity.

We also constructed cDNAs of ectoapyrase lacking one trans-

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² M. Handa, personal communication.
membrane segment. The COOH-terminal deletion mutant (CD39CT-Myc, Fig. 1), lacking the second transmembrane domain and the COOH-terminal cytoplasmic sequence, was generated by substituting the last 42 amino acid residues of the ectoapyrase with a Myc epitope (EQKLISEEDL; see “Experimental Procedures”). Accordingly, CD39CT-Myc should be a type II transmembrane protein (nomenclature of von Heijne (35)) with the NH2 terminus in the cytoplasm and the COOH terminus located extracellularly. This prediction is consistent with the results of immunofluorescence staining. Both intact (Fig. 3A) and detergent-permeabilized (Fig. 3B) COS-7 cells transfected with CD39CT-Myc cDNA stained with anti-Myc monoclonal antibody (9E10), suggesting that the COOH terminus is located extracellularly. In contrast, when CD39WT-Myc cDNA was transfected into COS-7 cells, the anti-Myc monoclonal antibody only stained detergent-permeabilized cells (Fig. 3D) but not intact cells (Fig. 3C), indicating that the COOH terminus of wild type ectoapyrase is indeed located on the cytoplasmic side of the plasma membrane. These results show that CD39CT-Myc has lost the second transmembrane domain present in CD39WT-Myc. The enzymatic activities, measured with intact cells, of the wild type and the COOH terminus-truncated ectoapyrases were 157 ± 15 and 66 ± 3 nmol of Pi/min/mg, respectively. The COOH terminus-truncated ectoapyrase has a $K_m$ for ATP (at 0.5 mM Ca$^{2+}$) of 300 $\mu$M. Either the COOH terminus-deletion mutant (CD39NT-Myc, Fig. 1), lacking the NH2-terminal cytoplasmic sequence and transmembrane domain, was constructed by replacing the first 38 amino acid residues with the CD4 signal peptide and cleavage site sequences, and by fusing the Myc epitope peptide right after the COOH-terminal cytoplasmic sequence (see “Experimental Procedures”). The use of the COOH signal peptide and cleavage sequence generates a type I integral membrane protein, with the NH2 terminus located extracellularly and the COOH terminus in the cytoplasm. Expression of CD39NT-Myc cDNA in COS-7 cells resulted in the appearance of ectoapyrase at the plasma membrane with an activity of 34 ± 2 nmol of Pi/min/mg, corresponding to about 21.5% of that obtained by expression of the wild type ectoapyrase cDNA. Since intact COS-7 cells transfected with CD39CT-Myc and CD39NT-Myc cDNAs have calcium-stimulated ecto-ATPase activity, we conclude that the products of these two cDNAs are targeted to the plasma membrane.

Like the wild type ectoapyrase, the NH2- or COOH terminus deletion mutants were also highly glycosylated; more than one glycosylated product was found when the wild type (Fig. 4, lane 1) and the mutant proteins (Fig. 4, lanes 2–3) were expressed in COS-7 cells, and a single deglycosylated polypeptide appeared after extensive deglycosylation with F. meningosepticum glycopeptidase F (see “Experimental Procedures”). Proteins were then separated on a reducing SDS gel and transferred to nitrocellulose membranes. The membranes were probed with anti-CD39 antibody. Positions of molecular size markers are shown in kDa. These results suggest that the presence of two transmembrane domains results in the appearance of two proteins in COS-7 cells.

The conclusion is that the mutant forms of ectoapyrase are enzymatically active and targeted to the proper location.

Effect of Detergents on the Enzymatic Activity of the Wild Type and Mutant Ectoapyrases—The effect of detergents on the various constructs is shown in Table II. As expected, one group of detergents, including Triton X-100, C12E9, dodecyl maltoside, and CHAPS, brings about a loss of 88% of the enzymatic activity of the wild type enzyme, whereas digitonin causes only a small decrease in activity (10%). On the other hand, Triton X-100 had a negligible effect on the enzymatic activity of the soluble ectoapyrase, and it caused an actual increase in the activity of the COOH- and NH2-terminal deletion mutants. The effect of Triton X-100 on these mutants is similar to that with potato apyrase; we believe that the increase in activity is due to the release of activity trapped in closed membrane vesicles.

These results suggest that the presence of two transmem-
TABLE II

| Soluble CD39, potato apyrase, and the crude membranes of COS-7 cells transfected with wild type (WT) CD39, CD39NT-Myc, or CD39CT-Myc cDNAs were treated with or without 1% detergents at 4 °C for 1 h and then assayed for calcium-dependent ATPase activity in the presence of 1 mM Na$_2$H$_2$PO$_4$, 0.5 mM Na$_2$VO$_4$, and 1 mM ATP. The calcium-dependent ATPase activities of fractions treated with detergents are presented as the percentage of the activities of control samples. Values are means ± S.D. of three independent experiments.

| Soluble CD39 | Triton X-100 | Digitonin |
|--------------|--------------|-----------|
| CD39 (WT) crude membrane | 100 ± 1 | 12 ± 1 | 90 ± 2 |
| Soluble CD39 | 100 ± 2 | 92 ± 3 | 95 ± 2 |
| Potato apyrase | 100 ± 1 | 155 ± 4 | ND$^a$ |
| CD39CT-Myc crude membrane | 100 ± 2 | 145 ± 1 | ND |
| CD39NT-Myc crude membrane | 100 ± 1 | 144 ± 2 | ND |

$^a$ Similar results were obtained with C12E9, dodecyl maltoside, CHAPS, and octyl glucoside.

$^b$ ND, not determined.

brane domains is necessary for the inhibitory effect of some detergents. We inquired whether detergents have an effect on the structure of the ectoapyrase.

Quaternary Structure of Wild Type and Mutant Ectoapyrases—The quaternary structure of the ectoapyrases expressed in COS-7 cells was determined by glutaraldehyde cross-linking and sucrose density gradient centrifugation.

Glutaraldehyde cross-linking experiments on crude membranes with wild type ectoapyrase resulted in a ladder of bands with $M_r$ of $\sim 150,000$, $\sim 230,000$, and $\sim 300,000$, corresponding to dimers, trimers, and tetramers of the $M_r 70,000$ polypeptide (Fig. 5A). These results, suggesting that wild type ectoapyrase is a tetramer in cell membranes, were supported by the sedimentation behavior of the enzyme solubilized with detergents. However, the size of the ectoapyrase depended on the type of detergent. As can be seen in Fig. 6, ectoapyrase solubilized with digitonin had a sedimentation coefficient of 9.8 S, whereas in Triton X-100 the protein has a sedimentation coefficient of 4 S. It is important to point out that digitonin had little effect on the activity of wild type ectoapyrase, while in Triton X-100 88% of the enzymatic activity was lost (Table II). Globular, hydrophilic proteins with these sedimentation coefficients would be expected to have $M_r$ values of 200,000 and 50,000, respectively. For membrane proteins solubilized by detergents, however, work from this laboratory (27, 30, 36, 37) has shown that protein-detergent complexes have smaller sedimentation coefficients than do water-soluble proteins. On the basis of the results obtained with other membrane proteins (30, 37), we estimate that the protein components of the 9.8 and the 4 S peaks have $M_r$ values of $\sim 330,000$ and $\sim 75,000$, respectively. Furthermore, the sedimentation coefficient of the ectoapyrase solubilized with cholate (Fig. 6B) is 6.7 S, corresponding to a dimer of the ectoapyrase with $M_r$ of $\sim 160,000$. The relative values of 9.8, 6.7, and 4 S for the three species of ectoapyrase are the predicted ones for proteins with relative sizes of 4:2:1 (38). The straightforward conclusion is that in digitonin the ectoapyrase is tetrameric, while in Triton X-100 it is monomeric. This conclusion is supported by cross-linking done on the gradient fraction and shown in Fig. 5, B and C. Glutaraldehyde cross-linking of the digitonin-solubilized ectoapyrase tetramers (fraction 20 in Fig. 6A) produced the ladder of bands indicative of monomers, dimers, trimers, and tetrasons after a 2-min exposure to 8 mM glutaraldehyde (Fig. 5C); whereas with Triton X-100-solubilized monomeric protein (fraction 27 in Fig. 6A), no cross-linked product was visible after a 10-min exposure to 8 mM glutaraldehyde (Fig. 5B). We conclude that ectoapyrase is a tetrameric protein, and detergents that inhibit ectoapyrase activity (e.g. Triton X-100) dissociate the ectoapyrase tetramer into monomers.

Since the native ectoapyrase is a tetramer, we inquired whether oligomerization is a result of interactions between the extracellular domain or the transmembrane domains. Accordingly, we determined the size of the soluble ectoapyrase that lacks the transmembrane and cytoplasmic domains. Soluble ectoapyrase sedimented more slowly than bovine serum albumin on a sucrose density gradient without detergents (Fig. 7A), and there were no cross-linked products found after treatment with glutaraldehyde (Fig. 7B). These results indicate that soluble ectoapyrase is a monomer. Accordingly, the extracellular domain of wild type ectoapyrase is not likely to be involved in ectoapyrase oligomerization.

Next, we investigated the role of the two transmembrane domains of the ectoapyrase in oligomerization. Fig. 8 presents the results of sucrose density gradient sedimentation of CD39CT-Myc and CD39NT-Myc mutant ectoapyrases. Like wild type ectoapyrase, CD39CT-Myc and CD39NT-Myc behaved as monomeric proteins after solubilization with Triton X-100 (Fig. 8, A and B). Interestingly, most of CD39CT-Myc...
and a fraction of CD39NT-Myc were tetrameric after solubilization with digitonin, indicating that either one of the two transmembrane domains of ectoapyrase is sufficient for ectoapyrase oligomerization. The presence of monomeric mutant ectoapyrases lacking one or both transmembrane domains are similar to that of the wild type tetramers or wild type monomers. We conclude that the transmembrane domains of ectoapyrase are responsible for the formation of ectoapyrase tetramers.

The Ectoapyrase Tetramer Is Stabilized by Noncovalent Bonds—It has been reported that chicken gizzard muscle ecto-ATPase (34) and rat lung ectoapyrase (39) are present as homotrimers and homodimers, respectively, stabilized by intermolecular disulfide bonds. To examine this possibility, detergent-solubilized ectoapyrase was analyzed by either reducing or nonreducing SDS-PAGE, followed by immunoblotting with anti-CD39 antibody. The molecular weights of detergent-solubilized mutant ectoapyrases were estimated by their sedimentation coefficients based upon the results of Liu and Guidotti (31).

FIG. 7. Soluble ectoapyrase is a monomeric protein. A, sucrose density gradient sedimentation of soluble ectoapyrase. The purified protein (20 μg) was sedimented on 5–25% (w/v) sucrose density gradient as described under “Experimental Procedures.” Fractions were collected and assayed for Ca²⁺-dependent ADPase activity in the presence of 1 mM NaN₃ and 0.5 mM Na₃VO₄. The positions of marker proteins were determined by SDS-PAGE. B, glutaraldehyde cross-linking of soluble ectoapyrase. 3 μg of soluble ectoapyrase were incubated with 0–8 mM glutaraldehyde for 10 min. The samples were separated by 7.5% nonreducing SDS-PAGE, and soluble ectoapyrase was detected by immunoblotting with anti-CD39 antibody. Positions of molecular size markers are shown in kDa.

FIG. 8. Sucrose density gradient sedimentation of detergent solubilized mutant ectoapyrase proteins. Crude membranes from COS-7 cells transfected with CD39CT-Myc (A) and CD38NT-Myc (B) cDNA were solubilized with 1% digitonin or 1% Triton X-100 for 15 min on ice and then separated on 5–25% (w/v) sucrose density gradients as described under “Experimental Procedures.” Each fraction was assayed for Ca²⁺-dependent ADPase activity in the presence of 1 mM NaN₃ and 0.5 mM Na₃VO₄. The positions of marker proteins were determined by SDS-PAGE. The molecular weights of detergent-solubilized mutant ectoapyrases were estimated by their sedimentation coefficients based upon the results of Liu and Guidotti (31).

FIG. 9. The enzymatic activity of wild type ectoapyrase tetramer is greater than those of wild type monomer, soluble ectoapyrase, CD39CT-Myc tetramer, and CD38NT-Myc tetramer. To compare the relative enzymatic activities of soluble ectoapyrase (lanes 1–4), CD39CT-Myc tetrarers (lanes 5–6; fraction 20 in Fig. 8A), CD38NT-Myc tetrarers (lanes 7 and 8; fraction 20 in Fig. 8B) ectoapyrase tetrarers (lanes 9 and 10; fraction 6 in Fig. 6B) and ectoapyrase monomers (lanes 11 and 12; fraction 12 in Fig. 6B), the amounts of protein in samples with a given Ca²⁺-dependent ADPase activity were determined by SDS-PAGE, followed by immunoblotting with anti-CD39 antibody. Anti-CD39 antibody only recognizes the extracellular domain of wild type CD39 (12). Positions of marker proteins are shown in kDa. These experiments were repeated three times with two independent sucrose density gradient fractions, and the results were similar.
Ectoapyrase (CD39) Tetramers

![Graph](image)

**Fig. 10.** Reconstitution of ectoapyrase tetramers from monomers by sucrose density gradient sedimentation. Triton X-100-solubilized ectoapyrase monomers (100 μl of fraction 13 in Fig. 6B) were sedimented on a 5–20% (w/v) sucrose density gradient containing 0.2% digitonin as described under "Experimental Procedures." Fractions were collected and assayed for Ca$^{2+}$-dependent ATPase activity in the presence of 1 mM Na$_3$N$_3$ and 0.5 mM Na$_3$VO$_4$.

The position of marker proteins was determined by reducing SDS-PAGE. The total Ca$^{2+}$-dependent ATPase activity of the reconstituted ectoapyrase tetramers was integrated (10–12 absorbance units) and compared with that of the ectoapyrase monomers applied to the gradient (2 absorbance units).

of P/min/mg) is lower than that of the digitonin-solubilized rat lung ectoapyrase (~1000 μmol of P/min/mg of protein) (39).

Next, we compared the catalytic activities of the tetramers of mutants with single transmembrane strands with that of wild type ectoapyrase tetramers. As shown in Fig. 9, greater amounts of CD39CT-Myc (lanes 5 and 6) and CD39NT-Myc (lanes 7 and 8) tetramers than wild type CD39 tetramers (lanes 9 and 10) were required to achieve the same Ca$^{2+}$-stimulated ADPase activity. These results indicate that wild type tetramers are more active than mutant tetramers. We suggest that the heterologous interactions between both transmembrane domains are more important than homologous interactions for enzymatic activity. This hypothesis is supported by the fact that both CD39CT-Myc and CD39NT-Myc are detergent-resistant, as indicated in Table II.

We conclude that interactions between the NH$_2$- and COOH-terminal transmembrane domains of the four monomers are required for stabilization of the tetramers in a conformation with an increased enzymatic activity compared with that of the monomers. Without these interactions, the conformation of the tetramer is altered so that the enzymatic activities of soluble CD39, CD39NT-Myc, and CD39CT-Myc are smaller than that of the wild type tetramer and similar to that of the wild type monomer. Accordingly, the enzymatic activities of the mutants are not affected by detergent solubilization.

**DISCUSSION**

This paper shows that ectoapyrase is a tetrameric membrane protein, that the tetramers have greater enzymatic activity than the monomers, and that the transmembrane domains of the protein are responsible for tetramerization.

In this study, we measured the size of ectoapyrase and found that native ectoapyrase is a homotetramer. First, expressed ectoapyrase is an oligomer (at least a tetramer) in cell membranes by glutaraldehyde cross-linking. Second, sucrose density gradient sedimentation profiles of both the expressed ectoapyrase in COS-7 cells and the native ectoaprases from LG2 cells (data not shown) are identical: ectoapyrase behaved as a tetramer after solubilization with digitonin, a detergent that has little effect on the enzymatic activity, and became monomeric after solubilization with Triton X-100, a detergent that inhibits the activity. It has been suggested that detergents may inactivate ectoapyrase by destabilizing an oligomeric state of the protein (33, 34), and it has been reported that chicken gizzard muscle ecto-ATPase (34) and rat lung ectoapyrase (39) are disulfide-linked homotrimers and homodimers, respectively. However, this work is the first demonstration that the ectoapyrase is a noncovalent tetramer.

Since oligomer formation does not involve the formation of intersubunit disulfide bonds, the interactions that promote oligomerization are either between the extracellular domains or the transmembrane and intracellular domains of the monomers. Since the soluble ectoapyrase is a monomer, the transmembrane domains rather than the extracellular domain of the ectoapyrase are likely to be involved in oligomerization. The observation that mutant ectoaprases with only one transmembrane domain (CD39CT-Myc and CD39NT-Myc) are still tetramers indicates that one transmembrane domain is sufficient for ectoapyrase oligomerization.

Interestingly, two other members of the E-ATPase protein family (1), human ecto-ATPase (CD39L1 (14)) and human Golgi luminal UDPase (40), are also tetrameric proteins. The conservation of quaternary structure in these enzymes suggests that oligomerization may have an important function. We propose that ectoapyrase tetramers have higher enzymatic activity than the monomers by virtue of interactions between the transmembrane domains. This supposition is consistent with the fact that monomeric ectoaprases, including Triton X-100-solubilized wild type ectoapyrase and mutant soluble ectoapyrase, have lower catalytic activity than ectoapyrase tetramers. Accordingly, detergent inhibition of the enzymatic activity is caused by the dissociation of ectoapyrase tetramers into monomers. Interestingly, other E-type ATPases that are not membrane-bound, such as potato apyrase (Table II), T. gondii NTPases (9), and Tetrahymena ecto-ATPase (32), are also not sensitive to detergents. This is a further indication that the presence of transmembrane segments is the key element in the effect of detergents on the activity of the enzyme. Furthermore, CD39CT-Myc and CD39NT-Myc tetramers are less active than wild type ectoapyrase tetramers, and their activities are not affected by detergents. We suggest that heterologous interactions between the transmembrane domains of the monomer in the tetramer are important for enzymatic activity. Without these interactions, the activities of mutant ectoaprases (CD39CT-Myc, CD39NT-Myc, and soluble CD39) are decreased and are no longer affected by detergents. Thus, the effect of detergents is to disrupt the interactions between the NH$_2$- and COOH-terminal transmembrane domains.

The membrane topology of ectoapyrase and ecto-ATPase is unusual for an ecto-enzyme, since these are usually attached to the membrane by a single transmembrane domain or lipid link (16, 17); however, it has been found in a class of channel proteins (for a review, see Ref. 18), including P2X purinergic receptors (41), inwardly rectifying K$^+$ channels (42), an epithelial sodium channel (ENaC) (43), and the mscL mechanosensitive channel of *Escherichia coli* (44). Interestingly, P2X receptors (45), inwardly rectifying K$^+$ channels (42), and the epithelial sodium channel (46) are also tetrameric proteins. We

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wonder whether the subunit interactions in these channels are similar to those in the ectoapyrase tetramer. Conversely, it is possible that the ectoapyrase also has channel or pore activity.

The principal conclusion of this work is that the transmembrane domains of the ectoapyrase mediate ectoapyrase oligomerization and affect enzymatic activity through heterologous interactions. Detergent inhibition of ectoapyrase activity is due to the dissociation of tetramers into monomers. Ectoapyrase mutants lacking one or both transmembrane domains have lower enzymatic activity than the wild type enzyme, and the activity is not affected by detergents.

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