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

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Ting-Fang Wang‡, Yvonne Ou§, and Guido Guidotti¶

From the Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

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 NH_2- 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 NH_2- 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 NH_2- 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 thyroglobulin, *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 meningosepticium* 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 (MCRAISLRRLLLLLLQLSQLLAVTQGKTLVLGKEGES (19)) was generated by polymerase chain reaction (PCR),[1] fused with the extracellular part of rat CD39 cDNA (amino acid residues 39–470). Two more amino acids, NS, were introduced by an *Eco*RI 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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¶ To whom correspondence should be addressed. Tel.: 617-495-2301; Fax: 617-495-8308; E-mail: guidotti@fas.harvard.edu.

[1] The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; [^{14}C]FSBA, 5′-(*p*-fluorosulfonyl)benzoyl-[8-^{14}C]adenosine.

vector (Invitrogen, San Diego, CA).

To construct the recombinant baculovirus transfer vector, the MaxBac 2.0 Baculovirus expression vector system from Invitrogen was used. The sCD39his DNA insert was excised from pcDNA3 and subcloned into pBlueBac4.5 vector downstream of the strong polyhedrin promoter. The recombinant transfer vector was cotransfected with a baculovirus that had deletions in essential parts of the virus genome (supplied by Invitrogen). Positive recombinant baculovirus with both blue color and occlusion body negative phenotype were selected by a standard plaque assay. The DNA of the recombinant virus was isolated and analyzed by PCR. Recombinant virus stock was amplified by culturing infected cells several times, and a high titer virus solution was harvested.

The COOH-terminal deletion mutant (CD39CT) was generated by PCR amplification of the rat brain ectoapyrase cDNA (amino acid residues 1–470). The NH_2-terminal deletion mutant (CD39NT), which lacks the NH_2-terminal cytoplasmic sequence and first transmembrane domain, was constructed by connecting the cDNA for the murine CD4 signal peptide and cleavage sequence with the cDNA for the ectoapyrase lacking the first 38 amino acids (amino acid residues 39–512) (12). Myc-tagged proteins (CD39WT-Myc, CD39CT-Myc, and CD39NT-Myc) were constructed by PCR with an oligonucleotide cassette encoding the Myc epitope (EQKLISEEDL) fused to the COOH terminus of wild type CD39 (CD39WT), CD39CT, and CD39NT. The resulting PCR products were subcloned into the pCI-neo expression vector (Promega, Madison, WI) for expression in COS-7 cells.

Purification of Soluble CD39 Protein—Spodoptera frugiperda Sf9 cells were grown in a serum-free insect medium (EX-CELL 401 from JRH Biosciences, Lenexa, KS) and infected with recombinant baculovirus at a multiplicity of infection of 3. Culture medium was collected between 72 and 90 h after infection, cells were pelleted by centrifugation at 1000 × *g* for 5 min, and the supernatant was applied to a 3-ml concanavalin A-Sepharose 4B column (Sigma) at a flow rate of 1 ml/min. The column was washed with 30 ml of buffer A (25 mM Tris-buffered saline, pH 7.0, and 50 μM $CaCl_2$). Ca^{2+} ion was added to maintain apyrase activity. The concanavalin A-binding glycoproteins were then eluted with 30 ml of 1 M α-methylmannoside (Sigma) in buffer A. The eluate was applied to a 1.5-ml HisBind resin column, washed with 15 ml of buffer A and then with 1.5 ml of buffer A containing 1 mM imidazole to remove nonspecific binding proteins. The hexahistidine-tagged sCD39his protein was eluted by 6 ml of elution buffer (25 mM Tris-buffered saline, 50 μM $CaCl_2$, and 0.5 M imidazole) and then dialyzed overnight against 40 mM Tris-HCl, pH 7.5, containing 50 μM $CaCl_2$. About 1 mg of protein, determined by Peterson's method (20), was isolated from 600 ml of insect cell culture. The purified protein preparation was analyzed by 7.5% reducing SDS-PAGE and blotted to a polyvinylidene difluoride membrane (Bio-Rad). The soluble ectoapyrase was visualized by Ponceau S staining (21) and then excised for trypsin digestion and NH_2-terminal peptide sequence determination. NH_2-terminal peptide sequencing was done by William S. Lane (Harvard University Microsequencing Facility, Harvard University, Cambridge, MA).

Labeling of Soluble Ectoapyrase Protein with 5′-(p-Fluorosulfonyl)-benzoyl-[8-^{14}C]Adenosine ([^{14}C]FSBA)—The purified soluble sCD39his protein (10 μg) was incubated at 37 °C for 1 h in 100 μl of 0.4 mM [^{14}C]FSBA (specific activity, 50 μCi/μmol), 40 mM Tris-HCl (pH 7.5) and 50 μM $CaCl_2$. FSBA is an ATP analogue (22) and an inhibitor of ectoapyrase (23). ATP (final concentration 4 mM) was added with [^{14}C]FSBA to block labeling. The reactions were stopped by adding 50 μl of reducing sample buffer (2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 65 mM Tris/HCl, pH 6.8). The samples were boiled, analyzed on 7.5% SDS-PAGE, and examined by autoradiography.

Antibodies and Immunoblotting—Rabbit anti-rat ectoapyrase (CD39) antiserum used in immunoblotting has been previously described (12). Anti-Myc monoclonal antibody (9E10) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Deglycosylation of Ectoapyrases—F. meningosepticium glycopeptidase F was used to deglycosylate asparagine-linked glycans (24). COS-7 cells transfected with wild type or mutant recombinant CD39 cDNAs were harvested and lysed with radioimmune precipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS). After incubation at 4 °C for 1 h, the insoluble fraction of the cell lysate was removed by centrifugation at 10,000 × *g* for 15 min. The COS-7 cell lysates were boiled for 10 min in 100 μl of a solution with 10 mM Na phosphate (pH 8.2), 20 mM EDTA (pH 8.0), 10 mM β-mercaptoethanol, and 0.1% SDS. The denatured protein mixtures were then incubated with or without *F. meningosepticium* glycopeptidase F (0.5 unit) for 20 h.

Immunofluorescence Staining—COS-7 cells grown on polylysine-coated culture chamber slides (Nunc Inc., Naperville, IL) were transfected at ~40–60% confluence with CD39CT-Myc or CD39WT-Myc cDNA, using the LipofectAMINE method. The immunofluorescence staining was done by modifying the method described by Yoon and Guidotti (25). For staining permeabilized cells, cells were fixed 2 days after transfection with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with 9E10 anti-Myc monoclonal antibody at 1 μg/ml concentration and with fluorescein isothiocyanate-labeled secondary antibodies (Jackson Immunoresearch Laboratory, West Grove, PA) at a dilution of 1:1000. Immunofluorescence was viewed with a Zeiss Axioskop microscope. For staining nonpermeabilized cells, the first antibody was incubated directly without fixation of cells, and fixation was done after incubation with the secondary antibody. All of the incubation and washing steps were done in the absence of detergents.

Preparation of COS-7 Crude Membranes and Membrane Protein Extraction—Crude membranes of the transfected COS-7 cells were prepared by the method of Coppi and Guidotti (26), except that *N*-ethylmaleimide was added to a final concentration of 2 mM to the hypotonic lysis buffer (10 mM HEPES, pH 7.4, 50 mM sucrose) to prevent intermolecular disulfide bond formation during the preparations. Crude membranes from four 100-mm plates of transfected COS-7 cells were resuspended in 0.8 ml of buffer (10 mM HEPES-Tris, pH 7.2, 5 mM EGTA, 10 μg/ml aprotinin). 100-μl crude membranes were solubilized with detergent (1% final concentration of digitonin or Triton X-100 or 2% final concentration of 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 Ca^{2+}-dependent apyrase activity in 200 μl of reaction buffer containing 1 mM ADP (Tris-buffered, pH 7.4), 20 mM HEPES-Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM NaN_3, 0.5 mM Na_3VO_4, and 1 mM EGTA. The Ca^{2+}-stimulated ADPase activity was determined by subtracting values obtained with EGTA alone from those with 1.5 mM $CaCl_2$ plus chelator and by measuring the inorganic phosphate released as described by Ames (28). The positions of protein markers were determined by 7.5% reducing SDS-PAGE and Coomassie Blue staining. The sedimentation coefficients of the detergent-solubilized ectoapyrases 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 estimate molecular weight based on the results of Liu and Guidotti (31).

Reconstitution of Ectoapyrase Monomers into Tetramers—Triton X-100-solubilized ectoapyrase monomers (100 μl of fraction 13 in Fig. 6*B*) were layered onto 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 Ca^{2+}-stimulated ATPase activity was determined as described above. The total Ca^{2+}-stimulated ATPase activity of tetrameric ectoapyrase was integrated and compared with that of the monomeric ectoapyrase 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) (Polyscience, Warrington, PA) was incubated with crude membrane (25 μl), soluble ectoapyrase (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 ectoapyrase products were separated by 5.5% nonreducing SDS-PAGE and then analyzed by immunoblotting with anti-CD39 antibody.

Ectoapyrase Assay—To measure ectoapyrase activity, COS-7 cells (10^5 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 EGTA) 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

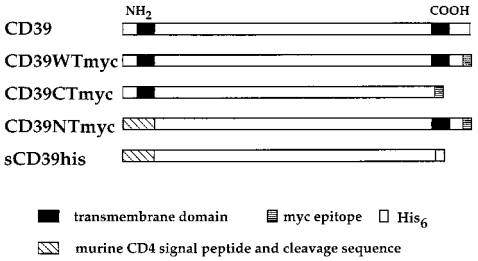

FIG. 1. **Construction of the soluble and mutant ectoapyrase proteins.** Schematic representation of wild type CD39, the Myc-tagged CD39 (CD39WT-Myc), the NH_2-terminal deletion mutant (CD39NT-Myc), and the COOH-terminal deletion mutant (CD39CT-Myc). The transmembrane domains of CD39, the CD4 signal peptide and cleavage sequence, the Myc epitope, and the hexahistidine peptide (His_6) are indicated.

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 $CaCl_2$ 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*) apyrase[2] 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 (MCRAISLRRLLLLLLQLSQLLAVTQGKTLVLGKEGES) (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 Ca^{2+}-dependent ATPase activity of 68 μmol of P_i/min/mg of protein. [^{14}C]FSBA, a competitive inhibitor of ectoapyrase (23), labeled a protein with M_r ~56,000 (Fig. 2*A*). Anti-CD39 antibody also recognized a protein with the same molecular weight by immunoblotting (Fig. 2*B*). The NH_2-terminal peptide sequence of this 56-kDa protein is *X*TLVL. 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. 2*C*). Accordingly, the apyrase activity of this 60-kDa protein was estimated to be ~100–200 μmol of P_i/min/mg of protein.

[2] M. Handa, personal communication.

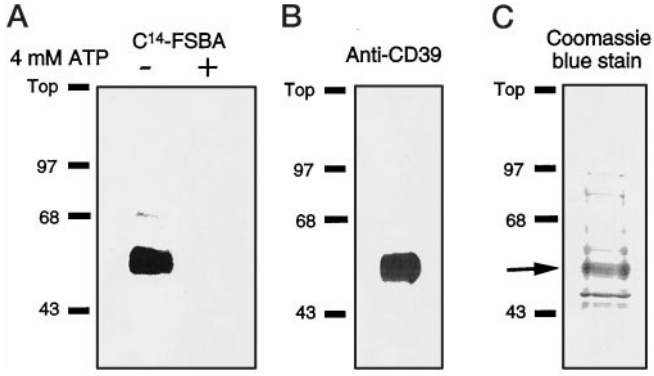

FIG. 2. **Expression of soluble ectoapyrase (sCD39his) in insect cells.** *A*, [^{14}C]FSBA cross-linking of soluble ectoapyrase. Soluble ectoapyrase was purified as described under "Experimental Procedures"; 10 μg of purified protein were incubated with 0.4 mM [^{14}C]FSBA in the presence or absence of 4 mM ATP at 37 °C for 1 h. The samples were analyzed by reducing 7.5% SDS-PAGE. The radioactive image was produced by a BSA 2000 Bio-Imaging Analyzer. *B*, immunoblot of soluble ectoapyrase. 3 μg of purified protein were separated by reducing 7.5% SDS-PAGE, transferred onto nitrocellulose, and probed with anti-CD39 antibody. Immunoreactivity was visualized with a horseradish peroxidase-conjugated goat anti-rabbit antibody and the Renaissance chemiluminescence reagent. *C*, Coomassie Blue staining of soluble ectoapyrase. 3 μg of purified protein products were separated on a reducing 7.5% SDS-PAGE, and the gel was stained with Coomassie Blue. The position of soluble ectoapyrase bands is marked with *arrowheads*.

TABLE I
Substrate specificity of wild type ectoapyrase (CD39) and soluble CD39

Soluble CD39 and human Epstein-Barr virus transformed LG2 cells were incubated in the standard solution with 1 mM NaN_3 and 0.5 mM Na_3VO_4. The reaction was started by adding the nucleotides to a final concentration of 1 mM and incubating at 37 °C. Ca^{2+}-stimulated apyrase activity was determined at the end of a 20-min incubation by measuring the inorganic phosphate released as described by Ames (28). The values are means ± S.D. of three independent experiments.

Substrate	Wild type CD39	Soluble CD39
ATP	100 ± 4	100 ± 2
ADP	58 ± 2	57 ± 4
AMP	0 ± 1	0 ± 1
dATP	95 ± 2	85 ± 5

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 LG2 cells (5) (Table I). The soluble ectoapyrase had a K_m of 220 μM for ATP (at 0.5 mM Ca^{+2}) compared with a K_m of 75 μM for the wild type enzyme. The enzymatic activity of soluble ectoapyrase was dependent on either Ca^{2+} or Mg^{2+} 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-

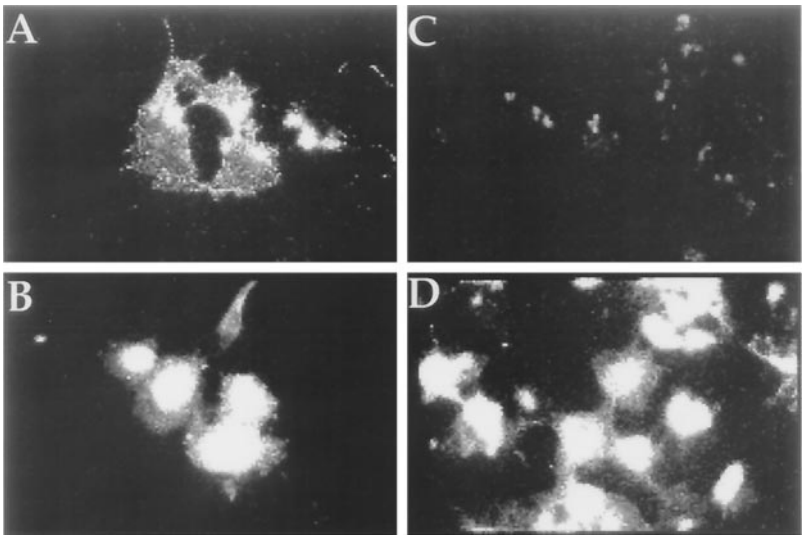

FIG. 3. **Immunofluorescence photographs of CD39CT-Myc- and CD39WT-Myc-transfected COS-7 cells.** COS-7 cells were transfected with CD39CT-Myc (*A* and *B*) or CD39WT-Myc (*C* and *D*). The intact cells (*A* and *C*) or detergent-permeabilized cells (*B* and *D*) were then stained with anti-Myc monoclonal antibody 9E10 and then with fluorescein isothiocyanate-labeled secondary antibody.

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 resides 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 NH_2 terminus in the cytoplasm and the COOH terminus located extracellularly. This prediction is consistent with the results of immunofluorescence staining. Both intact (Fig. 3*A*) and detergent-permeabilized (Fig. 3*B*) 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. 3*D*) but not intact cells (Fig. 3*C*), 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 P_i/min/mg, respectively. The COOH terminus-truncated ectoapyrase has a K_m for ATP (at 0.5 mM Ca^{+2}) of 300 μM.[3] Either the COOH terminus-truncated ectoapyrase has 42% of the activity of the wild type enzyme or its expression at the plasma membrane is lower than that of the wild type enzyme.

The NH_2 terminus deletion mutant (CD39NT-Myc, Fig. 1), lacking the NH_2-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 CD4 signal peptide and cleavage sequence generates a type I integral membrane protein, with the NH_2 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 P_i/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.

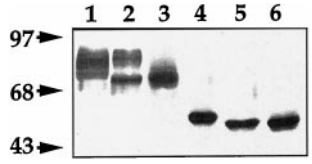

FIG. 4. **Expression of wild type and mutant ectoapyrases in COS-7 cells.** Immunoblots of CD39WT-Myc (*lanes 1* and *4*), CD39CT-Myc (*lanes 2* and *5*), and CD39NT-Myc (*lanes 3* and *6*) proteins in COS-7 cell lysates are shown. Cell lysates were denatured and treated with (*lanes 4–6*) or without (*lanes 1–3*) *F. meningosepticium* 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.

Like the wild type ectoapyrase, the NH_2- 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. meningosepticium* glycopeptidase F (Fig. 4, *lanes 4–6*). These results indicate that the mutant ectoapyrases are not only active but also posttranslationally modified in COS-7 cells. The apparent molecular weight of the deglycosylated CD39NT-Myc (*lane 6*) was similar to that of CD39CT-Myc (*lane 5*), and these were smaller than that of CD39WT-Myc (*lane 4*), confirming that the CD4 signal peptide of CD39NT-Myc was cleaved and that the COOH terminus of CD39CT-Myc was missing.

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 NH_2-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-

[3] A. Grinthal, personal communication.

TABLE II
Detergent effects on the enzymatic activities of various apyrase proteins

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 NaN_3, 0.5 mM Na_3VO_4, and 1 mM ATP (see "Experimental Procedures"). The calcium-dependent ATPase activities of samples treated with detergents are presented as the percentage of the activities of control samples. Values are means ± S.D. of three independent experiments.

	Control	Triton X-100[a]	Digitonin
		%	
CD39 (WT) crude membrane	100 ± 1	12 ± 1	90 ± 2
Soluble CD39	100 ± 2	92 ± 3	95 ± 3
Potato apyrase	100 ± 1	155 ± 4	ND[b]
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.

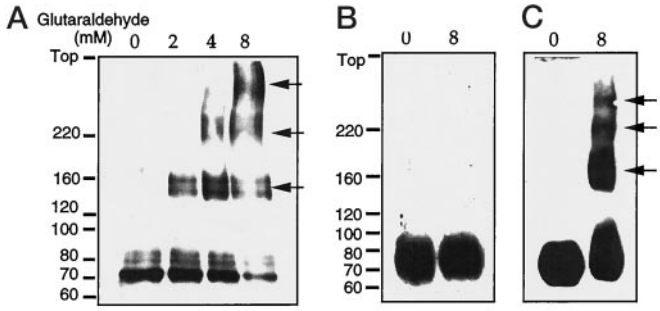

FIG. 5. **Glutaraldehyde cross-linking of wild type ectoapyrase before and after solubilization with detergents.** *A*, crude membranes (25 μl) of COS-7 cells transfected with wild type CD39 cDNA were incubated with 0–8 mM glutaraldehyde for 2 min. *B*, Triton X-100 solubilized ectoapyrase monomers (fraction 27 in Fig. 6*A*) were incubated with 0 or 8 mM glutaraldehyde for 10 min. The samples were separated by 5.5% nonreducing SDS-PAGE, and the protein was detected by blotting with anti-CD39 antibody. Positions of molecular size markers are shown in kDa. *C*, digitonin-solubilized ectoapyrase tetramers (fraction 20 in Fig. 6*A*) were incubated with 0 or 8 mM glutaraldehyde for 2 min and analyzed by SDS-PAGE as in *B*.

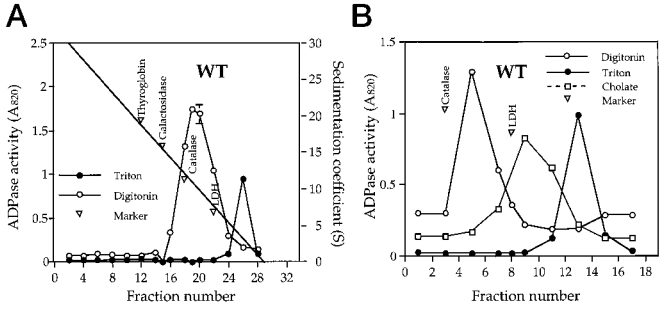

FIG. 6. **Sucrose density gradient sedimentation of detergent solubilized ectoapyrase proteins.** Crude membranes (40 μl) of COS-7 cells transfected with wild type CD39 cDNA were solubilized with 1% digitonin, 1% Triton X-100, or 2% cholate for 15 min on ice and then separated on 5–25% (w/v) (*A*) or 5–20% (w/v) (*B*) sucrose density gradients as described under "Experimental Procedures." Each fraction was assayed for Ca^{2+}-dependent ADPase activity in the presence of 1 mM NaN_3 and 0.5 mM Na_3VO_4. The positions of marker proteins were determined by reducing SDS-PAGE. The calibration curve was obtained by plotting the fraction number *versus* the sedimentation coefficients of the marker proteins. The molecular weights of detergent-solubilized ectoapyrases were estimated by their sedimentation coefficients based upon the results of Liu and Guidotti (31).

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 ~150,000, ~230,000, and ~300,000, corresponding to dimers, trimers, and tetramers of the M_r 70,000 polypeptide (Fig. 5*A*). 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 ~330,000 and ~75,000, respectively. Furthermore, the sedimentation coefficient of the ectoapyrase solubilized with cholate (Fig. 6*B*) is 6.7 S, corresponding to a dimer of the ectoapyrase with M_r of ~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. 6*A*) produced the ladder of bands indicative of monomers, dimers, trimers, and tetramers after a 2-min exposure to 8 mM glutaraldehyde (Fig. 5*C*); whereas with Triton X-100-solubilized monomeric protein (fraction 27 in Fig. 6*A*), no cross-linked product was visible after a 10-min exposure to 8 mM glutaraldehyde (Fig. 5*B*). 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. 7*A*), and there were no cross-linked products found after treatment with glutaraldehyde (Fig. 7*B*). 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

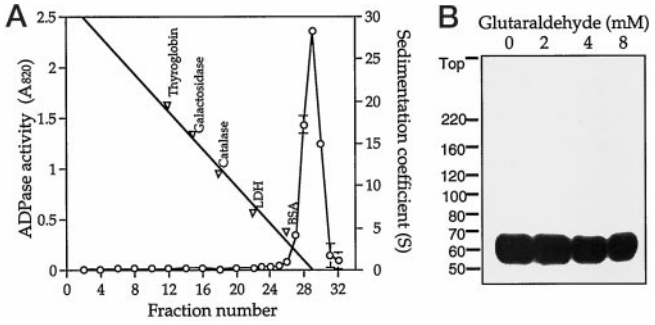

FIG. 7. **Soluble ectoapyrase is a monomeric protein.** *A*, sucrose density gradient sedimentation of soluble ectoapyrase. The purified protein (20 μg) was sedimented on 5–25% (w/v) sucrose density gradient as described under "Experimental Procedures." Fractions were collected and assayed for Ca^{2+}-dependent ADPase activity in the presence of 1 mM NaN_3 and 0.5 mM Na_3VO_4. 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.

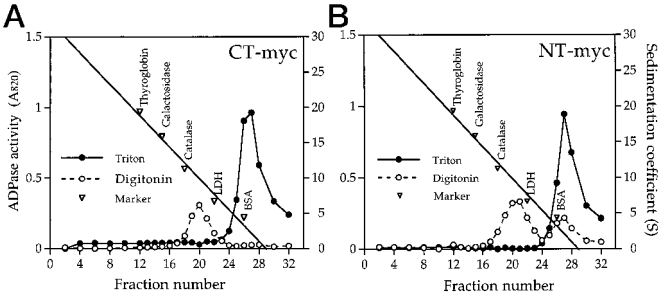

FIG. 8. **Sucrose density gradient sedimentation of detergent solubilized mutant ectoapyrase proteins.** Crude membranes from COS-7 cells transfected with CD39CT-Myc (*A*) and CD39NT-Myc (*B*) cDNA were solubilized with 1% digitonin or 1% Triton X-100 for 15 min on ice and then separated on 5–25% (w/v) sucrose density gradients as described under "Experimental Procedures." Each fraction was assayed for Ca^{2+}-dependent ADPase activity in the presence of 1 mM NaN_3 and 0.5 mM Na_3VO_4. 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).

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 CD39NT-Myc after solubilization with digitonin (Fig. 8*B*) suggests that the NH_2-terminal transmembrane domain has a stronger tendency to self-associate than the COOH-terminal transmembrane domain. 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. Both ectoapyrase tetramers (in digitonin) and monomers (in Triton X-100) are monomers by reducing or nonreducing SDS-PAGE. Moreover, no disulfide-linked ectoapyrase oligomers were detected after nonreducing SDS-PAGE of crude membranes (Fig. 5*A*, *left lane*). These results are consistent with the fact that Triton X-100 can

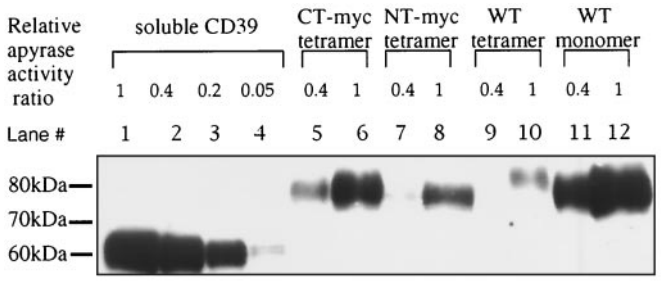

FIG. 9. **The enzymatic activity of wild type ectoapyrase tetramer is greater than those of wild type monomer, soluble ectoapyrase, CD39CT-Myc tetramer, and CD39NT-Myc tetramer.** To compare the relative enzymatic activities of soluble ectoapyrase (*lanes 1–4*), CD39CT-Myc tetramers (*lanes 5–6*; fraction 20 in Fig. 8*A*), CD39NT-Myc tetramers (*lanes 7* and *8*; fraction 20 in Fig. 8*B*), ectoapyrase tetramers (*lanes 9* and *10*; fraction 6 in Fig. 6*B*) and ectoapyrase monomers (*lanes 11* and *12*; fraction 12 in Fig. 6*B*), the amounts of protein in samples with a given Ca^{2+}-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.

dissociate ectoapyrase tetramers into monomers (Fig. 6), since Triton X-100 is unlikely to reduce intermolecular disulfide bonds. Since we added *N*-ethylmaleimide to the cell lysis buffer to prevent intermolecular disulfide bond formation during the preparation of crude membranes (see "Experimental Procedures"), we believe that the results obtained by other investigators may be the result of disulfide bond formation during membrane preparation.

The Enzymatic Activity of Tetramers Is Greater than That of Monomers—One of the central questions in this study is whether the specific activity of ectoapyrase tetramers is greater than that of monomers. To answer this question, we compared by immunoblotting with anti-CD39 antibody the amount of tetrameric protein with a given enzymatic activity with that of the monomer with a similar enzymatic activity. The tetramers were from fraction 5 of the sucrose density gradient with digitonin (Fig. 6*B*), while the monomers were from fraction 13 of the gradient with Triton X-100 (Fig. 6*B*). As can be seen in Fig. 9, the amount of protein present in *lanes 9* and *10* (tetramers) is substantially lower (by a factor of 5–10) than that in *lanes 11* and *12* (monomers). Importantly, the dissociation to monomer with a reduction in enzymatic activity is reversible. When the monomers in fraction 13 of the Triton X-100 gradient in Fig. 6*B* were sedimented through a sucrose density gradient containing 0.2% digitonin, the enzyme had the sedimentation behavior of the tetrameric proteins and the total activity of the applied monomer increased by 5–6-fold (Fig. 10). Together, these results suggest that ectoapyrase wild type tetramers have higher catalytic activity than do the wild type monomers.

Heterologous Interactions between Both Transmembrane Domains Are Important for Enzymatic Activity and Detergent Resistance—We asked whether the enzymatic activities of the mutant ectoapyrases lacking one or both transmembrane domains are similar to that of the wild type tetramers or wild type monomers.

As shown in Fig. 9, at least 10 times more soluble ectoapyrase (*lanes 1–4*) was required to obtain the same enzymatic activity as that of digitonin-solubilized ectoapyrase tetramers (*lanes 9–10*). This result indicates that soluble ectoapyrase has a lower enzymatic activity than the wild type ectoapyrase tetramer and is also consistent with the fact that the specific activity of purified soluble ectoapyrase (~200 μmol

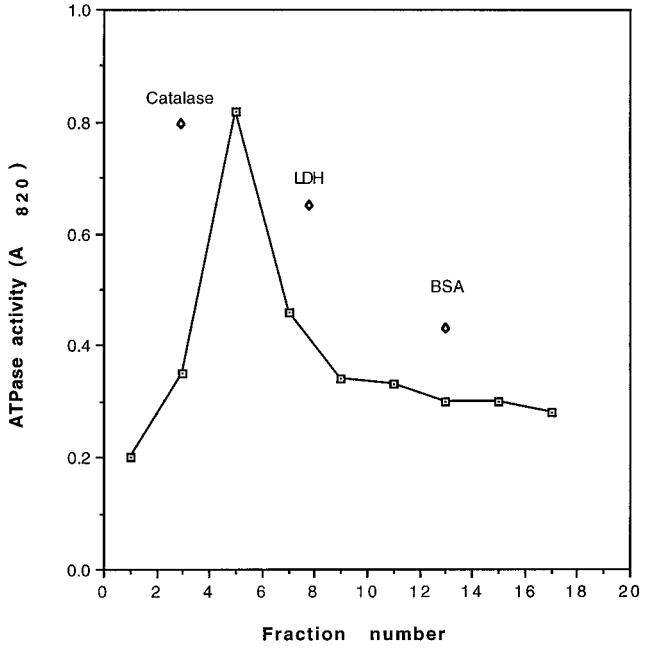

FIG. 10. **Reconstitution of ectoapyrase tetramers from monomers by sucrose density gradient sedimentation.** Triton X-100-solubilized ectoapyrase monomers (100 μl of fraction 13 in Fig. 6*B*) were sedimented on a 5–20% (w/v) sucrose density gradient containing 0.2% digitonin as described under "Experimental Procedures." Fractions were collected and assayed for Ca^{2+}-dependent ATPase activity in the presence of 1 mM NaN_3 and 0.5 mM Na_3VO_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_i/min/mg) is lower than that of the digitonin-solubilized rat lung ectoapyrase (~1000 μmol of P_i/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 ectoapyrases 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 ectoapyrases 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.[4] 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 ectoapyrases, 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 ectoapyrases (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

[4] T.-F. Wang, Y. Ou, and G. Guidotti, unpublished results.

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

Ting-Fang Wang, Yvonne Ou and Guido Guidotti

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