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Soluble Apyrases Release ADP during ATP Hydrolysis

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A soluble form of CD39 was expressed and purified from High-Five insect cells. The soluble CD39 is a monomer with a molecular weight of 54,000. The k_{cat} and K_m of the purified soluble CD39 were 4.6 s^{-1} and 12 μM for ATP and 1.3 s^{-1} and 7 μM for ADP as substrates, respectively. One nucleotide binding site was detected on the monomer only in the presence of Ca^{2+}. In contrast to the membrane bound CD39, soluble CD39 released ADP as an intermediate during ATP hydrolysis, as did the soluble potato apyrase. © 2001 Academic Press

Key Words: **CD39; E-NTPDase; apyrase; ADP; release; binding site; mechanism.**

The ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases, formerly called E-ATPases) (1) are involved in extracellular nucleotide catabolism and are characterized by dependence on divalent cations and insensitivity to inhibitors of P-type, F-type, and V-type ATPase (2, 3). The E-NTPDases can be distinguished by their activity with nucleoside triphosphates and diphosphates (4). E-NTPDase1 (CD39) hydrolyzes ATP and ADP about equally well. E-NTPDase2 (CD39L1) hydrolyzes ATP 10 times more actively than ADP. E-NTPDase3 (CD39L3) hydrolyzes ADP at 1/3 the rate of ATP hydrolysis. All three E-NTPDases contain five highly apyrase conserved domains (ACR1–ACR5); ACR1 and ACR4 may be involved in interactions with the β and γ-phosphates of ATP, respectively (5, 6). However, the mechanism for the substrate selectivity of these isoforms in not known.

CD39 was originally identified as a B cell surface marker (7, 8); it is also present on activated NK cells, T lymphocytes, and endothelial cells (9). CD39 is a glycoprotein (10) with six potential N-linked glycosylation sites (9, 11, 12, 13) and two transmembrane domains, one each at the N-terminal and C-terminal ends (9); its catalytic region is on the outer surface of the plasma membrane (14, 15). It is an oligomer whose quaternary structure depends on the presence of the N-terminal and C-terminal transmembrane domains (10). Disruption of the oligomeric structure decreased the ability of CD39 to hydrolyze ATP and ADP (10) and CD39 lacking the N- and C-terminal transmembrane strands had approximately 10% of the hydrolytic activity of the intact protein (10, 16).

Heine *et al.* (17) measured the products of hydrolysis of ATP by CD39 and reported that ATP was converted directly to AMP without the release of ADP as a stable intermediate. This was a surprising finding as ADP is an excellent substrate of CD39.

In this study, a recombinant soluble CD39 with a C-terminal histidine tag was successfully expressed by High-Five insect cells and purified. Investigation of the kinetics of the purified soluble CD39 suggests that the mechanism of hydrolysis by the soluble CD39 is different from that of the intact protein and similar to that of other soluble ectoapyrases

EXPERIMENTAL PROCEDURES

Reagents. ATP, ADP, ADPNP, ADPCP, AMPCP, ATPγS, ADPβS, and potato apyrase (Grade VII) were obtained from Sigma (St. Louis, MO). Triton X-100 was purchased from Calbiochem (La Jolla, CA). Fetal bovine serum, penicillin/streptomycin/L-glutamine, Dulbecco's modified Eagle medium (DMEM), and lipofectamine were from GIBCO/BRL (Gaithersburg, MD). Chemiluminescence reagents were from Pierce (Rockford, IL). Zeocin, High-Five medium were from Invitrogen (Carlsbad, CA).

Expression and purification of soluble CD39 (sCD39). The cDNA for rat CD39 was modified according to Wang *et al.* (12): both N-terminal (1–38) and C-terminal (471–511) transmembrane domains were truncated, a fragment encoding the murine CD4 signal peptide and cleave site sequence (MCRAISLRRLLLLLLQLSQLLAVTQGKTLVLGKEGES) was fused to the N-terminus of the truncated CD39 (amino acid residues 39–470), and a six histidine amino acid tag was placed at the C-terminus. The constructed cDNA fragment was subcloned into pIZ/V5-His vector from Invitrogen at *Hin*dIII and *Xho*I sites, generating pIZ-sCD39. Before transfection into High-Five insect cells, the region of pIZ-sCD39 encoding CD39 was sequenced; no point mutation was detected. The plasmid pIZ-sCD39 was transfected into High-Five cells using lipid-mediated transfection following InsectSelect System protocols (Invitrogen). After three weeks of selection with 100 μg/ml zeocin, a stable cell line that expresses sCD39 was established. High-Five serum-free medium was chosen to facilitate expression and purification of sCD39. The cell culture was scaled up from a 25-ml flask to an 1-liter spinner following the protocol provided by Invitrogen. The final medium contained

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50 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml zeocin, and 10 unit/ml heparin.

Purification of sCD39. 300 ml of conditioned medium was harvested 48 h after each transfer and, after removal of cells and debris, was concentrated to about 10 ml using an Amicon concentrator. The solution was applied to 12 ml of concanavalin A-Sepharose 4B resin (Sigma) equilibrated with Buffer A (25 mM Tris–HCl, pH 7.0, 80 g NaCl and 5 g KCl per liter) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. Buffer A containing 0.1 mM α-methylmannoside, 25 ml, was used to wash the column. Proteins were eluted with 30 ml of Buffer A including 1 M α-methylmannoside. An Amicon concentrator was used to replace Buffer A with 40 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.5 mM $CaCl_2$. The solution was loaded on a 4 ml Pro-bond nickel resin equilibrated with 24 mM KH_2PO_4, 16 mM K_2HPO_4, and 0.5 M NaCl (pH 7.8). The column was washed with 8 ml of 24 mM KH_2PO_4, 16 mM K_2HPO_4, 5 mM imidazole and 0.5 M NaCl (pH 6.0) to remove non-specifically bound proteins. sCD39 was eluted with the same buffer containing 500 mM imidazole. The elution buffer was changed to 40 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.5 mM $CaCl_2$ with an Amicon concentrator. Cold saturated $(NH_4)_2SO_4$ was added to a final concentration of 60% saturation. After overnight incubation at 4°C, the precipitated protein was removed by spinning for 5 min at 15,000*g*. The supernatant was adjusted to 80% saturated $(NH_4)_2SO_4$ and incubated for several hours at 4°C to allow sCD39 to precipitate completely. The precipitated sCD39 was collected and re-suspended in about 500 μl of 40 mM Tris–HCl (pH 7.5), 0.5 mM $CaCl_2$. This sample was loaded on a Superose-12HR gel filtration column from Pharmacia Biotech equilibrated with 40 mM Tris–HCl (pH7.5), 0.5 mM $CaCl_2$. The major peak was collected, and concentrated to about 50 μl with a YM30 Centricon concentrator from Millipore.

Relative molecular mass determination of sCD39. Superdex 200HR from Pharmacia Biotech was used to separate standard proteins (Pharmacia Biotech) and sCD39 in 40 mM Tris–HCl (pH 7.5), 0.5 mM $CaCl_2$, and 150 mM NaCl.

Nucleotidase activity assay. The reaction was carried out in 20 mM Hepes–Tris (pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM EDTA, 1mM HEDTA and 2.5 mM $CaCl_2$. It was started by adding nucleotides at 37°C. After incubation for 15 min, the reactions were stopped by adding 10% SDS. Inorganic phosphate was measured according to Ames (18). One unit of activity is defined as the release of 1 μmol Pi/min/mg.

Nucleotide binding assay. The purified sCD39 was applied to a 2-ml spin column of Chelex-100 equilibrated with 20 mM Hepes–Tris (pH 7.4), 120 mM NaCl, 5 mM KCl to remove bound Ca^{2+}. 2 μg of purified sCD39 was incubated with different nucleotides in the presence or absence of 1 mM Ca^{2+} in 50 μl of 20 mM Hepes–Tris (pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM EDTA, and 1mM HEDTA for several hours. Free nucleotides were removed by sequentially passing through two 1.5 ml Sephadex G-15 spin-columns. Bound nucleotides then were extracted from sCD39 with 0.4 M perchloric acid; precipitated sCD39 was removed by spinning at 8000*g* for 5 min and the supernatant was neutralized with 5 M potassium carbonate. Neutralized samples were frozen, thawed slowly at 4°C, and pressed through a 0.2 μm filter to remove the potassium carbonate. The samples were stored at −80°C.

Nucleotide separation by HPLC. Nucleotides were separated by anion exchange HPLC, according to Hartwick and Brown (19), on a SAX column (10 × 0.46 mm) from Rainin Instuments. The low concentration buffer (A) was 0.08 M $NH_4H_2PO_4$ (pH 3.8), and the high concentration buffer (B) was 0.25 M $NH_4H_2PO_4$ (pH 4.95) and 8 mM KCl. The sample was loaded on the column equilibrated with buffer (A); then a gradient of buffer (B) (4 min, 0–2.5% (B); 26 min, 2.5–25% (B)) was used for elution. The flow rate was 1 ml/min.

Immunoblotting. Rabbit anti-rat ectoapyrase (CD39) antiserum was obtained from Wang *et al.* (12). The immunoblotting procedure was according to the protocol provided by Bio-Rad.

Cell culture and crude membrane preparation. COS-7 cells were cultured as described by Coppi and Guidotti (20). Transfection of COS-7 cells with the plasmid (pCI_CD39) with wild-type CD39 and preparation of crude membrane were performed as described by Wang *et al.* (12).

Calculation of free calcium. Concentrations of free calcium or calcium nucleotide complexes were calculated using MaxChelator program with included calcium complex constants from Martell and Smith (21).

Protein concentration assay. Concentrations of proteins were determined using D_C Protein Assay from Bio-Rad following the provided protocol.

RESULTS

Expression and purification of sCD39. The recombinant soluble CD39 (sCD39) was secreted into the growth medium by High-Five cells at about 5 mg/liter (determined by measuring the specific activity of sCD39). Neither nucleotidase activity nor sCD39 protein was detected in the conditioned media from non-transfected High-Five cells (data not shown).

During the purification 2 mM NEM was included in the solutions to prevent the aggregation of sCD39. After concanavalin A–Sepharose 4B and nickel resin affinity purification, sCD39 was about 50% pure as can be seen by the major band observed at 54 kDa in Fig. 1A, lane 1. The level of purification increased to 80% by ammonium sulfate (60% w/v) precipitation (Fig. 1A, lane 2), and to 95% by gel filtration on Superose 12HR (Fig. 1A, lane 3). This purification procedure generated about 0.5 mg/liter of more than 95% pure sCD39.

Immunoblotting with anti-CD39 serum confirmed that the purified protein was derived from CD39 (Fig. 1B). Only one band was detected at 54 kDa. The molecular weight of sCD39 was further determined as 54 ± 2 kDa (Fig. 1C) by gel filtration on Superose 12HR. Protein sequencing of the purified protein further confirmed that it is derived from CD39.

Nucleotidase activities of purified sCD39. The ATPase and ADPase activities of the purified sCD39 were measured at different concentration of Ca^{2+}ATP or Ca^{2+}ADP (Fig. 2). The molar ratio of Ca^{2+} to ATP or ADP was 1:1. The concentrations of Ca^{2+}ATP or Ca^{2+}ADP were calculated using the MaxChelator program, as described under Experimental Procedures. The maximal specific activity of sCD39 was 4.96 ± 0.39 units for Ca · ATP hydrolysis, and 1.4 ± 0.1 units for Ca · ADP hydrolysis. The k_{cat} for Ca · ATP and Ca · ADP were 4.6 ± 0.36 s^{-1} and 1.3 ± 0.1 s^{-1}, respectively. The K_m's of sCD39 for Ca · ATP and Ca · ADP were 12 ± 2.1 μM and 7 ± 0.1 μM, respectively (Table 1). The ATPase activity of sCD39 was about 3.5 times greater than its ADPase activity.

The ATPase and ADPase activities were determined at different free Ca^{2+} concentration in the presence of 2

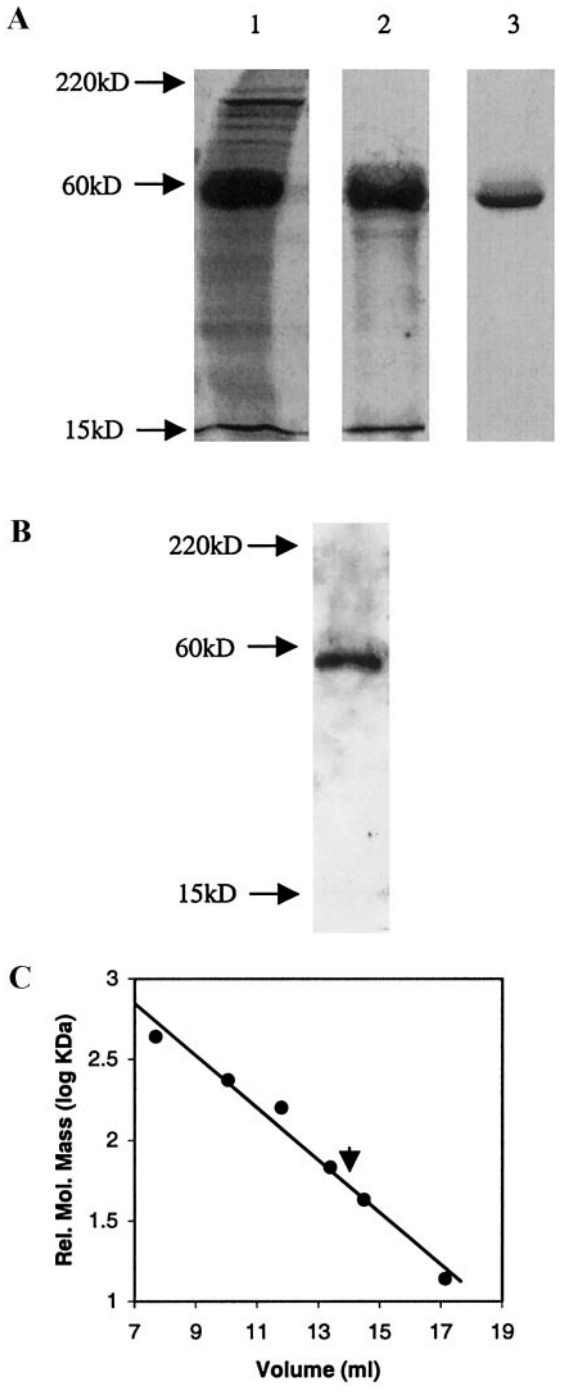

FIG. 1. Purification and characterization of the recombined soluble CD39 (sCD39). (A) Results of SDS–PAGE of sCD39 after concanavalin A–Sepharose and nickel affinity purification (Lane 1), ammonia sulfate precipitation (Lane 2), and Superose-12HR gel filtration (Lane 3). (B) Immunoblotting of purified sCD39 with anti-CD39 serum. (C) Relative molecular mass of purified sCD39 determined with HMW and LMW gel filtration calibration kits from Pharmacia Biotech. The arrow indicates the position of sCD39 elution.

mM ATP or ADP. The K_m for free Ca^{2+} was 1.1 μM in the presence of ATP, and 0.07 μM in the presence of ADP.

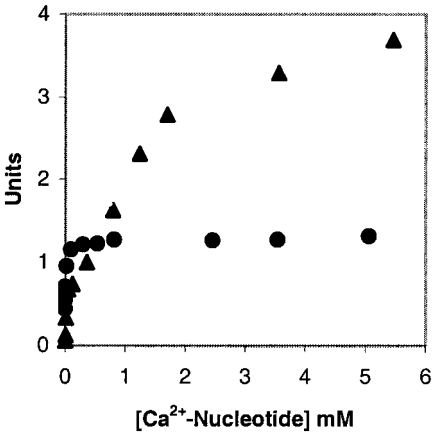

FIG. 2. The specific ATPase (▲) and ADPase (●) activities of purified sCD39. In each assay, 0.5 μg of sCD was added into the reaction buffer, and the released inorganic phosphate was quantified (see Experimental Procedures). The molar ratio of nucleotide to Ca^{2+} was 1:1, and the concentrations of the metal-nucleotide complexes were calculated as described under Experimental Procedures. These data were averaged from three independent assays.

Molar ratio of nucleotides bound to sCD39. The molar ratios of bound nucleotides to purified sCD39 in the presence and absence of Ca^{2+} were determined by the method described under Experimental Procedures (Table 1). This method was capable of removing all free nucleotide as indicated by the controls, which were done in the absence of enzyme. Purified sCD39 bound ATP analogs at a molar ratio between 0.54 and 0.72 in the presence of Ca^{2+}, while the molar ratios of bound ATP analogs to sCD39 were decreased to about 0.18 in the absence of Ca^{2+}. Only ADPβS, a non-hydrolysable ADP analog, was found to bind sCD39 at a molar ratio

TABLE 1

Molar Ratio of Bound Nucleotides over Purified sCD39 Determined by HPLC

Complex	Ca^{2+}	Molar ratio of nucleotide/enzyme
sCD39 + ATPγS	+	0.72 ± 0.13
	−	0.18 ± 0.09
sCD39 + ADPNP	+	0.54 ± 0.11
	−	0.19 ± 0.07
sCD39 + ADPCP	+	0.67 ± 0.12
	−	0.18 ± 0.10
sCD39 + ADPβS	+	0.17 ± 0.05
	−	0
sCD39 + AMPCP	+/−	0
ATP	+/−	0
ADP	+/−	0
sCD39	+/−	0

Note. This table summarizes the results of the nucleotide-binding assay as described under Experimental Procedures in the presence (+) or the absence (−) of Ca^{2+}. Most of the assays were repeated four times.

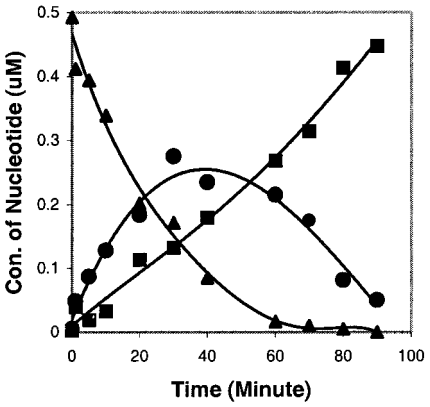

FIG. 3. Analysis of ATP hydrolysis of sCD39 by HPLC. 2 μg of sCD39 was included in each assay. The reaction was initiated by adding 0.5 mM Ca^{2+} · ATP (1:1). ATP is represented as ▲; ADP is represented as ●; and AMP is represented as ■.

of 0.17. These data suggest that only one detectable nucleotide binding site exists on the purified sCD39.

Mechanism of ATP hydrolysis by sCD39. The process of ATP hydrolysis by purified sCD39 was analyzed with HPLC to quantify the amounts of ADP and AMP present during the reaction (Fig. 3). The reaction consisted of 800 μl buffer containing 20 μg sCD39 and 0.5 mM ATP. At different times, 50 μl of reaction solution were removed and the reaction was stopped with perchloric acid. The ATP hydrolysis rate was calculated as 0.6 units/mg during the first 15 min after the reaction started, which is consistent with the result obtained by measuring the amount of released inorganic phosphate (Fig. 2).

The concentrations of ATP and ADP became equal at 21 min, and they were twice the concentration of AMP. This result reveals that ADP, the first product of ATP hydrolysis, was released from the enzyme before ADP cleavage to AMP.

It was reported that intact CD39 from transfected CHO cells did not produce ADP during ATP hydrolysis to AMP (16). It is interesting to know if the same process occurs when CD39 is expressed in COS-7 cells as tetramers. As shown in Fig. 4B, intact CD39 hydrolyzed ATP directly into AMP and orthophosphate at a rate 2.86 μmole · min^{-1} · mg^{-1} of total membrane protein, and no intermediate ADP was detected. Neither ATPase nor ADPase activity was detected on the crude membrane prepared from non-transfected COS-7 cells (Fig. 4A). CD39 is dissociated into monomers by solubilization with 1% Triton X-100 (12). This quarternary structure change affected the mechanism of hydrolysis because the solubilized enzyme released ADP during ATP hydrolysis (Fig. 4C). The rate of ATP hydrolysis by Triton X-100 solubilized CD39 was about 7% of that of intact CD39.

The results shown above raise the question as to whether the soluble apyrases may have a mechanism of ATP hydrolysis similar to that of sCD39. Since potato apyrase is a soluble protein with no transmembrane domains, we examined its mechanism of ATP hydrolysis. As shown in Fig. 5, ADP was formed before

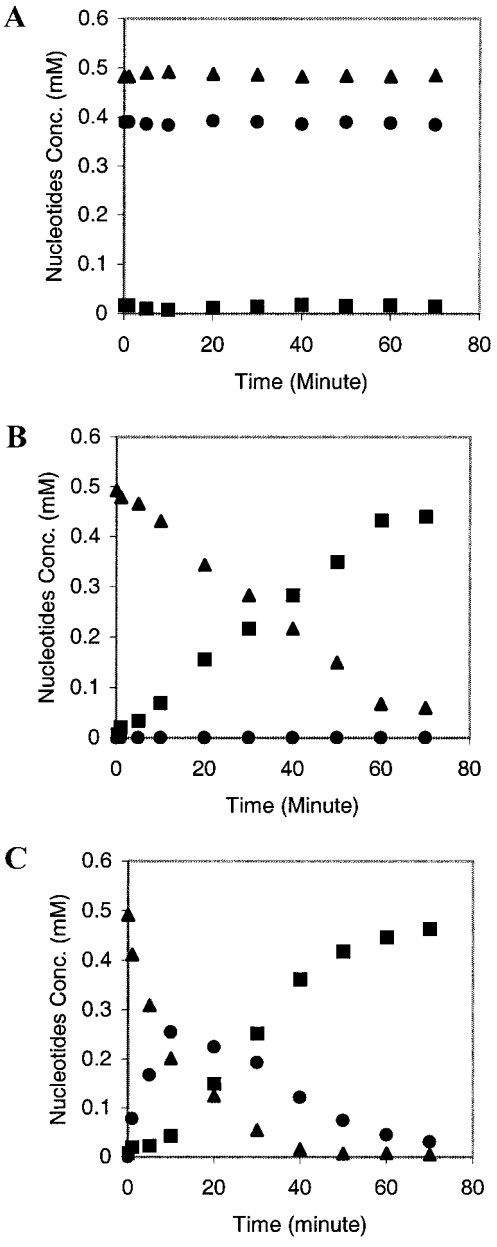

FIG. 4. Analysis of ATP hydrolysis of intact CD39 or Triton X-100 solubilized CD39 monomer. ATP (▲), ADP (●), and AMP (■) were separated as described under Experimental Procedures. (A) 0.12 μg total protein prepared as membrane vesicles from vector only transfected COS-7 cells was included in each assay in the presence of 0.5 mM Ca^{2+} · ATP (1:1). (B) 0.12 μg total protein prepared as membrane vesicles from CD39 transfected COS-7 cells was added in each reaction in the presence of 0.5 mM Ca^{2+} · ATP (1:1). (C) 2μg Triton X-100 solubilized membrane protein from CD39 transfected COS-7 cells was present in each assay, and the concentration of Ca^{2+} · ATP (1:1) was 0.5 mM.

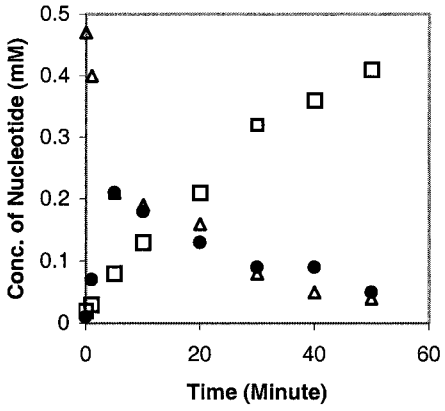

FIG. 5. Potato apyrase ATP hydrolysis analyzed by HPLC. 0.03 unit of potato apyrase was included in each reaction initiated by adding Ca^{2+} · ATP (1:1) at 0.5 mM. ATP (△), ADP (●), and AMP (■) were separated as described under Experimental Procedures.

AMP appeared, suggesting that ADP was released from soluble potato apyrase.

DISCUSSION

CD39 has two transmembrane segments, at the N- and C-terminal ends (9), that are required for oligomerization (10) and an extracytoplasmic catalytic domain (11, 14). A truncated CD39 (39–471 amino acid) with CD4 secretion signal and cleavage site previously was expressed in sf9 insect cells at a yield of 1 mg/liter (10). Another similar construct, CD39Flag (38–476 amino acid) with the IL-2 signal sequence, was expressed in CHO cells at a yield of 2 mg/liter (16).

Here we report that a recombinant CD39 (39–471 amino acid) with CD4 secretion signal and cleavage site has been successfully expressed in High-Five insect cells at a yield of 5 mg/liter. This high yield allows significant amounts of sCD39 to be purified. The molecular mass of the purified sCD39 from High-Five cells is 54 kDa, which is similar to the value obtained when the same recombinant sCD39 was expressed in sf9 cells (10). Immunoblotting confirms that only one band was detected at the 54 kDa position. Protein sequencing further indicated that the purified protein was derived from CD39.

The nucleotide binding experiments indicate that only one nucleotide-binding site was detected on sCD39 and that the presence of Ca^{2+} facilitated nucleotide binding to the catalytic site. Thus sCD39 most likely uses the complex of nucleotide and Ca^{2+} as substrate for catalysis. It is generally the case that complexes of nucleotides and metals serve as substrates for enzymes that carry out nucleotide hydrolysis, like the F_1-ATPase.

The K_m's of purified sCD39 are 12 μM for Ca · ATP and 7 μM for Ca · ADP, which are very close to the value characterized from a differently constructed soluble CD39 with Flag-tag (16). The k_{cat} of sCD39 for Ca · ATP (4.6 s^{-1}) and Ca · ADP (1.3 s^{-1}) also are in the same range as previously reported (16). The V_{max} of sCD39 for ATP is 3.5-fold greater than that for ADP. This ratio is similar to that obtained for Triton X-100 solubilized membrane CD39 (22). However, these data are different from those reported by Gayle III *et al.* (16) in which the V_{max} of sCD39 for ADP is 2.75-fold greater than that for ATP.

It has been reported that CD39 does not release intermediate ADP during the ATP hydrolysis (17). Our results indicate that this is true only when CD39 forms oligomers. Intermediate ADP is released from the enzyme by CD39 monomers and by the recombinant soluble CD39. Furthermore, another soluble apyrase, potato apyrase, also releases intermediate ADP during ATP hydrolysis. Thus the catalytic features of the purified sCD39 are more like potato apyrase than native CD39. It may be a feature of soluble apyrases to release intermediate ADP during ATP hydrolysis.

Two conclusions can be made from these results. One is that the mechanism of nucleotide hydrolysis by CD39 is strongly dependent on the interaction of the transmembrane domains with the active site. Previously, we had shown that the overall enzymatic activity and the ratio of ATPase to ADPase activities were also regulated by the transmembrane segments (22). Another conclusion is that the tissue localization of CD39 and of CD39L1 can have an important role on the signaling properties of ATP. Where CD39 is present, ATP is converted to AMP and then to adenosine by 5′-nucleotidase; ADP is not an appreciable product. On the other hand, in the presence of CD39L1, ATP is converted to ADP which will be relatively stable. With soluble apyrases, ADP and AMP are both present in solution during ATP hydrolysis; these enzymes act as a combination of CD39 and CD39L1.

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