Articles

Substitution of His59 Converts CD39 Apyrase into an ADPase in a Quaternary Structure Dependent Manner†

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ABSTRACT: The two transmembrane domains of CD39 ecto-apyrase regulate the formation of fully active homotetramers. We show that mutations in apyrase conserved region 1 (ACR1) have two dramatically different sets of effects determined by whether they occur in intact tetramers or in disrupted tetramers or monomers. In intact tetramers, substitution of H59 in the rat brain CD39 ACR1 with G or S abolishes more than 90% of the ATPase activity but less than 50% of the ADPase activity, converting the enzyme into an ADPase with relative ADP:ATP hydrolysis rates of 6:1 or 8:1, respectively. In contrast, the same substitutions in tetramers lacking either transmembrane domain, in monomers lacking both transmembrane domains, or in detergent-solubilized full-length monomers have no effect on ATPase activity and increase ADPase activity approximately 2-fold, resulting in equal ATPase and ADPase activities. N61R substitution has a much smaller effect on the ADPase:ATPase ratio in both cases. While the data for truncated and monomeric constructs are consistent with the proposed role of ACR1 as the β-phosphate binding domain by analogy with the actin/hsp70/hexokinase superfamily, the finding that H59 substitutions in full-length CD39 primarily diminish the ATP hydrolysis rate suggests that ACR1 may play a different role in intact tetramers. We propose that CD39 uses different ATPase and ADPase mechanisms in different quaternary structure contexts, and that H59 in ACR1 plays a central role specifically in ATP hydrolysis in intact tetramers.

In addition to its role as an energy source in the cytoplasm, ATP is regularly released into the extracellular space and the lumen of intracellular organelles, where it and its derivatives, ADP and adenosine, modulate neuronal signaling (*1*), platelet clotting (*2*−*4*), immune function (*5*), and other processes through interaction with extracellular receptors (*6*). In several cases, particularly in neuronal and platelet signaling, adenosine inhibits the processes activated by ATP or ADP (*2, 6*−*8*), so that the overall activity of the system reflects the relative as well as the total concentrations of each molecule. Since ATP must be broken down to adenosine before it can be transported back into the cytoplasm (*9, 10*), the rate of conversion from ATP to adenosine may also determine the survival of cells that secrete a significant amount of their cytoplasmic ATP stores, such as multidrug resistant cancer cells that release ATP in conjunction with drug expulsion (*11*).

A set of enzymes with active sites outside the cell, or ecto-enzymes, is responsible for the extracellular metabolism of ATP. In particular, ecto-ATPases and ecto-apyrases hydrolyze the terminal phosphate of trinucleotides and of both tri- and dinucleotides, respectively (*12*), and 5′-nucleotidase converts AMP to adenosine (*13*). The ecto-ATPases and apyrases are divalent cation-activated, high activity enzymes expressed on a variety of cell types including neurons (*14*), endothelial cells (*15*), activated lymphocytes (*16*), and cells of skeletal muscle (*17*), secretory organs (*18*−*20*), and several types of tumors (*21, 22*). Cloning and sequencing of a growing number of these enzymes has led to their designation as a single family including both the ATPases, which hydrolyze ATP at a rate at least 10-fold greater than that of ADP (*23*), and the apyrases, which hydrolyze ATP and ADP at similar rates (*12*). Both subfamilies contain soluble forms as well as membrane-bound forms which in most cases are anchored in the membrane by two terminal transmembrane domains. Given the homology between the ATPases and the apyrases, as well as the different physiological concentrations of ATP, ADP, and adenosine resulting from their activities, it is of interest to understand the structural features that determine whether primarily ATP or both ATP and ADP can be hydrolyzed.

While a specific active site has not been determined for any of the family members, attention has focused on four conserved domains, termed apyrase conserved regions (ACR) 1−4 (*24*). In particular, ACR1 and -4 are homologous to the β- and γ-phosphate binding domains of the actin/hsp70/hexokinase superfamily (*25, 24*), suggesting that these

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Table 1: Comparison of ACR1 Sequences in Ecto-ATPase/Apyrase Family Members with Zero or One Transmembrane Domain vs Those with Two Transmembrane Domains[a]

soluble/single transmembrane domain		two transmembrane domains	
protein	sequence	protein	sequence
potato apyrase	DAGSTGSR	rat CD39	DAGSSHTN
T. p. gondii 1	DAGSSSTR	rat eATPase	DAGSSHTS
T. p. gondii 3	DAGSSSTR	hCD39	DAGSSHTS
pea GDPase	DAGSTGSR	hCD39L1	DAGSSHTS
yeast GDPase	DAGSTGSR	mCD39	DAGSSHTN
yeast Apy1p	DAGSSGSR	ch ATPase	DAGSSHTA
hCD39L2	DAGSTGTR	ch ATPDase	DAGSTHTA
hCD39L4	DAGSTGTR	human HB6	DAGSSRTT

[a] Two transmembrane domain forms, with the exception of HB6, have H and N/S/A in positions 59 and 61 (numbered according to rat CD39), and soluble and single transmembrane domain forms have G/S and R in the corresponding positions.

domains may play a key role in nucleotide binding and hydrolysis. The crystal structures of actin with ATP and with ADP suggest that both nucleotides bind at approximately the same site (*26*), while the finding that mutagenesis of conserved amino acids in either ACR1 or -4 of human apyrase diminishes ATPase and ADPase activities (*27*) is consistent with a role of the ACRs in both ATP and ADP hydrolysis. However, sequence comparisons between same species pairs of ecto-apyrases and ecto-ATPases reveal identity in all four ACRs, suggesting that the basis for discrimination between ATP and ADP lies somewhere other than the ACRs.

On the other hand, the apyrase and ATPase subfamilies exhibit conserved differences in their transmembrane domains (*17, 28*). Although unlikely to contact nucleotides directly, the transmembrane domains have been shown to regulate tetramer formation and activity in the mammalian apyrase CD39 (*29*). Dissociation or deletion of the transmembrane domains prevents the formation of fully active tetramers (*29*), suggesting that intersubunit interactions influence interactions between the active site and substrates. Comparison between the ACRs of soluble or single transmembrane domain family members and those with two transmembrane domains (*17, 24, 28*) points to two residues in ACR1, H59 and N61 in rat brain CD39, which correlate with the presence or absence of transmembrane domains. Family members with two transmembrane domains have H and N/S/A at these positions, with the exception of the apyrase HB6 which has R and T (*40*), while forms with zero or one transmembrane domain have G/S and R (Table 1). The presence of these residues in a putative phosphate binding domain and their correlation with the presence or absence of transmembrane domains, in conjunction with the transmembrane domain sequence differences between ATPases and apyrases, led us to investigate the roles of these amino acids in ATP, ADP, and relative ADP:ATP hydrolysis rates as well as the influence of quaternary structure on their roles.

MATERIALS AND METHODS

Reagents. ATP and ADP were purchased 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's medium (DMEM),

Table 2: Summary of Kinetic Parameters of Activation and Inhibition

	K_m(free Ca^{2+}) (μM)		inhibition by high Ca^{2+}	
	ATP	ADP	ATP	ADP
wt	30 ± 3	52 ± 12	+	+
wtH59G	25 ± 6	36 ± 15	−	+
wtH59S	19 ± 2	100 ± 35	−	+
wtN61R	130 ± 9	277 ± 67	+	+
wt Triton	2.7 ± 0.1	0.73 ± 0.09	+	−
wtH59G Triton	2.4 ± 0.7	0.38 ± 0.03	+	+
CT	3.1 ± 0.2	0.64 ± 0.01	+	−
CTH59G	3.6 ± 0.6	0.34 ± 0.02	+	+
CTH59S	5.2 ± 0.5	0.38 ± 0.02	+	+
CTN61R	20 ± 2	3.1 ± 0.3	+	−
NT	4.4 ± 0.3	1.6 ± 0.1	+	+
NTH59G	5.2 ± 0.5	0.63 ± 0.05	+	+
sol	3.8 ± 0.2	0.83 ± 0.06	+	−
solH59G	5.2 ± 0.6	0.68 ± 0.03	+	+

and lipofectamine were purchased from GIBCO/BRL (Gaithersburg, MD). Chemiluminescence reagents were purchased from Pierce (Rockford, IL).

DNA Construction. Cloning and plasmid insertion of rat brain CD39 cDNA are described in ref *14*. Construction of the soluble, carboxy-terminal truncated (CT), and amino-terminal truncated (NT) CD39 plasmids for mammalian cell transfection is also described in ref *29*. H59G, H59S, and N61R mutations were originally made in CT by Mas Handa (unpublished) and were transferred directly from CT into the wild type by replacement of an *Nhe*I/*Sac*II fragment including the ACR1 coding region. The H59G mutation was introduced into soluble CD39 by PCR amplification of the extracellular domain of CTH59G and subcloning into a vector previously constructed by T.-F. Wang containing the CD4 signal sequence and hexa-His tag. NTH59G was constructed by replacement of a *Hin*dIII/*Sac*II fragment containing the CD4 signal sequence and the ACR1 coding region of CD39 with the corresponding fragment from soluble H59G.

Preparation of COS7 Cell Crude Membranes. COS7 cells were transfected at 50−70% confluency using the lipofectamine method and harvested 72 h after transfection. Crude membranes were prepared by the method described in ref *30* and resuspended in 50 μL of 50 mM Tris-HCl, pH 7.8, per 100 mm plate.

Detergent Solubilization. Crude membranes were solubilized for 30 min on ice in assay buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM *N*-(2-hydroxyethyl)-ethylenediaminetriacetic acid (HEDTA), 1 mM NaN_3, and 1% Triton X-100. Following solubilization, membranes were centrifuged at 4 °C at high speed for 10 min, and the supernatant was used for assays immediately.

Purification of Soluble CD39. Conditioned medium from three plates of transfected COS7 cells was centrifuged for 10 min at 3000 rpm in a clinical centrifuge and passed over a 1 mL ConA Sepharose column. The column was then washed with 20 mL of a Tris-buffered saline (TBS) solution containing 1 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$. Glycoproteins including soluble CD39 or soluble CD39-HG were eluted with 2 mL of 1 M α-methyl-D-mannoside in the same solution. Protein was concentrated by centrifuging in a Centricon-30 for 90 min at 5000 rpm and diluted 1:200

for nucleotidase assays.

Immunoblot and Antibody. A 7.5% SDS−polyacrylamide gel was run as described (*31*) and transferred to nitrocellulose at 200 mA for 4 h. The membrane was probed with rabbit anti-rat CD39 antiserum (*14*) in 1% milk in TBS followed by secondary goat anti-rabbit horseradish peroxidase antibody in 1% milk in TBS and visualized by chemiluminescence. A control with wild-type CD39 was performed to verify that readings were within the range in which protein concentration was proportional to band density (not shown).

Nucleotidase Assays. Nucleotidase reactions were carried out in a 200 μL solution containing 50 mM Tris-HCl, pH 7.5, 1 mM NaN_3, 1 mM EDTA, 1 mM HEDTA, 2 mM ATP or ADP, and varying concentrations of $CaCl_2$. NaN_3 was included in order to be consistent with earlier experiments in which it was used to inhibit background F-type ATPase activity in cell lysates; at this concentration, it does not affect wild-type or mutant CD39 activities under these conditions (unpublished results). Reaction solutions containing enzyme, $CaCl_2$, EDTA, HEDTA, and NaN_3 were preincubated for 10 min at 37 °C. Reactions were started by the addition of nucleotide and stopped after 20 min by removal from the 37 °C bath and addition of 300 μL of 10% SDS. Phosphate concentration was determined by the colorimetric method (*32*).

Calculation of Free Calcium. Concentrations of free calcium were calculated using the MaxChelator program with included calcium complex constants from Martell and Smith (*33*, *34*).

Plotting and Curve Fitting. The graphing program IGOR was used to display calcium titration curves and to calculate K_m for free calcium.

RESULTS

Full-Length and Truncated CD39 Families with Intact and Mutant ACR1. Four families of rat brain CD39 mutants were constructed by introducing ACR1 point mutations into four parent cDNAs: wild-type CD39 and CD39 lacking either the carboxy-terminal transmembrane domain and cytoplasmic tail (CT), the amino-terminal transmembrane domain and cytoplasmic tail (NT), or both transmembrane domains and cytoplasmic tails (sol). H59G was introduced into all four constructs, and H59S and N61R were introduced into wild type and CT, resulting in the wild-type family (wt, wtH59G, wtH59S, wtN61R), the CT family (CT, CTH59G, CTH59S, CTN61R), the NT family (NT, NTH59G), and the soluble family (sol, solH59G). All constructs were expressed in COS7 cells and either extracted by crude membrane preparation (wt, CT, and NT families) or secreted into the culture medium and partially purified on a ConA column (sol and solH59G).

Quantitation of Relative Activity per Unit of Protein. The relative amounts of protein required for the same amount of ATPase activity, 10 nmol of phosphate release per minute, were determined by densitometric analysis of an immunoblot with an antibody specific for the CD39 extracellular domain (Figure 1). For the wild-type and CT families, two bands are visible. The bands were not reducible by tripling the amount of β-mercaptoethanol in the sample loading buffer and therefore likely represent different degrees of posttranslational modification, possibly glycosylation. Since the relative contribution of each band to the total activity is unclear, both bands were included in the quantitation. The relative activity for each mutant was calculated by taking the ratio of the wild type to the mutant band density. All activities are reported as percent of wild-type ATPase activity. The activity of wild-type CD39 was 2 nmol of P_i (μg of protein)$^{-1}$ min^{-1}. Although comparisons of activity between members of different families may not be exact, the fact that relative band intensities are consistent within each family suggests that comparisons within families are accurate.

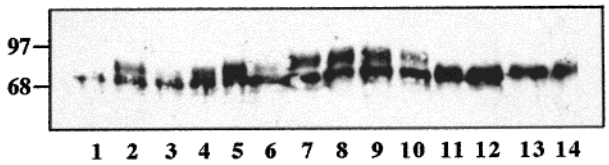

FIGURE 1: Expression in COS7 cells. Crude membranes from COS7 cells transfected with each construct were run on a 7.5% SDS−PAGE gel and immunoblotted with anti-CD39. Samples were loaded according to the amount of membranes required for a maximal ATPase activity of 10 nmol of P_i/min, or for a multiple or fraction of the given activity where indicated. The activity of the wild-type protein was 2 nmol of P_i (μg of protein)$^{-1}$ min^{-1}. Lane 1, wild-type CD39; lane 2, wild-type × 4; lane 3, wtH59G × 1/8; lane 4, wtH59G × 1/4; lane 5, wtH59S × 1/4; lane 6, wtN61R; lane 7, CT; lane 8, CTH59G; lane 9, CTH59S; lane 10, CTN61R; lane 11, NT; lane 12, NTH59G; lane 13, sol; lane 14, solH59G.

As described in detail below, removal of either or both transmembrane domains reduces activity, consistent with the previous report (*29*), and alters the kinetic profiles of ATP and ADP hydrolysis. The effects of ACR1 mutations on ADPase, ATPase, and relative ADPase:ATPase activities vary dramatically with the presence or absence of intact interactions between the two transmembrane domains. Sucrose density gradient sedimentation of the wild-type, NT, and CT enzymes and their corresponding ACR1 mutants verified that ACR1 point mutation did not significantly affect tetramer formation (data not shown).

H59 and N61 Mutations in Full-Length CD39. CD39 activity varies with free calcium concentration according to a biphasic curve consisting of an activation component followed by an inhibitory component at high free calcium concentration (Figure 2A). The activity for each protein was therefore determined by performing calcium titrations at a saturating substrate concentration. This approach makes it possible to distinguish between a change in maximal rate and a change in calcium affinity or inhibition.

As shown in Figure 2, replacement of H59 with either G or S dramatically reduced ATPase activity to 6.3% or 4.4% of wild type, respectively. ADPase activity was also reduced, but only to 51% or 54% of wild type. The differential effects on ATPase and ADPase activities converted the enzyme from an apyrase to an ADPase with an ADP:ATP hydrolyis ratio of 6:1 or 8:1, respectively. N61R mutation reduced ADP hydrolysis to a similar extent as did the H59 mutations, to 43% of wild type, but had a less severe effect on ATPase activity than did the H59 mutations, reducing it to 23% of wild type. wtN61R therefore remained an apyrase, although the ADP:ATP hydrolysis ratio rose from 0.7:1 in wild type to 1.3:1 in wtN61R.

All mutants in this family also exhibited alterations in the kinetic profile. wtH59G and wtH59S lost the high free

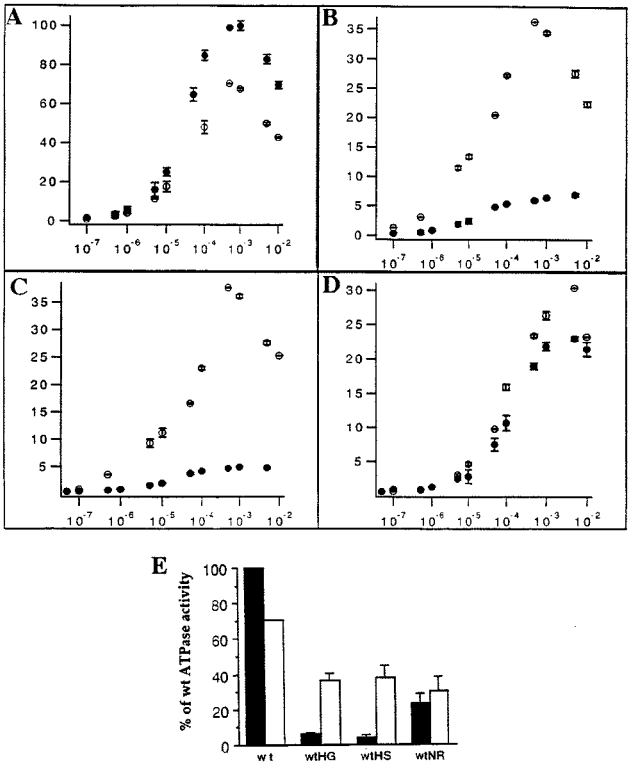

FIGURE 2: ATPase and ADPase activities of wild-type CD39 with intact and mutant ACR1. (A−D) Free calcium titrations with 2 mM ATP (filled circles) and 2 mM ADP (open circles). Activities are expressed as percent of wild-type maximal ATPase activity. (A) Wild-type CD39, (B) wtH59G, (C) wtH59S, (D) wtN61R. (E) Comparison of maximal ATPase (black) and ADPase (white) activities.

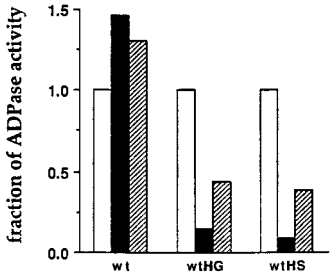

FIGURE 3: ATP competition in wild-type and H59 mutants. The extent of ATP competition with ADP was determined for wild type, wtH59G, and wtH59S by comparing relative phosphate production in the presence of 2 mM ADP (white), 2 mM ATP (black), and 2 mM ADP + 2 mM ATP (hatched) at 0.5 mM free calcium. Activities are expressed as fractions of ADPase activity for each construct.

calcium inhibitory component for ATP but not for ADP. In contrast, wtN61R showed a substrate-independent 5-fold increase in the K_m for free calcium activation.

To determine whether the loss of ATPase activity in wtH59G and wtH59S was due to a defect in binding or in hydrolysis, competition experiments to assess the ability of ATP to interfere with ADP hydrolysis were performed (Figure 3). With both mutants as well as with wild-type CD39, phosphate release was measured in the presence of 2 mM ADP, 2 mM ATP, and 2 mM ADP combined with 2 mM ATP, in each case with the total calcium adjusted to give a free calcium concentration of 0.5 mM. wtH59G and wtH59S showed significantly lower phosphate production in the presence of ADP plus ATP than with ADP alone, while

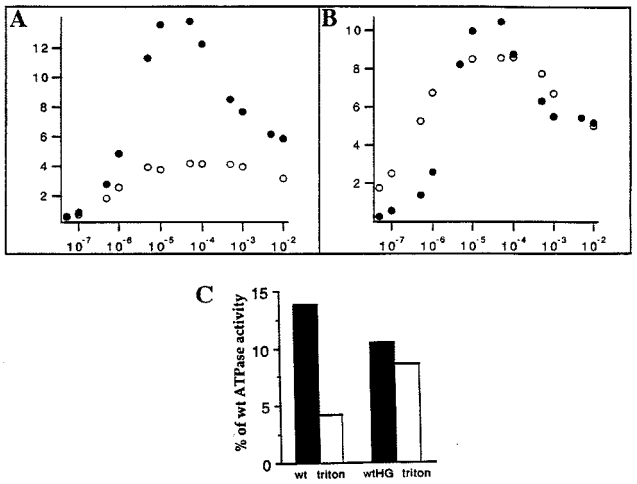

FIGURE 4: Triton-solubilized wild-type CD39 and wtH59G. Free calcium titrations in the presence of 2 mM ATP (filled circles) or 2 mM ADP (open circles) on wild type (A) and wtH59G (B) after solubilization in 1% Triton. Activities are expressed as percent of maximal ATPase activity in wild-type crude membranes. (C) Maximal ATPase (black) and ADPase (white) activities.

wild-type CD39 released more phosphate with ADP plus ATP than with ADP alone, as would be expected from its faster hydrolysis rate with ATP than with ADP. This suggests that a change in the hydrolysis rate, rather than in relative substrate binding, is responsible for the observed change in activity. Additional experiments, in which the ATPase activities of wtH59G and wtH59S were not increased by using 10 or 20 mM ATP (data not shown), are consistent with a hydrolysis rather than a binding deficiency.

Change in Activity and Kinetics with Detergent Solubilization. To determine the relevance of the intact quaternary structure for the observed wild-type and mutant properties, crude membranes were solubilized with 1% Triton X-100, a procedure that was previously shown to cause dissociation of rat brain CD39 tetramers to monomers (*29*). Calcium titrations were performed as for the crude membrane experiments, and activities are scaled according to the amount of expressed protein added to the reaction, as determined for the crude membranes. As shown in Figure 4, detergent solubilization significantly changes the properties of wild-type CD39. Maximal ATPase activity is reduced to 13.8% and ADPase to 5.9% of the respective crude membrane activities. The greater loss in ADPase activity lowers the ADP:ATP ratio from 0.7:1 in intact tetramers to 0.3:1 in solubilized monomers. In addition to the more dramatic loss in ADPase activity, detergent solubilization also abolishes the inhibitory component of the ADPase calcium titration curve. Finally, the K_m for free calcium is reduced for both ATP and ADP.

In contrast to its effect on wild-type CD39, detergent solubilization of wtH59G reduces ADPase activity only to 24% of the wtH59G ADPase activity in crude membranes and increases ATPase activity approximately 2-fold relative to crude membranes (compare Figure 2B with Figure 4B). As a result, in detergent wtH59G becomes an apyrase with nearly equal ATPase and ADPase activities. Solubilization thus has opposite effects on the ADPase to ATPase ratio in wild type compared to wtH59G; it increases the difference between the two activities in wild type and brings them closer together in the mutant. Comparison of the V_{max} activities of

wtH59S and wtN61R in detergent suggests that wtH59S follows the same pattern as wtH59G, with ATPase and ADPase activities becoming nearly equal, while wtN61R has a ratio similar to that of solubilized wild type (data not shown).

In addition to the changes in hydrolysis rates, wtH59G exhibits a reduction in the K_m for free calcium with either ATP or ADP as the substrate, similar to the wild type. In contrast to the wild type, the wtH59G calcium profile for ADP retains its inhibitory component upon solubilization. Finally, solubilization of wtH59G restores the ATPase inhibitory component that was missing in wtH59G crude membranes.

Truncation of C-Terminal Transmembrane Domain. While detergent solubilization is known to disrupt quaternary structure by breaking apart tetramers, the possibility that detergent has an additional effect on the active site cannot be ruled out. Removing the carboxy-terminal transmembrane domain has been shown to yield tetramers with significantly reduced activity compared to wild-type tetramers (*29*), suggesting that although subunits can interact, presumably through the amino-terminal transmembrane domain, the intersubunit contacts necessary for full activity are lost. To study the relationship between quaternary structure and ACR1 under detergent-free conditions, we therefore expressed constructs without the carboxy-terminal transmembrane domain and with either intact ACR1 or the H59G, H59S, or N61R mutations. As in the full-length CD39 experiments, calcium titrations on crude membranes were used to compare activity and kinetic properties. Activity is expressed relative to maximal ATPase activity in wild-type CD39 and is scaled according to protein expression.

C-terminal truncated CD39 with intact ACR1 (CT), despite its sedimentation as a tetramer, behaves more like the detergent-solubilized full-length CD39 monomer than like the intact full-length tetramer (Figure 5). Like the monomer, CT has lower ATPase and ADPase activities, with a greater loss of ADPase relative to ATPase activity to yield an ADPase to ATPase ratio of 0.4:1. Also similarly to the solubilized monomer, CT exhibits a reduction in the K_m for free calcium in the presence of both substrates. The inhibitory component of the calcium titration curve is lost for ADP, but not for ATP, as in the solubilized monomer.

Calcium titrations with CTH59G and CTH59S reveal that the effect of mutating H59 to G or S depends significantly on the presence of the carboxy-terminal transmembrane domain. Rather than diminishing ATPase activity and having a more modest effect on ADPase activity, as in the full-length protein, H59 substitution in CT increases the maximal ADPase activity approximately 2-fold and has no effect on the maximal ATPase activity, both in comparison to CT. Changes in the kinetic profile are consistent with the H59 mutations having an effect on ADPase activity. In particular, the inhibitory component for ADPase that is lost in CT is restored by the H59 mutations, possibly similar to the restoration of ATPase inhibition in full-length H59G in conjunction with the increase in ATPase activity upon detergent solubilization. All of these differences between CT and the CTH59 mutants mimic the differences between solubilized full-length wild-type and H59 mutants, indicating that the disruption of quaternary structure, rather than the presence of detergent or of the transmembrane domain itself, is responsible for the context dependence of H59 substitution effects.

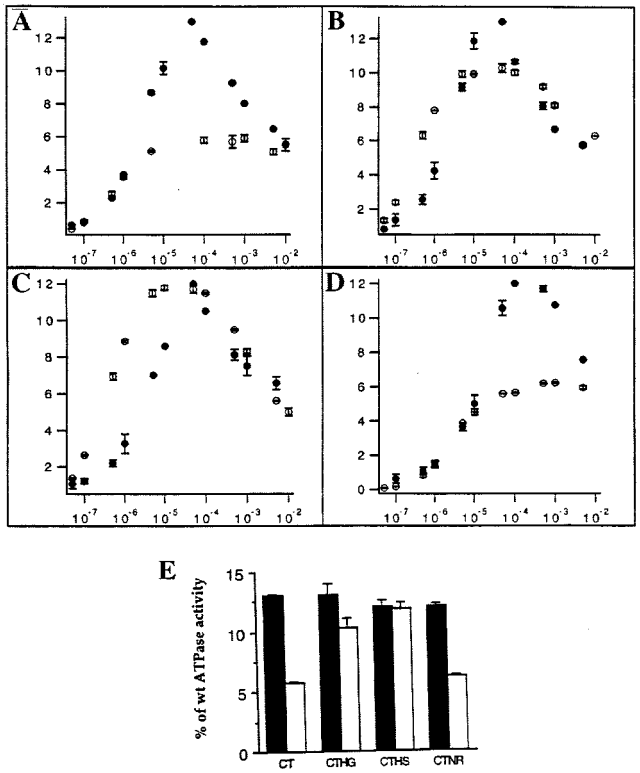

FIGURE 5: ATPase and ADPase activities of CD39 lacking the C-terminal transmembrane domain with intact and mutant ACR1. (A−D) Free calcium titrations with 2 mM ATP (filled circles) and 2 mM ADP (open circles). Activities are expressed as percent of maximal wild-type ATPase activity. (A) CT, (B) CTH59G, (C) CTH59S, (D) CTN61R. (E) Comparison of maximal ATPase (black) and ADPase (white) activities.

As in the full-length protein, N61R substitution resulted in an increase in the K_m for free calcium in the presence of both ATP and ADP. However, in contrast to the loss of activity resulting from the substitution in full-length CD39, N61R substitution in CT had no effect on ATPase activity and slightly increased ADPase activity. As with the CTH59 mutants, the ADPase to ATPase ratio resembles the solubilized rather than the crude membrane form of wtN61R.

Truncation of N-Terminal Transmembrane Domain. To explore further the role of the two transmembrane domains in regulating activity at ACR1, we expressed a version of CD39, NT, that lacks the amino-terminal rather than the carboxy-terminal transmembrane domain. Previous studies of NT showed that while it is not entirely tetrameric, the tetramers that form have higher activity than CT tetramers (*29*), suggesting that intersubunit contacts in NT may retain certain features of the wild type that are not retained in CT.

As previously reported (*29*), NT with intact ACR1 exhibited reduced ATPase and ADPase activities (Figure 6). Like CT, the K_m for free calcium in the presence of both substrates was lower than in wild-type CD39. However, unlike CT, NT retains the inhibitory component for ADPase activity, and the ADPase to ATPase ratio of 0.6 to 1 is closer to that of wild type.

Given the V_{max} ratio intermediate between those for intact tetramers and for CT or solubilized monomers, we asked whether the quaternary interactions mediated by the carboxy-

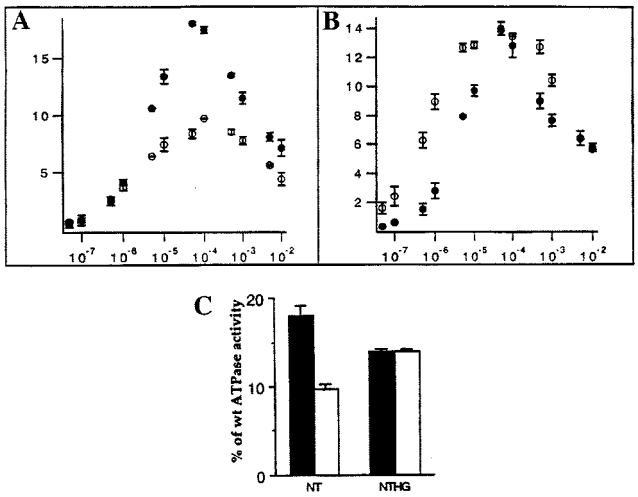

FIGURE 6: ATPase and ADPase activities of CD39 lacking the N-terminal transmembrane domain with intact and mutant ACR1. Free calcium titrations with 2 mM ATP (filled circles) and 2 mM ADP (open circles) using (A) NT and (B) NTH59G. (C) Comparison of maximal ATPase (black) and ADPase (white) activities.

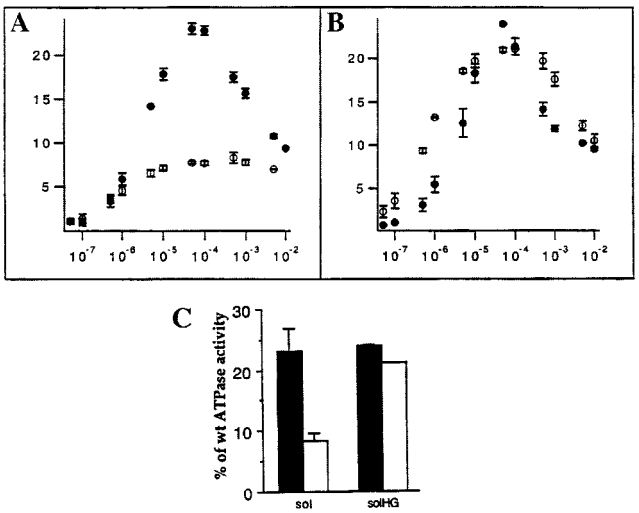

FIGURE 7: ATPase and ADPase activities of soluble CD39 lacking both transmembrane domains with intact and mutant ACR1. Free calcium titrations with 2 mM ATP (filled circles) and 2 mM ADP (open circles) using (A) soluble CD39 and (B) soluble CD39 with H59G mutation. (C) Comparison of maximal ATPase (black) and ADPase (white) activities.

terminal transmembrane domains alone might retain some of the character of the wild-type tetramers and thus have a similarly intermediate effect on interactions at ACR1, as measured by differences between proteins with intact or mutant ACR1. We therefore expressed a version of NT with the H59G mutation. As shown in Figure 6, V_{max} for ADP was increased approximately 1.5-fold while V_{max} for ATP was slightly decreased. As a result, ADPase activity was equal to ATPase activity rather than slightly lower as observed for CTH59G and solubilized wtH59G. Nevertheless, the activity differences between NT and NTH59G were similar to those between CT and CTH59G. H59G substitution restores ADPase inhibition in CT while having no obvious effect on ADPase inhibition in NT.

Soluble CD39 Lacking both Transmembrane Domains. Finally, we expressed a soluble form of CD39 in order to compare the above results with a detergent-free system in which absolutely no quaternary contacts are maintained. As previously described, soluble CD39 lacks both transmembrane domains, has activity similar to the solubilized full-length monomer, and is exclusively monomeric (*29*). Accordingly, the activity, the V_{max} ratio, the reduced K_m for free calcium in the presence of either ATP or ADP, and the loss of the inhibitory component of the ADPase calcium profile were all identical to the properties observed for solubilized full-length CD39 (Figure 7). The corresponding soluble CD39 with the H59G mutation behaved identically to the detergent-solubilized wtH59G; ATPase activity was unaffected, and ADPase activity was 2.5-fold higher than in soluble CD39.

DISCUSSION

These studies demonstrate that quaternary structure, as regulated by transmembrane domain interactions, influences the total and relative ATPase and ADPase activities of the ecto-apyrase CD39 through H59 in ACR1. In intact tetramers, H59 appears to play a key role in ATP and a smaller role in ADP hydrolysis, as indicated by the loss of more than 90% of ATPase activity but less than 50% of ADPase activity upon substitution with G or S. In contrast, when the quaternary structure is lost or disrupted, H59 appears to play a minor, if any, role in ATP hydrolysis and to hinder ADP hydrolysis. These results suggest that ATP and ADP hydrolysis occur by different mechanisms distinguished by different substrate interactions with ACR1, and that these interactions are each dramatically altered by changes in quaternary structure.

While the importance of H59 is consistent with a previous report implicating a histidine residue in hydrolysis (*35*, *36*), the finding that H59 affects primarily the ATP rather than ADP hydrolysis rate is counterintuitive in light of the putative role of ACR1 as the β-phosphate binding domain (*25*). The crystal structures of actin with ATP and with ADP (*26*), as well as the proposed mechanism for hsp70 ATPase activity (*37*) based on the hsp70 (*38*) and actin structures (*26*), suggest that ACR4, the putative γ-phosphate binding domain, contains the residues involved in ATP hydrolysis and that ACR1 plays a more direct role in ADP hydrolysis. In fact, these predictions are consistent with the behavior of truncated or monomeric CD39, in which ADP rather than ATP hydrolysis is affected by H59 substitution. The fact that substitution with G or S results in an increase in ADPase activity in monomeric or truncated backgrounds can be understood in terms of the presence of a G or S in all other soluble apyrases; if, as suggested by the actin−ADP crystal structure, the backbone of the residue at that position contacts the β-phosphate, the presence of a large histidine might distort the substrate orientation. We suggest that oligomerization creates an altered active site such that the roles of each ACR suggested by analogy with the actin/hsp70/hexokinase superfamily may no longer be valid. The presence of H59, which occurs only in membrane-bound family members, appears to be central to the increase in ATPase activity gained upon oligomerization, although the basis for increased ADPase activity in oligomers remains unclear.

The role of N61 in ATP and ADP hydrolysis also appears to depend on the intact tetramer structure; N61R substitution

decreases both ATP and ADP hydrolysis rates by more than 50% in the wild-type protein but causes only slight or no change in maximal activity in CT. On the other hand, in both the tetrameric and the monomeric contexts, N61R substitution has a much smaller effect on the ADP:ATP activity ratio than do the corresponding H59 mutants. These results suggest that while quaternary structure regulates the roles of both H59 and N61, H59 is more central to the distinction between ATPase and ADPase mechanisms and to the relation between quaternary structure and ADP:ATP rate ratio. The findings that mutations of the completely conserved ACR1 residues D54 and G56 in CT (Mas Handa, personal communication) and of the equivalent residues in the human wild-type CD39 (*27*), as well as deletion of the entire ACR1 (*39*), all abolish both ATPase and ADPase activity further highlight H59 as a key to structure-dependent ADP:ATP ratio determination.

In this study we used calcium titrations rather than single calcium concentrations to analyze changes in total and relative activities. We showed that the kinetic profile for calcium is biphasic, with one activation and one inhibition component, and that changes in maximal activity caused by truncation or point mutation are in many cases accompanied by changes in the K_m for activation and in the presence of the inhibitory component (summarized in Table 1). The use of titrations avoids the problem of comparing activities for different proteins or substrates at different stages of activation or inhibition and thus masking or obtaining misleading information on changes in total and relative activities. In addition, changes in the kinetic profile provide further insight into the relation among quaternary structure, ACR1 residues, and activity.

First, the truncation-regulated difference in ACR1 point mutation effects is correlated with a decrease in the K_m for calcium activation by 1 or more orders of magnitude. The size of the decrease in K_m is independent of whether one or both transmembrane domains are removed or dissociated. The link between the correlated sets of changes is unknown but underscores the idea that the specific quaternary structure governed by interactions between both transmembrane domains gives rise to a specific set of active site−substrate interactions and that disruption of this structure results in a different specific set of substrate interactions characteristic of an individual subunit. As discussed below, these observations raise the question of whether a different set of transmembrane domains, such as those found in the ecto-ATPase, might give rise to a third specific set of active site−substrate interactions.

Comparison of kinetic profiles also gives further insight into the contribution of ACR1 residues to ADP and ATP hydrolysis mechanisms. In the case of H59 substitution, the differential effects on ADPase and ATPase V_{max} are paralleled by differential effects on the high calcium inhibitory component, with the same quaternary structure dependence for both features. In contrast, N61R substitution increases K_m in a manner which is the same for ADP and ATP, does not necessarily correlate with changes in V_{max}, and is independent of quaternary structure. Unfortunately, not enough is known about the molecular bases for K_m and the inhibitory component to interpret these results in detail. Since it cannot be determined from our data whether activation and inhibition with increasing calcium are due to the increase in free calcium concentration, to the increase in calcium−nucleotide complex, or to the decrease in free nucleotide concentration, changes in K_m or inhibition may reflect changes in affinity for calcium, nucleotide, or calcium−nucleotide complex at one or more sites. Nevertheless, the kinetic results complement the V_{max} data in support of a quaternary structure-dependent role for H59 in distinguishing between ATPase and ADPase mechanisms.

How quaternary structure might regulate a change in hydrolysis mechanisms is unclear. One possible model for the relationship among transmembrane domains, quaternary structure, and active site may be the two transmembrane domain, tetrameric potassium channels. Like CD39, the inward rectifier potassium channel $K_{ir}2.1$ has two transmembrane domains which bridge an extracellular domain and which regulate formation of a tetramer. As shown in ref *41*, the transmembrane domain sequences limit their packing to an arrangement in which each transmembrane domain lies near one C-terminal and one N-terminal transmembrane domain. In contrast, Kcsa, another tetrameric potassium channel with two transmembrane domains, has been shown to pack in a completely different form (*42*). The finding that two channels each with two transmembrane domains and tetrameric structure but different transmembrane sequences can form such different types of tetramers raises the possibility that different members of the ecto-apyrase/ecto-ATPase family might also form different types of tetramers. Formation of different tetramer structures might in turn result in different tertiary structures within each subunit, resulting in different active site−substrate interactions. In this way, different ADP:ATP ratios might be observed for family members with similar ACR sequences but different transmembrane sequences, such as the ecto-apyrases and the ecto-ATPases.

This hypothesis does not explain the basis for the different ADP:ATP hydrolysis ratios observed among soluble family members with similar ACR sequences. Of the soluble forms that have been both sequenced and characterized, all have S or G in place of H59 (Table 1), yet human CD39L4 has a 20:1 ADPase:ATPase ratio (*43*) while *Toxoplasma gondii* NTPase1 and NTPase3 have ratios of 1:1 and 1:100, respectively (*44*). Differences between these activity ratios and that of the soluble H59G construct described in this paper may be at least partially related to the presence of the conserved R among the soluble family members (Table 1) in place of N61 in our soluble H59G construct. Whether any of the inherently soluble enzymes oligomerize or form complexes with other proteins is unknown, but it is interesting that the substrate specificities of *Toxoplasma gondii* NTPase1 and NTPase3 have been found to be determined by a block of residues near the C-terminus (*45*).

In this study, we find that the quaternary structure of CD39 regulates differential roles of ACR1 in ATP and ADP hydrolysis. In particular, we identify ACR1 residues, H59 and N61, whose roles are regulated by quaternary structure. Through mutation of each residue, we uncover differences in the mechanisms of ADP and ATP hydrolysis in intact tetramers and identify H59 as central to ATP hydrolysis and to the distinction between the mechanisms. We suggest that quaternary structure, which is determined by transmembrane domain interactions, affects the ADP:ATP activity ratios by altering the roles of key ACR residues. This might explain

how ecto-apyrases and ecto-ATPases with similar ACR sequence and different transmembrane sequences have different ADP:ATP activity ratios.

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