Dynamic Motions of CD39 Transmembrane Domains Regulate and Are Regulated by the Enzymatic Active Site†

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ABSTRACT: The two transmembrane domains flanking the active site of CD39 regulate its activity, but little is known about the structural and dynamic features underlying their importance. Here we use a disulfide crosslinking strategy to examine transmembrane helix interactions and dynamics and to correlate these features with activity and substrate binding. We find strong intrasubunit TM1−TM2 interactions, as well as TM1−TM1′ and TM2−TM2′ interactions between dimer subunits, near the extracellular side of the membrane but only weak interactions near the cytoplasmic end. The specific helix faces that constitute each interface are highly flexible, indicating a significant degree of rotational mobility within the packed structure. Analysis of activity after locking the helices in various orientations via disulfide bonds suggests that not only the arrangement but also the ability of the helices to move relative to each other is crucial for enzyme function. Helix mobility is in turn modulated by substrate binding. These results suggest that rather than playing a static structural role to support an optimal active site conformation, the transmembrane domains undergo dynamic motions that underlie their functional relationship with the active site.

A variety of cell types secrete nucleotides into the extracellular space constitutively and in response to mechanical, biochemical, and electrical stimuli (*1, 2*). Once outside the cytoplasm, nucleotides and their derivatives act via purinergic receptors to regulate processes such as platelet clotting, vascular homeostasis, cell size maintenance, neuronal signaling, immune function, and cell growth (*1*−*3*). Since nucleotides cannot be transported back into the cytoplasm until their phosphates have been removed (*4*), local concentrations of nucleotides and their derivatives are regulated primarily by hydrolytic ectoenzymes. In particular, the ubiquitous family of ectoenzymes known as the ecto-nucleoside triphosphate diphosphohydrolases (eNTPDases)[1] specifically hydrolyzes the terminal phosphoanhydride bonds of tri- and dinucleotides (*5*−*7*). The family includes membrane-bound and -secreted enzymes, both at the cell surface and in intracellular organelles, with a range of substrate specificities (*5*−*7*). At the cell surface, the membrane-bound enzyme CD39 (eNTPDase1) hydrolyzes both tri- and dinucleotides and has been shown to play a primary role in regulating a variety of vascular and inflammatory processes (*8*−*10*). Unlike most other ectoenzymes, CD39's function depends on its transmembrane regions (*11*), suggesting the potential for cross talk between the active site and the membrane or cytoplasmic parts of the protein.

A CD39 molecule consists of a large extracellular domain flanked by one amino-terminal and one carboxy-terminal transmembrane domain, each of which has a small cytoplasmic tail (*12*). The active site is thought to reside in the extracellular domain, based on the presence of five apyrase conserved regions (ACRs) which are shared with other members of the eNTPDase family and which alter activity and substrate specificity when mutated (*13*−*17*). Although the specific functions of the ACRs are unknown, the homology of ACR1 and ACR4 to the phosphate binding domains of the actin−hsp70 ATPase superfamily (*13*) suggests a similar role in phosphate binding and hydrolysis in the eNTPDases. At least one of these regions, ACR1, is expected to be near the membrane based on its primary sequence location being fewer than 20 amino acids downstream of the first transmembrane domain. ACR5 is similarly located near the second transmembrane domain.

The original evidence that the transmembrane domains are required for full enzymatic activity was the loss of activity upon solubilization with most detergents (*11, 18*). Wang *et al.* (*11*) subsequently showed that removing both transmembrane domains to create a secreted extracellular domain mimicked the detergent effect and rendered the protein insensitive to detergent. Furthermore, removal of either the first (TM1) or second (TM2) transmembrane domain produced the same result as detergent or double truncation despite insertion in the membrane. These experiments led to the conclusion that maximal CD39 activity requires both of its transmembrane domains to be present and in the membrane.

Subsequent studies have demonstrated that the interplay between the transmembrane domains and the active site governs not only total activity but also substrate specificity (*15*), the role of the ACRs in the hydrolysis mechanism (*19*), and whether ADP is released or retained for further degrada-

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[1] Abbreviations: eNTPDase, ecto-nucleoside triphosphate diphosphohydrolase; ACR, apyrase conserved region; TM1, transmembrane domain 1; TM2, transmembrane domain 2; CuP, copper phenanthroline; malPEG, mPEG−maleimide.

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tion following hydrolysis of ATP (*20*). CD39 has also been proposed to act as an ATP channel (*21*) and to bind ran-binding protein with its cytoplasmic tail (*22*), raising the possibility that the transmembrane domains might also mediate transport or signaling functions and potentially provide a link between such functions and enzymatic activity.

Nevertheless, a structural picture of what exactly the transmembrane domains do that makes them so important remains to be developed. Most discussion of their role has revolved around oligomer formation, which they are thought to mediate due to the fact that the full-length protein is dimeric in certain detergents while the soluble extracellular domain alone is a monomer (*11*). However, activity does not correlate with oligomer formation per se; both the TM1- and TM2-truncated constructs, as well as the full-length protein when solubilized in cholate, form dimers but have low activity (*11*, *15*). Therefore, while oligomerization may be part of the transmembrane domains' function, it alone does not explain the connection between transmembrane domains and activity. Further understanding of how or whether TM1 and TM2 interact both within and between monomers, as well as the dynamics of their organization, is essential to any explanation of how the transmembrane domains regulate activity.

We have used oxidative crosslinking of introduced cysteines to study transmembrane domain interactions and mobility as well as how these features relate to activity. Oxidative sulfhydryl crosslinking has been used to map intra- and intersubunit transmembrane helix interactions in a variety of proteins, including the bacterial chemoreceptors Tar (*23*), Trg (*24*−*26*), and McpB (*27*), the V-ATPase (*28*) and F_1F_0-ATPase (*29*), G-protein coupled receptors (*30*−*32*), potassium channels (*33*), and the influenza M2 channel (*34*). The technique is based on the principle that if cysteine pairs are substituted one at a time for residues in the transmembrane domains, only those that are oriented toward each other and within the length of a disulfide bond will be crosslinked upon addition of an oxidizing agent. Since the reaction is performed over a period of time, helix mobility can be revealed by disulfides expected to represent different orientations of the helices. Since the protein can be maintained in its native environment, the relationship between orientation and function can be analyzed by examining both the effect of ligands or other conditions on crosslinking pattern and the effect of crosslinking on function. Using this strategy, we identify TM1 and TM2 interactions near the extracellular side of the membrane both within and between dimer subunits, and find that the helices exhibit a significant degree of relative mobility which is critical for enzymatic activity and modulated by the substrate.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. C13 in the amino-terminal cytoplasmic tail of rat brain CD39 was changed to serine using the QuikChange multi site-directed mutagenesis kit (Stratagene). An *Xho*I site was introduced at the site of the substitution for colony screening. All further substitutions were performed in this background using the same kit. Cysteines were introduced either one at a time or in pairs, and colonies were screened for each mutation using silently introduced restriction sites. The residues changed to cysteine and the corresponding restriction sites that were introduced are as follows: L18, I19, and I20 with *Avr*II; L21 with *Nde*I; A28−V35 with *Hpa*I; G36 with *Ase*I; L37 with *Apa*LI; Y479−L488 with *Sca*I; and I495, T496, G497, and L498 with *Nru*I. Mutagenesis reactions were performed in pBluescript, and the CD39 coding region was transferred to pCIneo containing a 3′ HA tag and sequenced following screening. Cysteines not paired during the original mutagenesis reactions were paired by transfer of *Nhe*I−*Sac*II or *Sac*II−*Not*I fragments in pCIneo.

Preparation of COS7 Cell Crude Membranes. COS7 cells were transiently transfected with 6 μg plasmid per 100 mm dish using Lipofectamine (Invitrogen). For cotransfection of wild-type CD39 and G36C, 3 μg of each plasmid was used per 100 mm dish. Cells were harvested 72 h after transfection, and crude membranes were prepared by the method described in ref *35*. Membranes were resuspended in 50 μL of 50 mM Tris-HCl (pH 7.8) per 100 mm plate, and aliquots were flash-frozen in liquid nitrogen and stored at −80 °C.

Oxidative Crosslinking. Cysteine-substituted protein was crosslinked in crude membranes using copper phenanthroline as the oxidizing agent. Copper phenanthroline (CuP) was prepared by combining cupric sulfate and 1,10-phenanthroline (Sigma) at a 1:3 molar ratio in water and used at a final concentration of 0.1 mM (expressed in terms of Cu^{2+} concentration). Reactions were carried out in 12 mM Tris-HCl (pH 7.5) at 4, 18, or 37 °C as indicated, for 5 min unless otherwise indicated. For samples loaded directly onto a gel, reactions were stopped by adding an equal volume of nonreducing SDS loading buffer containing 20 mM *N*-ethylmaleimide (NEM) and 20 mM EDTA. Reactions in samples to be further treated with mPEG−maleimide or to be used for sucrose gradients or nucleotidase assays were stopped as described below.

Reaction with mPEG−Maleimide. Crosslinking reactions were stopped by adding 0.5 volume of 20 mM Tris-HCl (pH 7.5) containing 20 mM EDTA and 1% SDS instead of by adding SDS loading buffer. An additional 0.5 volume of 60 mM mPEG−maleimide, molecular weight of 5000 (Nektar Therapeutics), dissolved in water was added, for a final concentration of 15 mM. The mixture was vortexed and incubated at 18 °C for 20 min, and the reaction was stopped with an equal volume of nonreducing SDS loading buffer containing NEM and EDTA as described above.

Western Blot Analysis. Samples mixed with nonreducing loading buffer as described above were heated for 10 min at 65 °C, resolved on a 5.5% SDS−polyacrylamide gel as described previously, and transferred to nitrocellulose at 250 mA for 2.5 h. Nitrocellulose membranes were probed with anti-HA11 monoclonal antibody (Covance) in 2% milk in a Tris-buffered saline/0.1% Tween 20 mixture, followed by a secondary anti-mouse horseradish peroxidase-conjugated antibody (Sigma) in 2.5% milk in a Tris-buffered saline/0.1% Tween 20 mixture. The protein was visualized by chemiluminescence (substrate from Pierce). Anti-CD39 polyclonal antibody was generated and used as described in ref *36*.

Sucrose Density Gradient Sedimentation. Crosslinking reactions were stopped by adding EDTA to a final concentration of 1 mM, and membranes were solubilized with 2% digitonin (Calbiochem) on ice for 1 h. Each sample was layered on top of a 4.5 mL continuous 5 to 25% sucrose gradient containing 50 mM Tris-HCl (pH 7.8), 1 mM EDTA,

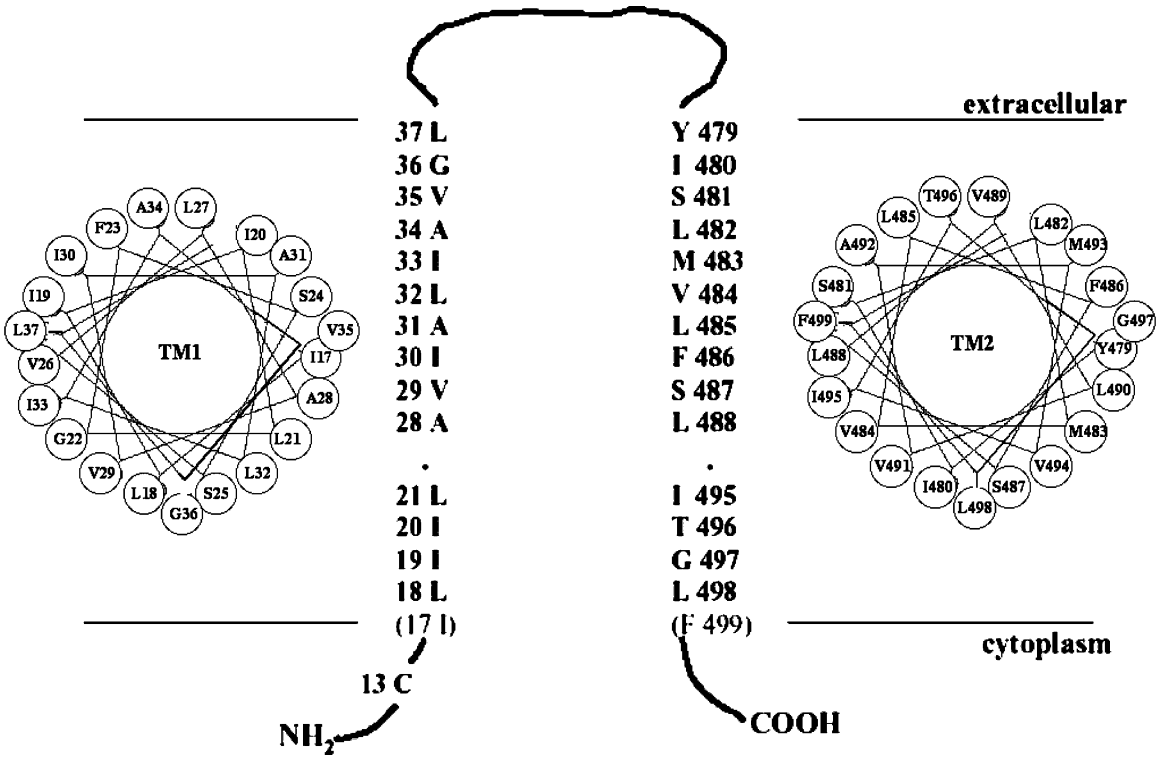

FIGURE 1: Residues changed to cysteine. Cysteine substitutions were introduced at the indicated positions into a CD39 parent molecule containing a C13S substitution. Helical wheel projections of TM1 and TM2, arranged clockwise from the N- to C-terminus, are shown next to the primary sequences.

1 mM HEDTA, 4.2 mM $CaCl_2$, and 0.2% digitonin. Gradients were centrifuged in a SW50.1 rotor at 40 000 rpm and 4 °C for 14 h. Fractions (300 μL) were collected from the top of the gradient, and activity was determined by adding 8 μL of 50 mM ATP to 192 μL of each fraction and following the reaction protocol described below.

Measurement of Activity after Crosslinking. Crosslinking reactions were stopped by 10-fold dilution of the mixtures with assay buffer containing 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, and 1 mM HEDTA. ATPase reactions were carried out in 200 μL containing the same buffer, a 25 μL aliqout of the diluted crosslinked sample, 4.2 mM $CaCl_2$, and 2 mM ATP. Samples were incubated for 20 min at 37 °C and the reactions stopped with 300 μL of 10% SDS. The amount of phosphate released was measured by the Ames colorimetric method (*37*).

RESULTS

Wild-type CD39 contains 10 extracellular cysteines, all of which are assumed to be oxidized to disulfides, as well as one unpaired cysteine at position 13 in the amino-terminal cytoplasmic tail. Although the wild-type protein did not crosslink in the presence of CuP, we nevertheless changed C13 to serine to avoid potential interference with cysteines introduced into the transmembrane domains. C13 has been suggested to be palmitoylated (*38*), but C13S substitution had no effect on the activity, detergent sensitivity, or dimerization of the protein. C13S was therefore used as the parent molecule and control for all transmembrane domain cysteine substitutions.

Single-Cysteine Substitutions. TM1 and TM2 residues at the positions shown in Figure 1 were individually changed to cysteine. Cysteine substitution had minimal effects on activity at all positions except L32 (65% of C13S activity), M483 (55%), and Y479 (20%). Dimerization of L32C and M483C was indistinguishable from C13S, as assessed by sucrose density gradient sedimentation, while Y479C was constitutively crosslinked (see below). All proteins except Y479C, which already had detergent level activity, retained sensitivity to Triton X-100.

Since each monomer contains only one free cysteine, disulfides can form only between oligomer subunits at potential TM1−TM1′ or TM2−TM2′ interfaces, where TM1′ and TM2′ refer to subunits different from TM1 and TM2. Crosslinked proteins can thus be identified by an increase in the apparent molecular weight corresponding to disulfide-linked dimers. As shown in Figure 2, strong crosslinking occurs via both TM1 and TM2 residues in the membrane region near the extracellular domain. The monomer runs at 80 kDa, as previously observed, and the disulfide-linked dimer runs at ~130 kDa. The minor 54 kDa band corresponds to a C-terminal proteolytic fragment routinely observed by us and others. At 37 °C, crosslinking is complete at positions L32−L37 on TM1 as well as V484−Y479 on TM2, despite the fact that sequential positions are predicted to reside on different faces of an α-helix. Some variation in crosslinking propensity consistent with helical organization is observed at 18 °C, but all positions are still 50−100% crosslinked. Only at 4 °C is a clear pattern observed; L32, V35, and G36 define a TM1−TM1′ interface, and M483, L482, and potentially I480 and Y479 define a TM2−TM2′ interface. Dissociation of CD39 to monomers by solubilization with Triton X-100 completely prevents crosslinking at all positions (not shown), verifying that crosslinking occurs between intact membrane-bound dimers rather than nonspecifically in solution.

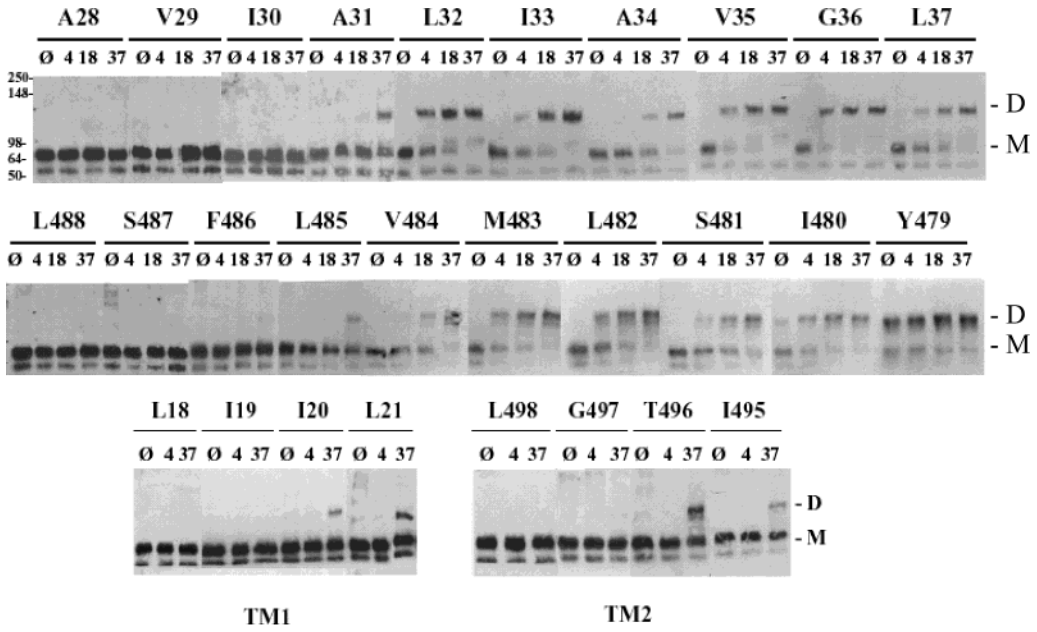

FIGURE 2: Single cysteines in TM1 and TM2. Crude membranes containing single-cysteine substitutions in either TM1 or TM2 were exposed either to buffer alone (Ø) or to 0.1 mM copper phenanthroline at 4, 18, or 37 °C: D for dimer and M for monomer.

In contrast, residues near the center of the membrane do not crosslink, and residues near the cytoplasmic end crosslink weakly at 37 °C, not at all at low temperature, and on one side of the helix. An assay for membrane sidedness and permeability based on oubain-inhibitable Na,K-ATPase activity has indicated that the membranes are unsealed; therefore, differences in CuP accessibility are unlikely to account for the difference in crosslinking propensity between the cytoplasmic and extracellular sides. While the lack of crosslinking near the center of the membrane might reflect a difference in reactivity as well as interhelical distance, the results highlight the extracellular end as the region of the strongest interaction.

While the crosslinking pattern at 4 °C suggests specific helical surfaces that predominate at the intersubunit interface, the ability of residues on all faces to crosslink extensively at higher temperatures is unexpected based on comparison with the transmembrane regions of other proteins studied under similar conditions. Some proteins do exhibit minor crosslinking at positions other than the primary interfaces, but the interfacial positions generally stand out much more clearly at physiological temperatures (*26*, *30*). When significant crosslinking at other positions has been observed, it has often been consistent with physiologically relevant features of the protein, such as higher-order oligomers (*27*) or conformational flexibility (*28*, *39*). We therefore sought to understand the basis for crosslinking at multiple interfaces and its relevance to enzyme function.

The simplest scenarios that might explain crosslinking at multiple interfaces are rotational mobility, higher-order oligomers, or transient collisions between dimers. As an initial test for the latter case, we measured the size of crosslinked proteins by sucrose density gradient sedimentation, following solubilization in digitonin to preserve subunit interactions. If dimer−dimer collisions were responsible for disulfide formation at certain positions, the sedimentation position would be expected to shift due to trapping of the protein as a tetramer. As shown in Figure 3, proteins crosslinked via positions I33, A34, and G36, which exhibit different temperature dependences and are expected to be on different faces of TM1, are all dimeric, as indicated by their sedimentation occurring at the same rate as that of C13S. Similar results were obtained for TM2, as well as with cholate, another detergent that preserves dimers (not shown). These results suggest that crosslinking does not change the native oligomerization state.

TM1−TM2 Cysteine Pairs. While CD39 is dimeric in certain detergents (*11*) and on a native blue gel (*40*), the possibility of higher-order oligomers occuring in native membranes has not been ruled out. For any single-cysteine mutant, the highest oligomer that can be observed on an SDS gel is a disulfide-linked dimer, but constructs with cysteines in both TM1 and TM2 have the potential to form disulfide-linked tetramers or even larger oligomers. If CD39 does form higher-order oligomers, they should be revealed by double-cysteine constructs in which the two cysteines face different subunits. On the other hand, if the multiple interfaces observed for single-cysteine constructs reflect rotational mobility within a dimer, double-cysteine constructs should form only monomers or dimers when crosslinked.

To distinguish between these possibilities, as well as to map potential TM1−TM2 interactions, we created a series of TM1−TM2 cysteine pairs in the region near the extracellular domain. To ensure observation of as many potential interfaces as possible, each position was paired with a set of positions on the opposite transmembrane domain that corresponds to at least one turn of a helix. As shown in Figure 4A, higher-order oligomers are never observed when A34 or S481 (both temperature sensitive) or L482 or G36 (both less sensitive to temperature) is sequentially paired with residues representing a turn of the helix on the opposite transmembrane domain. Furthermore, little or no dimer is observed for any of the pairs, despite the fact that each position alone crosslinks completely to dimers under the same conditions. The presence of two cysteines does not disrupt dimer formation as measured by sucrose density

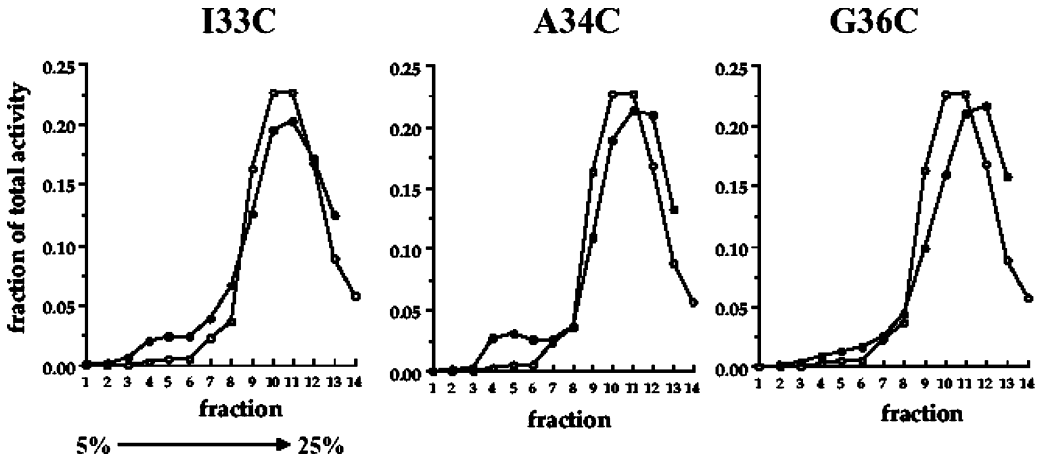

FIGURE 3: Sucrose density gradient sedimentation of crosslinked species. Constructs containing single-cysteine substitutions (●) or the C13S substitution only (○) were treated with copper phenanthroline in crude membranes, solubilized with digitonin, and centrifuged in a 5 to 25% sucrose gradient containing 0.2% digitonin. Fractions were collected from the top of the gradient and assayed for ATPase activity. Fraction 1 represents the top of the gradient.

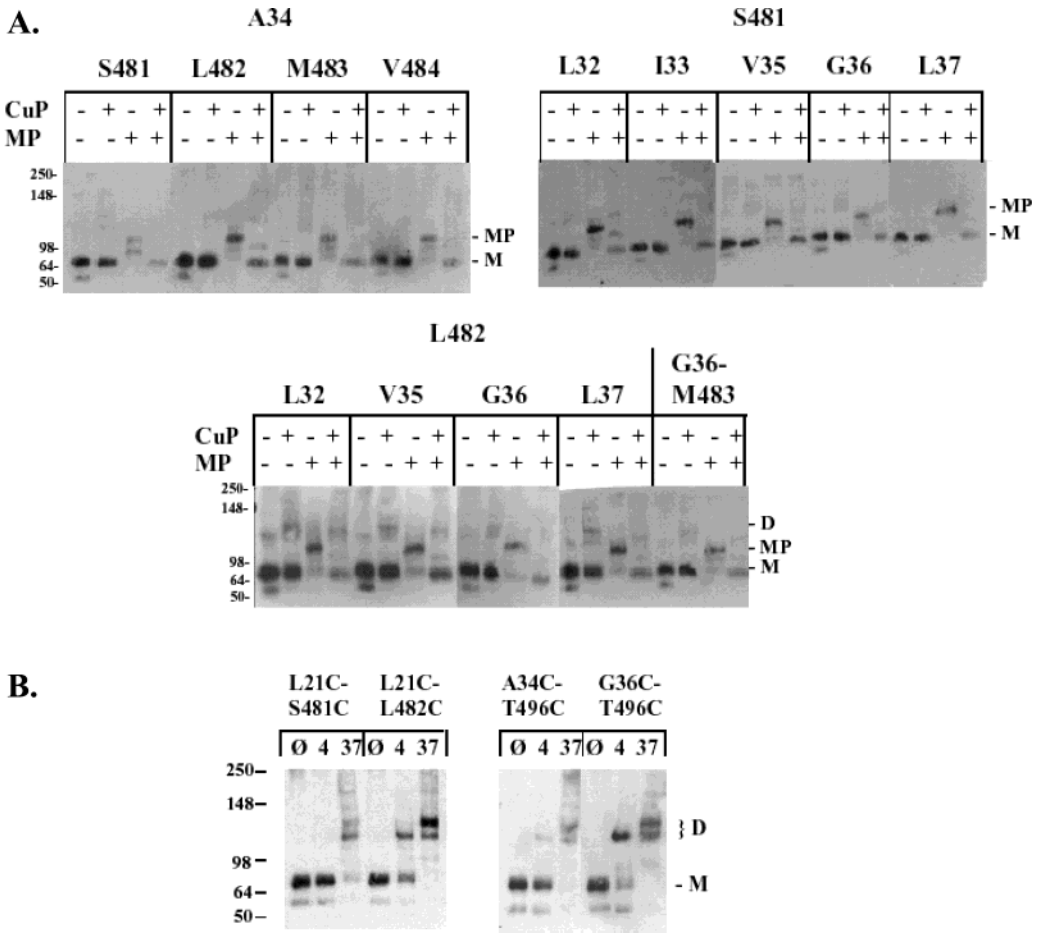

FIGURE 4: TM1−TM2 cysteine pairs. (A) Constructs containing the indicated pairs of cysteines in TM1 and TM2 were treated in duplicate with either buffer alone or copper phenanthroline (CuP) at 37 °C. One set of −/+ CuP samples was stopped with SDS loading buffer, and the other set was stopped with SDS and EDTA and treated with 15 mM mPEG−maleimide (MP) before addition of loading buffer: M for monomer, MP for malPEG-conjugated monomer, and D for dimer. (B) Constructs containing translationally separated TM1−TM2 cysteine pairs were treated with buffer alone (Ø) or with copper phenanthroline at 4 or 37 °C: M for monomer and D for dimer.

gradient sedimentation (not shown), suggesting that the lack of dimers results from intramolecular TM1−TM2 disulfides forming at the expense of intermolecular disulfides.

While intramolecular crosslinking often changes mobility on a gel, the CD39 monomers observed after CuP treatment are indistinguishable from untreated samples. We therefore used reactivity with mPEG−maleimide (malPEG) to determine whether the CuP-treated monomers contain TM1−TM2 disulfides. MalPEG is a 5000 MW PEG derivative with a maleimide group that reacts with free sulfhydryls and consequently slows the protein's mobility on a gel by an observable amount. Since the only free sulfhydryls in the double-cysteine constructs are the two in the transmembrane domains, non-crosslinked proteins would be expected to

react with two malPEG molecules, while those with a TM1−TM2 disulfide bond would be expected to have no free cysteines and not to react with malPEG. As Figure 4A shows, each protein reacts with malPEG when not previously treated with CuP. Treatment with a series of malPEG concentrations revealed an intermediate size band corresponding to one conjugated malPEG (not shown), verifying that the band shown in Figure 4A represents malPEG bound to both cysteines. In contrast, none of the proteins show any change in gel mobility when treated with CuP prior to reaction with malPEG. These results indicate that TM1−TM2 disulfides form regardless of the helix faces of the two cysteines.

The ability of a cysteine on either transmembrane domain to crosslink with cysteines successively placed around an entire turn of the opposite helix indicates substantial rotational mobility of both TM1 and TM2. To verify that this mobility is responsible for the ability of the single cysteines to form intersubunit disulfides regardless of helix face, we created a set of TM1−TM2 pairs with one cysteine near the extracellular end and the other near the cytoplasmic end. Such pairs should be unable to form TM1−TM2 disulfide bonds and should therefore be free to crosslink within higher-order oligomers that might otherwise be masked by faster-forming intramolecular disulfides. To maximize the potential of observing larger oligomers, we paired T496, the most strongly crosslinked position near the cytoplasmic end of TM2, with A34 or G36, each of which represents a different face at the extracellular end of TM1. In addition, we paired L21 near the cytoplasmic end of TM1 with S481 or L482 on different faces of TM2. Figure 4B shows that for each protein at 37 °C, the monomer is converted to two bands in the dimer size range. The lower of the two bands corresponds to TM1−TM1′ or TM2−TM2′ dimers crosslinked via positions at the extracellular end, as shown by comparison with dimers formed at 4 °C where cytoplasmic positions do not crosslink. The higher band is also a dimer, as verified by sucrose density gradient sedimentation of the Triton X-100-solubilized protein, and most likely contains both TM1−TM1′ and TM2−TM2′ disulfides. These results (1) verify that intramolecular crosslinking is limited by translational distance between the two positions and (2) suggest that rotational mobility rather than higher-order oligomers or transient dimer collisions is responsible for the diversity of helix surfaces at the dimer interface.

Dimer Stability. While the experiments described above establish that the transmembrane domain interactions are highly dynamic, the almost complete predominance of intramolecular over intermolecular crosslinking in Figure 4A raises the possibility that dimer partnerships themselves may be dynamic. Assuming TM1 and TM2 rotate rapidly in a packed dimer, it would be expected that for different double-cysteine constructs TM1 and TM1′, TM2 and TM2′, or potentially TM1 and TM2′ would collide before TM1 and TM2 at least some of the time. The fact that this rarely occurs suggests that TM1 and TM2 interact more closely with each other than with the other subunit. This situation might be accommodated within a stably packed dimer, but it also raises the possibility that monomers may be in equilibrium with dimers so that TM1 and TM2 of a given monomer interact constantly with each other but only transiently with a partner subunit. To distinguish between stable dimers, comparable to the stably interacting subunits of oligomeric proteins such as channels, and labile, rapidly exchanging dimer pairs, we cotransfected equal amounts of the HA-tagged G36C with untagged wild-type CD39. We expected that if CD39 forms stable dimers, half of the G36C would be stably paired with CD39 containing no free cysteines and would be unable to form disulfide-linked dimers. If dimers are labile, all of the G36C molecules would be expected to find and crosslink to each other, resulting in complete conversion to disulfide-linked dimers.

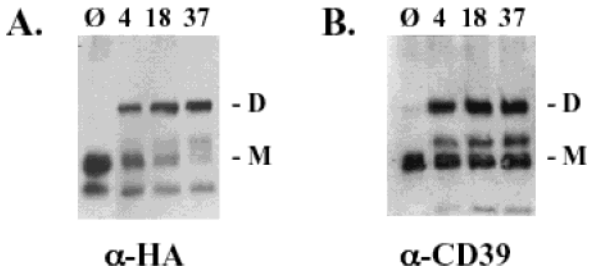

FIGURE 5: Cotransfected G36C and wild-type CD39. CD39 containing a G36C substitution and an HA tag was cotransfected with an equal amount of untagged wild-type CD39. Crude membranes were treated with buffer (Ø) or with copper phenanthroline at 4, 18, or 37 °C and subjected to Western analysis using (A) anti-HA or (B) anti-CD39 antibody: M for monomer and D for dimer.

The immunoblot in Figure 5A, which is probed with an HA tag antibody and thus shows only G36C, shows that G36C is completely crosslinked at 37 °C. The anti-CD39 immunoblot in Figure 5B, which shows both G36C and wild-type CD39, verifies that wild-type CD39 is expressed at the same level as G36C and remains monomeric after CuP treatment. At 4 °C, more G36C remains monomeric than does G36C transfected alone as in Figure 2A. Together, these results suggest that dimers are not stable entities but instead may be labile structures that rapidly dissociate and re-form while changing subunit partnerships in the process. The lability of dimer partnerships is consistent with the instability observed in most detergents (*11*, *18*) as well as in native blue gel buffer (*40*) and suggests that dimers may be in equilibrium with monomers in native membranes.

Enzymatic Activity of Crosslinked Proteins. The dynamic nature of both intra- and intersubunit transmembrane domain interactions raises the question of how such flexibility fits with the crucial structural role of the two helices. One possibility is that the particular arrangement of the transmembrane domains is less relevant than previously thought. Alternatively, function may require a specific transmembrane domain structure and simply not occur during the deviations observed by crosslinking. Another possibility is that flexibility itself is critical to nucleotide binding and hydrolysis.

To investigate the relationship between transmembrane domain structure and enzymatic activity, we analyzed activity following disulfide formation at each of the TM1−TM1′, TM2−TM2′, and TM1−TM2 interfaces discussed above. If transmembrane domain organization is unimportant, all crosslinked proteins should be as active as the un-crosslinked protein. If a specific structure is optimal, then crosslinking between certain helix surfaces would be expected to preserve or enhance activity while disulfides between other surfaces would reduce activity. Finally, if the helices are required to move relative to each other, activity would be expected to be compromised if the helices were locked in any single

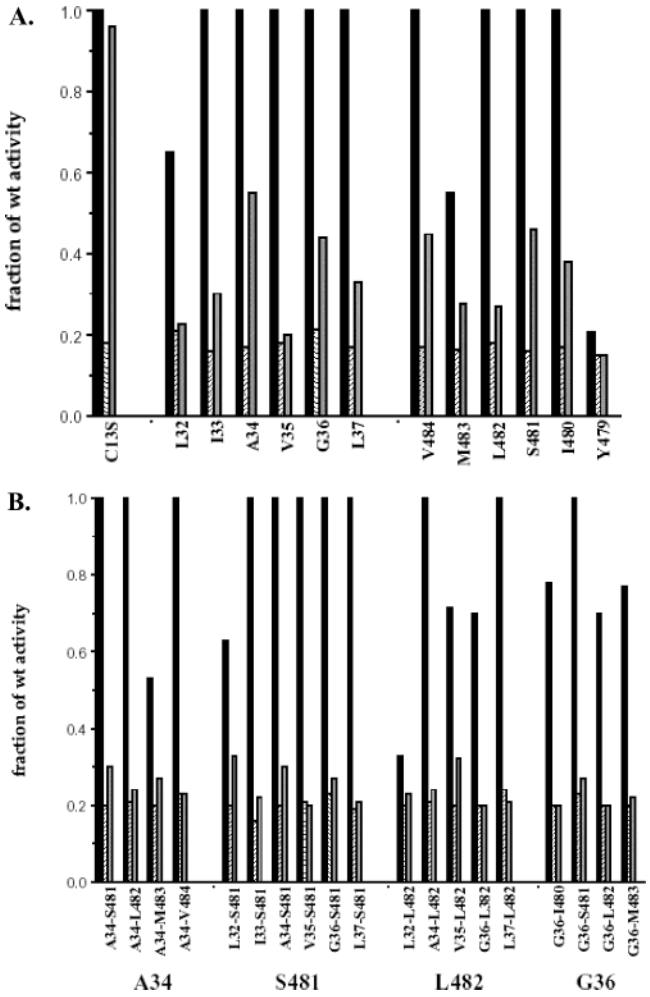

FIGURE 6: ATPase activity of crosslinked proteins. ATPase activity was measured following treatment of membranes with buffer (black bars), 0.5% Triton X-100 (hatched bars), or 0.1 mM copper phenanthroline (gray bars). Activity is expressed as a fraction of untreated wild-type ATPase activity: (A) C13S and single cysteines and (B) TM1−TM2 cysteine pairs.

position. A similar strategy has been used to demonstrate the functional requirement for a fixed intersubunit interface and a mobile intrasubunit interface in bacterial chemoreceptors Tar (*39*, *41*) and Trg (*25*).

As shown in Figure 6A, treatment with 0.1 mM CuP at 37 °C has only a minor effect on C13S but significantly reduces the activities of each of the single-cysteine constructs. Activities of non-crosslinked proteins following Triton X-100 solubilization are shown for comparison. Activity loss following crosslinking ranges from 50 to 80%, compared to approximately 80% with Triton X-100. With the exception of G36, the positions at which the most activity is lost are the ones that crosslink most readily at low temperatures, suggesting that these positions do not represent fixed interfaces in the functioning protein. The slightly higher retention of activity at the temperature sensitive positions may result from a small fraction of non-crosslinked protein. These positions might also interact more closely in the transition state of the enzyme. Nevertheless, none of the crosslinked proteins that retain any activity above the Triton-solubilized level become resistant to further Triton-induced activity loss (not shown), indicating that none of the disulfides stabilize an optimally active structure. These data suggest that TM1 and TM1′, as well as TM2 and TM2′, must be able to move relative to each other to support optimal enzyme function, although the possiblity that each crosslink distorts an optimal structure has not been ruled out.

Figure 6B shows that, despite the fact that intrasubunit TM1−TM2 disulfides form more readily than intersubunit disulfides, crosslinking between any of an exhaustive set of TM1−TM2 pairs reduces activity to approximately the same extent as Triton X-100 solubilization. Although the possibility that every combination distorts a highly specific structure cannot be ruled out, it seems less likely, given the comprehensive combinations of helix surfaces, the readily formed crosslinks, the fact that disulfide bonds allow a small amount of flexibility, and the high tolerance of the transmembrane domains for amino acid substitutions (unpublished data). Rather, these results suggest that relative movement between TM1 and TM2 within one subunit is critical for enzymatic function. Together with Figure 6A, these data indicate that (1) the orientation and interactions of both transmembrane domains, both within and between dimer subunits, are important to enzyme function and (2) transmembrane helix movement at intra- and intersubunit interfaces is essential to active site function.

Modulation of Crosslinking by the Substrate. The observed relationship between transmembrane domain dynamics and active site function suggests the possibility that substrate binding might in turn modify transmembrane domain structure. While ATP causes no significant changes in the regions that show little or no crosslinking (not shown), Figure 7 shows that including ATP in the crosslinking reaction abolishes crosslinking at specific positions in the region near the extracellular domain. For this set of experiments, we used 3 mM CuP to avoid potential disruption of the CuP complex by millimolar ATP concentrations; this concentration has been used in several studies with both bacterial and mammalian membrane proteins and gives CD39 crosslinking results identical to those obtained with 0.1 mM CuP. As Figure 7A shows, ATP inhibits intersubunit crosslinking at position A34 in a concentration-dependent manner. Disruption of the CuP complex does not explain the inhibition, since ATP is not predicted to compete significantly with phenanthroline at these relative concentrations, and even if it did, the remaining CuP concentration would still be higher than 0.1 mM. As shown in Figure 7B, ATP selectively inhibits intersubunit crosslinking via either TM1 or TM2 with the same helical pattern as low temperature does. Thus, the same TM1−TM1′ and TM2−TM2′ interfaces that predominate at 4 °C are stabilized at 37 °C in the presence of ATP.

As shown in Figure 7C for position A34, this effect is mimicked by the substrate ADP but not by AMP or phosphate, neither of which is a substrate or has any effect on activity. The experiment shown in Figure 7D, also for position A34, suggests that free ATP is more effective than Mg^{2+}-bound ATP. In the absence of divalent cations, the nonhydrolyzable ATP analogue AMP-PNP inhibits crosslinking with the same dose dependence as ATP. However, the presence of 1 mM Mg^{2+} causes the dose dependence to shift to higher AMP-PNP concentrations, and 5 mM Mg^{2+} prevents inhibition by AMP-PNP at all doses up to 5 mM. The relation between Mg^{2+} and AMP-PNP concentrations suggests that Mg^{2+} interferes by binding directly to AMP-PNP

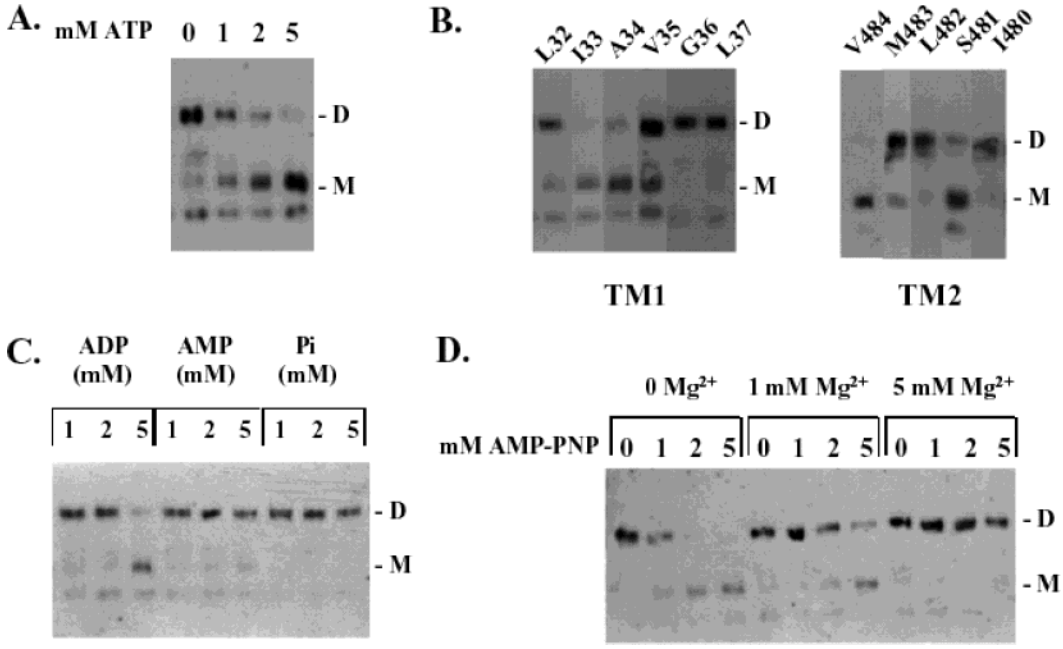

FIGURE 7: Crosslinking in the presence of ATP. (A) A34C was treated with 3 mM copper phenanthroline at 37 °C in the presence of the indicated concentrations of ATP. (B) Single-cysteine constructs were treated with 3 mM copper phenanthroline at 37 °C in the presence of 5 mM ATP. (C) A34C was treated with 3 mM copper phenanthroline at 37 °C in the presence of the indicated concentrations of ADP, AMP, and phosphate. (D) A34C was treated with 3 mM copper phenanthroline at 37 °C in the presence of the indicated concentrations of AMP-PNP and $MgCl_2$: M for monomer and D for dimer.

rather than to the enzyme. The same results were obtained at position S481 on TM2 as well as with calcium (not shown). The role, or even the presence, of divalent cations at the nucleotide binding site is not sufficiently understood to allow detailed correlation of these results with the hydrolysis mechanism. However, these results indicate that ATP in its non-metal-complexed form binds to the enzyme and stabilizes specific TM1−TM1′ and TM2−TM2′ interactions by reducing flexibility at the intersubunit interface.

DISCUSSION

This study demonstrates that CD39's transmembrane domains regulate its enzymatic activity via a set of highly dynamic intra- and intersubunit interactions. The helices interact predominantly in the region near the extracellular domain, in particular near putative active site components ACR1 and ACR5 located a short distance from TM1 and TM2, respectively. Crosslinking data support a model in which dimer subunits interact via both TM1−TM1′ and TM2−TM2′ interfaces that are well-defined at 4 °C but highly variable at physiological temperatures. TM1 and TM2 also interact strongly within a subunit, also with a high degree of variability in which surfaces form the interface. The variability in helix surfaces at each interface arises from rotational mobility of each helix around it axis. Dimers are the largest oligomers observed and appear to undergo frequent dissociation and reassociation rather than existing as fixed entities. Locking TM1, TM2, or both in place via any of a series of inter- or intrasubunit disulfides inhibits enzyme function, suggesting that both the organization and the mobility of both helices are critical to active site function. The ability of a substrate to modulate transmembrane helix flexibility further demonstrates a reciprocal relationship between transmembrane domain and active site dynamics.

Prior to this work, it was unknown whether the transmembrane domains interact, or, if so, whether they contribute to active site function via such interactions or simply via the positional restrictions imposed by their location in the membrane. The partial retention of native properties upon replacement of CD39's transmembrane domains with those of CD39L1, a related eNTPDase with similar topology but unrelated transmembrane domain sequences, called into question the requirement for specific sequences and thus for specific interactions (*19*). The data described here reveal not only that the transmembrane domains do interact but also that their arrangement, rather than just their placement in the membrane, mediates their functional relationship with the active site. At the same time, the variation of helix faces at each interface is consistent with the previously observed low requirement for sequence specificity. Together, the current and previous results suggest that (1) helix interactions and positional restrictions due to membrane insertion both contribute to native enzyme function and (2) TM1 and TM2 sequences promote but are not optimized for structural stability.

These results suggest that the transmembrane domains play a more dynamic role than previously thought. On the basis of prior studies, we proposed that the extracellular active site can exist in a tense or relaxed conformation, and that the transmembrane domains play a structural role in constraining the active site in the tense, higher-activity state (*19*). The unexpectedly high degree of flexibility observed here, and the fact that locking the helices in place is as detrimental to enzyme activity as removing them, suggests that the mobility of the transmembrane domains may be at least as important as stability. The apparent lack of sequence optimization for stability may thus be a critical feature of the transmembrane domains' ability to do their job, as has been proposed for other proteins (*42*). As suggested by the

better-defined structure at 4 °C and by the disturbance in activity by mutation at certain positions, the transmembrane domains must strike a balance between stability and mobility. Further investigation into how the transmembrane domains regulate activity should therefore include a more quantitative understanding of this balance. In particular, what fraction of time the protein spends in each of the observed conformations and which are physiologically relevant remain crucial questions.

The requirement for mobility may indicate that the transmembrane domains undergo coordinated motions during the process of nucleotide binding and hydrolysis, similar to the transmembrane domain rearrangements caused by binding of a ligand to a receptor. If so, coordination between active site and transmembrane domain dynamics would suggest a means by which the transmembrane domains might translate changes in the membrane or cytoplasm into changes in enzyme function, or by which nucleotide hydrolysis might regulate the proposed ATP conductance through the transmembrane domains (*21*). Such coupling between transmembrane domain movements and stages of nucleotide hydrolysis has been observed for P-glycoprotein, in which drug-induced transmembrane domain motions can stimulate or inhibit ATP hydrolysis and ATP hydrolysis promotes transmembrane domain movement (*43*, *44*). Crystal structures of hsp70, actin, and hexokinase, with which CD39 shares ATP binding site homology, predict a capacity for hingelike motion between the two subdomains of the active site (*45*), suggesting that CD39's transmembrane domains might be coupled to an analogous interdomain motion.

However, the relationship between transmembrane domain dynamics and active site function may not be as simple as conversion between discrete structures. The high degree of mobility and the fact that ATP modulates this mobility per se rather than the average structure suggest that ongoing mobility may be as important as any specific induced rearrangements. Substrate modulation of mobility but not of average structure has been observed for other proteins, such as the bacterial galactose receptor (*46*). In addition, changes in dynamic motions rather than in average structure have been proposed as the mechanism by which mutations alter the function of the *E. coli* trp repressor (*47*) and by which anesthetics alter gramicidin ion channel activity (*48*). At enzyme active sites, flexibility observed as ongoing dynamic motions is thought to contribute to the stability of the substrate complex and to allow product release (*47*). Ongoing dynamic motions of CD39's transmembrane domains might similarly be a crucial element of their regulation of the active site, with increases or decreases in mobility potentially causing changes in activity.

The ability of CD39's transmembrane domains to interact via a variety of helix interfaces also highlights a fundamental question concerning the flexibility and specificity of packed transmembrane helices. The membrane protein folding process and the lack of randomly aggregated helices in the membrane require a reasonable degree of sequence specificity at helix interfaces. Such a specificity requirement would be expected to allow fluctuations in lateral distance between two interacting helices, but not rotational mobility that places different residues at the interface. However, a disulfide bond requires the α-carbons of two cysteines to be within 5 Å of each other, and the average distance between interacting helices is ~9 Å. The readily formed disulfide bonds between different helix faces observed in this study therefore indicate that the transmembrane domains can and do pack in multiple orientations. A similar situation has been observed for the influenza M2 channel, which consists of a tetramer of single transmembrane domains; both crosslinking (*34*) and NMR hydrogen−deuterium exchange experiments (*49*) indicate that different helix faces can face the pore of the packed tetramer. For both M2 and CD39, the transmembrane domains interact in the absence of other parts of the protein, suggesting that they are held together primarily by their own interactions. This combination of factors raises the question of how transmembrane helices that mediate their own interactions can remain packed while simultaneously changing the face that constitutes the packing interface. A possible explanation might be that the helices can twist rather than rotating as rigid rods so that only part of the standard interface is disrupted at a time.

Among membrane proteins, a variety of biochemical and spectroscopic methods have revealed that different proteins exhibit different degrees of transmembrane helix mobility (*50*), suggesting that such motions are characteristic of the protein and may provide mechanistic insight. The results described here reveal that CD39's transmembrane helices are more dynamic than previously recognized and that transmembrane domain motions represent a parameter that can both regulate and be regulated by the enzyme active site. Further understanding of transmembrane domain mobility and of how such mobility is transmitted to the active site may provide a new direction for the study of function of CD39 and regulation of CD39 by its environment.

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