Identification of Cysteine Residues Responsible for Oxidative Cross-linking and Chemical Inhibition of Human Nucleoside-triphosphate Diphosphohydrolase 3*

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Cysteine-to-serine mutations were constructed to test the functional and structural significance of the three non-extracellular cysteine residues in ecto-nucleoside-triphosphate diphosphohydrolase 3 (eNTPDase3). None of these cysteines were found to be essential for enzyme activity. However, Cys10, located on the short N-terminal cytoplasmic tail, was found to be responsible for dimer formation occurring via oxidation during membrane preparation as well as for dimer cross-linking resulting from exogenously added sulfhydryl-specific cross-linking agents. The resistance to further cross-linking of these dimers into higher order oligomers by lysine-specific cross-linkers suggests that this enzyme may form its native tetrameric structure as a “dimer of dimers” with nonequivalent interactions between subunits. Cys501, located in the hydrophobic C-terminal membrane-spanning domain of eNTPDase3, was found to be the site of chemical modification by a sulfhydryl-specific reagent, p-chloromercuriphenylsulfonic acid (pCMPS), leading to inhibition of enzyme activity. The effect of pCMPS was negligible after dissociation of the enzyme into monomers by Triton X-100, suggesting that the mechanism of inhibition is dependent on the oligomeric structure. Because Cys501 is accessible for modification by the membrane-impermeant reagent pCMPS, we hypothesize that eNTPDase3 (and possibly other eNTPDases) contains a water-filled crevice allowing access of water and hydrophilic compounds to at least part of the protein’s C-terminal membrane-spanning helix.

The eNTPDases† (1), also known as ecto-ATPases/ecto-apyrases and E-type ATPases (2), rapidly hydrolyze a variety of extracellular nucleoside 5′-triphosphates and 5′-diphosphates. There are six members of the vertebrate eNTPDase family known to date (1). eNTPDase1–4 are integral membrane proteins with two membrane-spanning segments, one near each end of their respective primary structures. eNTPDase5 and 6 each have a single hydrophobic signal sequence near their N termini, which, when cleaved, results in the release of a soluble enzyme to the outside of the cell (3–6). eNTPDase1–4 are all glycosylated and contain 10 conserved cysteine residues located in the extracellular region. All 10 of these conserved residues are likely to be involved in the formation of five conserved disulfide bonds. Different members of the eNTPDase family contain various numbers of nonconserved cysteine residues located either in membrane-spanning helices or in the N- or C-terminal cytoplasmic tails. Some of these cysteine residues may exist as free sulfhydryls and are not expected to be involved in disulfide bond formation. The lone non-extracellular cysteine residue in human CD39 (eNTPDase1) is located on the N-terminal cytoplasmic tail and has been shown to be palmitoylated (7).

The primary structure and enzymatic properties of eNTPDase3 (HB6 (8) and CD39L3 (9)) are intermediate between two other eNTPDases, eNTPDase1 (CD39 ecto-apyrase) and eNTPDase2 (ecto-ATPase) (8). Site-directed mutagenesis of eNTPDase3 has revealed many amino acids essential for nuclease activity as well as for expression and proper folding of the enzyme (10–14). In this study, we mutated singly and in combination all three of the non-extracellular eNTPDase3 cysteine residues (not involved in disulfide bonds) to serine. These residues are localized to the N-terminal cytoplasmic tail (cysteine 10), the C-terminal membrane-spanning helix (cysteine 501), and the interface of the C-terminal membrane helix and the cytoplasm (cysteine 509), respectively. Although none of these cysteine residues were found to be essential for nuclease activity, cysteine 10 was identified as the residue responsible for oxidative dimer formation as well as for sulfhydryl-specific intermolecular cross-linking, and cysteine 501 was identified as the residue responsible for inhibition of the enzyme by the sulfhydryl-specific reagent pCMPS. pCMPS has been shown to inhibit several eNTPDases (15–17). This sulfhydryl-specific reagent is negatively charged under physiological conditions (pK_a = 1.5) (18) and is membrane-impermeant, being incapable of partitioning into or passing through biological membranes (19–22). Therefore, its ability to react with cysteine 501, which is located in the C-terminal membrane-spanning region of the protein, was surprising. We interpret this result as indicating that the part of the C-terminal membrane-spanning helix containing residue 501 must be solvent-accessible, possibly suggesting a water-filled crevice in the membrane-spanning region involved in the formation of the quaternary structure of eNTPDase3, and perhaps in the other membranous eNTPDases as well.

EXPERIMENTAL PROCEDURES

Materials—The QuikChange™ site-directed mutagenesis kit was purchased from Stratagene. Oligonucleotides were synthesized by the DNA Core Facility at the University of Cincinnati. LipofectAMINE Plus reagent, Dulbecco’s modified Eagle’s medium, calf serum, antibiotics/
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antimycotics, and the mammalian expression vector pcDNA3 were obtained from Invitrogen. The chemical cross-linking reagents DSS and BMOE and the SuperSignal West Dura Extended Duration chemiluminescence reagents were purchased from Pierce, Per-cast SDS 4–15% polyacrylamide gradient minigels and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were obtained from Bio-Rad. Ampicillin, nucleotides, pCMPS, p-hydroxymercuribenzoate, iodoacetic acid, iodoacetamide, NEM, 25% glutaraldehyde, malachite green, and other reagents were from Sigma.

Site-directed Mutagenesis of Human Brain eNTPDase3—eNTPDase3 cDNA was isolated as described (8). Mutagenesis of wild-type eNTPDase3 in the pcDNA3 vector was performed using the QuikChange site-directed mutagenesis kit as described (11). The sense oligonucleotides used for mutagenesis are as follows, with the substitution sites underlined: C10S, 5'-GACCCGCCAACCCTTCGAGAAACAGGC-3'; C501S, 5'-GGCAGCCTTGCTGTCTCTGGCATTTCTTGCG-3'; and C509S, 5'-GACATTCTTCGTACCTGTCACCGACCCAGAAG-3'. The complementary antisense oligonucleotides also necessary for the mutagenesis are not shown. The presence of the correct mutation and lack of unwanted mutations were confirmed by DNA sequencing using fluorescent dye automated sequencing in the Department of Molecular Genetics of the University of Cincinnati College of Medicine.

Transient Transfection—COS-1 cells were grown and transfected with wild-type and mutant eNTPDase3 cDNAs as described (23). Transfection with an empty pcDNA3 vector was also performed as a control. Approximately 46 h post-transfection, the COS-1 cells were harvested and used for analyses as described previously (23). Crude cell membrane preparations were obtained as described previously (8). In some experiments, COS cells were harvested and homogenized in buffer (30 mM MOPS, 2 mM EDTA, and 0.25% sucrose, pH 7.4) containing one of the following additives: 2 mM iodoacetamide, 2 mM NEM, 25% glutaraldehyde, malachite green, and other reagents were from Sigma.

Protein Assay—Protein concentrations were determined using the Bio-Rad CB-250 dye binding technique according to the modifications of Stoscheck (24, 25) using bovine serum albumin as the standard.

Nucleotidase Assays—Nucleotidase activities were determined by measuring the amount of inorganic phosphate released from nucleotide substrates in the presence of Mg2+ at 37 °C using a modification of the technique of Fiske and SubbaRow (26) as described previously (27). The values were corrected for pcDNA3/COS cell background (COS cells transfected with an empty pcDNA3 vector) as well as differences in expression levels as determined by Western blotting. In some experiments requiring increased sensitivity and/or the inclusion of Triton X-100, an inorganic phosphate assay utilizing malachite green (28) was employed due to the turbidity caused by the Triton X-100 detergent in the ammonium molybdate/inorganic phosphate assay (26).

Western Blot Analysis—For Western blotting, proteins were resolved on an SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, and the resulting Western blot was probed with an anti-C-terminal eNTPDase3 peptide antibody as described previously (23). Immunoreactivity was detected by chemiluminescence, and quantification of the resultant bands was accomplished using the AlphaImager™ 2000 documentation and analysis system (Alpha Innotech Corp., San Leandro, CA). Serial dilutions of the wild-type enzyme were used to generate a standard curve to quantify the expression levels of the mutants (relative to the wild-type enzyme) for each of the three transfactions.

Chemical Cross-linking—COS cell membranes (0.1 mg/ml) were incubated for 10 min at room temperature in buffer (20 mM MOPS and 5 mM MgCl2, pH 7.4) in the presence or absence of one or two of the following cross-linkers: 50–500 μM DSS, 50–100 μM BMOE, and/or 0.5–5 mM glutaraldehyde. Cross-linking stock solutions were freshly prepared in water (glutaraldehyde) or dry MgSO4 (DSS and BMOE). The cross-linking reactions were stopped by adding an excess (10 mM) of lysine (DSS and glutaraldehyde) or cysteine (BMOE) and incubating for 5 min prior to adding SDS-PAGE sample buffer. After heating the samples for 10 min at 60 °C, SDS-PAGE and Western blotting were performed as described above.

pCMPS Modification and Inhibition of eNTPDase3—COS cell membranes (0.2 mg/ml) containing expressed wild-type or mutant eNTPDase3 were incubated in buffer (20 mM MOPS and 5 mM MgCl2, pH 7.4) for 30 min at 22 °C in either the presence or absence of 1 mM pCMPS. Nucleotidase activity was measured as described previously.

RESULTS

Rationale for Selection of Mutations—The three non-extra-cellular cysteine residues of eNTPDase3 (cysteines 10, 501, and 509) (Fig. 1) were targeted for mutagenesis due to evidence of their possible involvement in other eNTPDases in the inhibition of nucleotidase activity by pCMPS and by other sulfhydryl-selective chemical modifications (15–17). In addition, one or more of these cysteine residues are likely to be involved in the dimer formation seen in eNTPDase3 on nonreducing SDS-polyacrylamide gels as well as in dimer formation resulting from sulfhydryl-selective chemical cross-linking agents (12, 13). The remaining 10 cysteine residues in eNTPDase3 are located in the large extracellular loop containing the active site and are very likely to form five extracellular disulfide bonds.

Nucleotidase Activity and Expression Level—The level of protein expression relative to the wild-type enzyme as well as the ATPase and ADPase activities (normalized for expression level) of all of the mutations are given in Table I. It is evident from Table I that no mutation of the three cysteine residues, either singly or in combination, resulted in inactivation of nucleotidase activity or a substantial reduction in the expression level of the mutant proteins. In addition, all mutants displayed ATPase/ADPase activity ratios not statistically different from that displayed by the wild-type enzyme (wild-type ATPase/ADPase ratio = 3.7 ± 0.2) (ratios not shown in Table I). However, we noted that all mutants containing the C10S mutation (i.e., C10S, C10S/C501S, C10S/C509S, and C10S/C501S/C509S) were expressed at higher levels than the other mutants and the wild-type enzyme (144, 140, 162, and 229% of the wild-type expression level, respectively) while maintaining normalized nucleotidase activities close to that of the wild-type enzyme. Because nucleotidase activities were not substantially decreased in any of the eNTPDase3 mutants, we did not probe for changes in tertiary and quaternary structures, as was done previously for other eNTPDase3 mutations with reduced activities (12–14).
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Oxidative and Sulfhydryl-specific Chemical Cross-linking—All seven cysteine mutants and wild-type eNTPDase3 were subjected to reducing SDS-PAGE and Western blot analysis after sulfhydryl group-directed cross-linking using BMOE as well as to nonreducing SDS-PAGE of the untreated samples (Fig. 2). All mutants lacking Cys\textsuperscript{10} were incapable of either being cross-linked by BMOE into a dimer, as shown by the Western blot results obtained after running the samples on a reducing SDS-polyacrylamide gel (Fig. 2A), or forming dimers on nonreducing gels (Fig. 2B). The samples used in Fig. 2 (A and B) were identical (harvested from the same set of transfections); but because this particular harvest of transfected cells displayed a decreased degree of oxidative cross-linking of eNTPDase3 (as evidenced by the large extent of dimer formation induced by BMOE) (Fig. 2A), oxidation was increased by exposure of the membrane preparations to air during freeze-thaw cycles in open containers prior to the nonreducing gel blot shown in Fig. 2B.

The expressed eNTPDase3 proteins containing cysteine 10 might be oxidatively cross-linked as part of their natural processing in COS cells, suggesting that the cross-linking is functionally significant; or they might be cross-linked as a result of oxidation during membrane harvesting and manipulation, as was previously suggested (29). To determine at what stage the expressed eNTPDase3 proteins were oxidized into dimers via cysteine 10, we homogenized the transfected COS cells under several sets of conditions. These included the presence of an alkylating agent (2 mM iodoacetamide or 2 mM NEM) or a reducing agent (0.5 mM dithiothreitol) in the homogenization buffer. The Western blot of the resultant samples is shown in Fig. 3 and reveals that treatment with either the alkylating agents (iodoacetamide and N-ethylmaleimide) or the reducing agent (dithiothreitol) during cell harvesting and homogenization resulted in a greatly diminished formation of dimers observed upon nonreducing SDS-PAGE (lanes 2–4). This indicates that the oxidative cross-linking of cysteine 10 did not occur in the intact COS cells, but instead during harvesting, homogenization, and centrifugation of COS cells to obtain crude membranes, prior to SDS-PAGE and Western blotting. In addition, treatment of wild-type eNTPDase3 with alkylating agents during the homogenization process eliminated the BMOE-induced, cysteine 10-mediated cross-linking seen upon Western analysis of reducing SDS-polyacrylamide gels (lanes 6 and 7).

**Table I**

Expression level and nucleotidase activities of all eNTPDase3 cysteine-to-serine mutations

Values given represent means ± S.E. of three separate transfections (each set of transfections were matched to their own wild-type and pcDNA3 vector controls, transfected in parallel). All nucleotidase activities are in micromoles of Pi released per mg of total COS cell membrane protein/h in the presence of 5 mM magnesium and 2.5 mM nucleotide. Normalized values were calculated by dividing the specific activity in micromoles of Pi/mg/h in the presence of 5 mM magnesium and 2.5 mM nucleotide.

| Enzyme            | % Wild-type expression level | Normalized ATPase activity (% wild-type activity) | Normalized ADPase activity (% wild-type activity) |
|-------------------|------------------------------|--------------------------------------------------|--------------------------------------------------|
| Wild-type eNTPDase 3 | 100                          | 100 ± 0 (100)                                   | 18 ± 2 (100)                                     |
| C10S              | 144 ± 10                     | 97 ± 14 (112)                                    | 22 ± 2 (105)                                     |
| C501S             | 77 ± 2                       | 116 ± 25 (130)                                   | 32 ± 5 (130)                                     |
| C509S             | 65 ± 8                       | 130 ± 25 (148)                                   | 32 ± 4 (155)                                     |
| C10S/C501S        | 144 ± 17                     | 104 ± 12 (148)                                   | 28 ± 3 (133)                                     |
| C10S/C509S        | 162 ± 8                      | 69 ± 10 (103)                                    | 18 ± 2 (99)                                      |
| C501S/C509S       | 62 ± 17                      | 94 ± 19 (138)                                    | 25 ± 4 (134)                                     |
| C10S/C501S/C509S  | 229 ± 28                     | 53 ± 7 (79)                                      | 14 ± 2 (77)                                      |

**A** BMOE cross-linking

**B** non-reducing gel

**Fig. 2.** Sulphydryl-specific (BMOE) cross-linking and nonreducing SDS-PAGE/Western blot analyses of wild-type and mutant eNTPDases. A, samples treated for 10 min at 22 °C with 100 μM BMOE prior to reducing SDS-PAGE and Western blotting; B, noncross-link samples subjected to SDS-PAGE under nonreducing conditions and Western-blotted. Note that all samples lacking cysteine 10 (with the C10S mutation) did not form dimers either after sulphydryl-specific chemical cross-linking (A) or upon nonreducing SDS-PAGE (B). The *wt* control in A is non-BMOE-cross-linked, reduced, wild-type eNTPDase3, and that in B is reduced, wild-type eNTPDase3.

**Sequential Treatment with Sulphydryl- and Amino-selective Cross-Linking Agents**—We next attempted to determine whether both the cysteine-specific (via Cys\textsuperscript{10} in the short cytoplasmic N-terminal domain) and the lysine-specific (via multiple lysine residues located mostly in the extracellular loop) cross-linking agents preferentially cross-link the native tetramer into dimers consisting of the same two monomers. Wild-type eNTPDase3 was cross-linked first with a sulphydryl-specific reagent and then with lysine-specific reagents, run on a reducing SDS-polyacrylamide gel, and Western-blotted (Fig. 4). The predominance of the dimer band is obvious in all cases. The results in Fig. 4 are consistent with our hypothesis that the same two sets of two monomers in the native tetrameric structure are efficiently cross-linked into dimers by both sulphydryl group-directed reagents (via cysteine 10) and the lysine group-directed reagents DSS and glutaraldehyde. This is especially evident in lanes 4–6, which compare the amount of cross-linking using BMOE alone, BMOE followed by DSS, and BMOE followed by glutaraldehyde, respectively. Only a small amount of oligomers larger than dimers were seen in these samples, although it is evident that the amount of monomer remaining after BMOE cross-linking was decreased by the subsequent lysine-specific cross-linking reagents. Comparing lanes 2, 4, and 5, it is evident that DSS treatment after BMOE treatment resulted in more dimers formed, but almost no
alkylating agents), or in the presence of 0.5 mM dithiothreitol (sulfhydryl-specific cross-linking reagent) for 10 min at 22°C to reduce disulfide bonds. The proteins were subjected to reducing SDS-PAGE after cross-linking with 100 μM BMOE (sulfhydryl-specific cross-linking reagent) for 10 min at 22°C and quenching with excess cysteine. The resultant gel was Western-blotted (sulfhydryl-specific cross-linking reagent) for 10 min at 22°C to glycosylation heterogeneity.

Results:

1. Effect of membrane preparation conditions on oxidative cross-linking of wild-type eNTPDase3. COS cell transfectants with wild-type eNTPDase3 cDNA were harvested and homogenized in the absence of additives (lanes 1 and 5), in the presence of 2.0 mM iodoacetamide (lanes 2 and 6) or NEM (lanes 3 and 7) (sulfhydryl-alkylating agents), or in the presence of 0.5 mM dithiothreitol (lanes 4 and 8) (reducing agent). Lanes 1–4 resulted from 1.0-μg samples subjected to nonreducing SDS-PAGE after air-induced oxidation via three freeze-thaw cycles, and lanes 5–8 represent wild-type samples subjected to reducing SDS-PAGE after cross-linking with 100 μM BMOE (sulfhydryl-specific cross-linking reagent) for 10 min at 22°C and quenching with excess cysteine. The resultant gel was Western-blotted and processed as described under “Experimental Procedures.”

2. Higher order cross-linked species than was seen with either reagent used alone. These data suggest that the native tetrameric quaternary structure is formed by the association of two dimers, with the contacts between the two monomers composing each dimer being more extensive than the contacts between the two dimers composing the tetramer.

Identification of Cysteine 501 as the Site of Inhibition by pCMPS—Mercurial sulfhydryl group-directed chemical modification reagents such as pCMPS have been reported to inhibit the eNTPDases (15–17). To determine which cysteine residues might be involved in this inhibition of enzyme activity by pCMPS, we tested wild-type eNTPDase3 and its cysteine mutants for susceptibility of both ATPase and ADPase activities to inhibition by 1 mM pCMPS. As evident from Fig. 5, pCMPS inhibited all mutants containing cysteine 501 by 50–60%, but did not substantially inhibit any mutant lacking cysteine 501 (C501S mutants). This indicates that cysteine 501 is the target for pCMPS-induced inactivation of the ATPase and ADPase activities of eNTPDase 3.

The effects of other sulfhydryl-directed reagents on the nucleotidase activity of eNTPDase 3 were examined. p-Hydroxymercuribenzoate (pHMB), another mercurial sulfhydryl-specific chemical modification reagent, also inhibited nucleotidase activity (Table II). However, several other sulfhydryl reagents, including NEM, iodoacetamide, and iodoacetic acid, did not inhibit nucleotidase activity under identical conditions (Table II). This lack of inhibition could be the result of either lack of reactivity of cysteine 501 with these reagents or lack of inhibition of enzyme activity after reaction of cysteine 501 with these sulfhydryl-selective reagents. To discriminate between these possibilities, we pretreated wild-type eNTPDase 3 with a sulfhydryl reagent that did not inhibit activity (NEM, iodoacetamide, or iodoacetic acid) prior to treatment with the inhibitory reagent pCMPS. Statistical analyses of the data in Table II showed no significant difference between the extent of pCMPS inhibition of samples pretreated with NEM, iodoacetamide, or iodoacetic acid and those samples not pretreated with these non-inhibitory sulfhydryl-selective agents. Thus, under the conditions used, cysteine 501 is not reactive with these three sulfhydryl-selective reagents because, if it is, subsequent modification of cysteine 501 by pCMPS would be blocked. As a result, there would be a protection of eNTPDase3 activity from pCMPS-induced inactivation by these three reagents, and this was not observed (Table II).

Mechanism of Inhibition of Nucleotidase Activity by pCMPS—It is interesting and somewhat surprising that the site of inhibition by pCMPS is cysteine 501, located in the C-terminal transmembrane domain (see Fig. 1). This location should be far from any extracellular active-site residues, indicating that inhibition by this reagent is not due to modification of a cysteine residue directly involved in the enzymatic site. Because the membrane-spanning helices and the quaternary structure have been shown to be important for the activity of eNTPDases (17, 29, 30), a reasonable mechanism for allosteric inhibition of eNTPDase3 caused by pCMPS is that introduction of the charged pCMPS molecule into Cys501 in the C-terminal membrane-spanning helix results in a weakening of the native tetrameric quaternary structure. To test this possibility, we examined the oligomeric structure of eNTPDase3 before and after solubilization with Triton X-100 detergent. This detergent has been reported to completely disrupt the native tetrameric structure of eNTPDase1 into monomers (29), thereby dramatically decreasing nucleotidase activity. To determine whether Triton X-100 also dissociates eNTPDase3 into monomers, C10S eNTPDase3 was treated with lysine-specific cross-linkers (DSS and glutaraldehyde) before and after solubilization of membranes with Triton X-100 (Fig. 6). Although the membrane-associated enzyme was cross-linked into dimers and higher oligomers (lanes 1 and 2), the enzyme solubilized in Triton X-100 did not demonstrate any intermolecular cross-linking, as evidenced by the absence of dimer or higher order oligomer bands upon Western blotting (lanes 3 and 4). These results are consistent with those described for CD39 (eNTPDase 1) (29).

Having established that Triton X-100 disrupts eNTPDase3 into monomers, we then investigated whether the inhibitory effect of pCMPS is additive to that of Triton X-100. In these experiments, we used C10S eNTPDase3, which behaves the same as the wild-type enzyme with respect to inhibition by pCMPS (Fig. 5) because it contains Cys501, but is devoid of Cys10, and therefore does not form oxidation-dependent dimers...
FIG. 5. Effect of pCMPS on the nucleotidase activities of wild-type and mutant eNTPDase 3. Crude membrane samples of COS cells transfected with wild-type (wt) or mutant eNTPDase 3 cDNAs were treated with or without 1 mM pCMPS and then assayed for nucleotidase activities as described under “Experimental Procedures.” The nucleotidase activities of samples treated with pCMPS are given as a percentage of the activities of control (untreated) samples and are given as a mean of three independent experiments. A question mark indicates data that are significantly different from the percentage of inhibition of nucleotidase activities in wild-type samples (Student’s t test, p < 0.05).

TABLE II
Effect of sulfhydryl-reactive compounds on nucleotidase activities of wild-type eNTPDase 3
Wild-type eNTPDase 3 (WT) was incubated in buffer (20 mM MOPS and 5 mM MgCl₂, pH 7.4) containing 1 mM sulfhydryl reagent for 30 minutes at 22°C with or without additional treatment with 1 mM pCMPS for 30 min at 22°C. Following incubation with sulfhydryl reagents, the nucleotidase activities of samples were assayed as described under “Experimental Procedures.” Values represent the percentage of the activities of control (untreated) samples and are given as means ± S.E. of three independent experiments. Asterisks indicate data that are significantly different from the percentage of inhibition of nucleotidase activities in wild-type samples (Student’s t test, p < 0.05).

|          | Control ATPase activity | Control ADPase activity |
|----------|------------------------|-------------------------|
| WT (control) | 100                    | 100                     |
| WT + pHMB | 65.6 ± 1.6             | 50.3 ± 2.1              |
| WT + NEM  | 102.6 ± 4.2            | 93.1 ± 3.4              |
| WT + NEM + pCMPS | 42.2 ± 10.9          | 47.6 ± 2.5              |
| WT + IAM  | 109.3 ± 6.8            | 93.2 ± 2.7              |
| WT + IAM + pCMPS | 40.2 ± 7.4         | 44.7 ± 5.7              |
| WT + IAA  | 100.7 ± 3.8            | 96.7 ± 1.4              |
| WT + IAA + pCMPS | 37.2 ± 4.5         | 37.8 ± 3.6              |

(Fig. 2), which would interfere in the ability of a detergent to disrupt the quaternary structure into monomers. As shown in Fig. 7, after solubilization with Triton X-100 and centrifugation at 100,000 × g for 1 h, treatment with 1 mM pCMPS resulted in almost no inhibition of the remaining enzyme activity, in contrast to the membrane-associated enzyme that was inhibited by ~50% with respect to both ATPase and ADPase activities. Conversely, we examined the effect of Triton X-100 after pCMPS treatment. As shown in Fig. 8, Triton X-100 inhibited ATPase activity by ~93% and inhibited ADPase activity by ~77%. Remarkably, inhibition of nucleotidase activities under (+pCMPS/+Triton X-100) conditions was only slightly more pronounced than that under (~pCMPS/+Triton X-100) conditions. This demonstrates that the inhibitory effects of pCMPS and Triton X-100 are not additive and therefore suggests they may occur via a similar mechanism. Taken together, the data in Figs. 7 and 8 suggest that pCMPS inhibition of eNTPDase 3 nucleotidase activities is mediated by a change in the quaternary structure, resulting in less favorable monomer-monomer interactions.

FIG. 6. DSS and glutaraldehyde cross-linking of C10S eNTPDase 3 before and after solubilization with Triton X-100. C10S eNTPDase 3 was used in these experiments because it, unlike the wild-type enzyme, does not exhibit the complication of being variably cross-linked into dimers by oxidation occurring during membrane preparation (Fig. 2B). Crude membrane samples of COS cells transfected with C10S eNTPDase 3 cDNA were either directly incubated with cross-linkers (lanes 1 and 2) or solubilized with 1% Triton X-100 (100,000 × g supernatant) prior to the cross-linking reaction (lanes 3 and 4). Cross-linking was performed with either 500 μM DSS (lanes 1 and 3) or 5 mM glutaraldehyde (lanes 2 and 4) as described under “Experimental Procedures,” and the samples were analyzed by Western blotting. The positions of monomers and dimers are indicated.

DISCUSSION
In this study, we used site-directed mutagenesis to examine the role of three non-extracellular cysteine residues in...
eNTPDase3. None of the cysteines were essential for nucleotidase activity; however, the C10S mutation did result in an increased expression level for the enzyme in transfected COS cells, and the triple cysteine-to-serine mutant, although expressed at a higher level, was slightly less active than the wild-type enzyme when normalized for expression level.

The cysteine residue present in the N-terminal cytoplasmic tail (Cys10) was found to be the residue responsible for both dimer formation occurring via oxidation of the wild-type enzyme during membrane preparation and dimer formation due to sulfhydryl-specific chemical cross-linking with BMOE (Fig. 2). There is a cysteine residue in the short N-terminal cytoplasmic tail of CD39 (eNTPDase1) that is analogous to cysteine 10 in eNTPDase3. In eNTPDase1, this residue is palmitoylated (7). This is clearly not the case for Cys10 of eNTPDase3, at least not for the enzyme expressed in COS cells, because this cysteine must be present as a free sulfhydryl to be cross-linked and oxidized to a disulfide. It is clear that dimers of wild-type eNTPDase3 observed on nonreducing SDS-polyacrylamide gels are due to oxidation of the Cys10 sulfhydryl to disulfides during harvesting and homogenization of cells because only a small amount of dimers was formed in eNTPDase3 COS cell membranes prepared in the presence of either alkylating or reducing agents (Fig. 3). In addition, this oxidative cross-linking of the wild-type enzyme can be increased by intentional exposure to air during freeze-thaw of the COS membrane preparations, as was done to generate the data in Fig. 2B.

Experiments on sequential cross-linking of eNTPDase3 with sulfhydryl- and amino-specific reagents suggest that, in a native tetramer (25, 26), the monomers are more efficiently cross-linked within a dimer than between two dimers. In other words, the four monomers in a tetramer are not equally likely to be cross-linked to each other. This is evident in Fig. 4, where amino group cross-linking (DSS and glutaraldehyde), subsequent to sulfhydryl-specific cross-linking via cysteine 10 (BMOE), resulted in the formation of very little additional trimers, tetramers, and higher oligomers, leaving the dimer as the predominant cross-linked form. Considering these data and previous work, both in our laboratory (30) and by Knowles and co-workers (17) using chicken muscle eNTPDase2 (ecto-ATPase), as well as evidence presented by Wang et al. (29) demonstrating that CD39 (eNTPDase1) is dimeric after solubilization with the detergent sodium cholate, we propose that, although all of the membranous eNTPDases...
exist as native tetramers (29, 31), these tetramers are not 4-fold symmetric, but instead are composed of an asymmetric “dimer of dimers,” where the interaction between two monomers in each dimer is different and stronger than the interaction between the two dimers forming the tetramer.

It has been previously demonstrated that some eNTPDases are inhibited by mercurial sulfhydryl group-selective reagents such as pCMPS (15–17). By examining pCMPS inhibition of wild-type and mutant eNTPDase3, we showed in this study that modification of cysteine 501 is primarily responsible for inhibition of nucleotidase activity by pCMPS in eNTPDase3 (Fig. 5). This result is somewhat surprising due to the location of cysteine 501 in the membrane-spanning region near the C-terminal end of the protein. Under physiological conditions and the conditions used in this study, pCMPS is a permanently charged and therefore membrane-impermeant reagent (18–22). Therefore, it was unexpected that this reagent could react with a cysteine residue predicted to be inside the plasma membrane. We interpret this result as indicating the presence of an aqueous cavity or crevice in the vicinity of cysteine 501; and thus, the interior of the membrane-spanning region surrounding cysteine 501 must be solvent-accessible. This interpretation is consistent with other systems in which pCMPS was used to determine the solvent accessibility of cysteine residues in membrane-spanning regions of proteins containing aqueous pores or crevices (19–21, 32).

The question remains as to how modification of cysteine 501 in the membrane-spanning domain inhibits nucleotidase activity that is catalyzed by a large extracellular domain of eNTPDase3, far removed from the membrane-spanning segments and cysteine 501. One possibility is that modification of cysteine 501 in a membrane-spanning region results in decreased or modified interactions of the membrane-spanning segments in the native tetrameric structure. This is a reasonable postulate because it has been shown that the membrane-spanning regions are important for maintaining the tetrameric structure and the enzyme activity of eNTPDase1 (CD39) (29). To test this possibility for mechanism of inactivation, we dissociated tetrameric eNTPDase3 into monomers by solubilization with Triton X-100 (Fig. 6) and examined the effect of pCMPS on solubilized monomeric enzyme. The fact that pCMPS no longer substantially inhibited the residual nucleotidase activity after solubilization with Triton X-100 (Fig. 7) suggests that this inhibition is dependent upon the oligomeric structure of eNTPDase3. The non-additive inhibitory effects of pCMPS and Triton X-100 on the nucleotidase activities of eNTPDase3 (Fig. 8) also suggest a common mechanism of enzyme inactivation by these two reagents, mediated by a weakening or disruption of the oligomeric structure.

Knowles and co-workers (17) have reported that pCMPS inhibits chicken muscle ecto-ATPase (eNTPDase2). These authors hypothesized that a cysteine residue located at the interface between the N-terminal membrane-spanning region and the large extracellular loop (cysteine 23) is the most likely target for modification by pCMPS, leading to inhibition of nucleotidase activity by interfering with that enzyme’s oligomerization. The data presented in our study, using site-directed mutagenesis of eNTPDase3, are consistent with their hypothesis that the pCMPS-induced inhibition of eNTPDase3 is mediated by interfering with monomer-monomer interactions in the native tetrameric quaternary structure. However, the proposed location of the cysteine residue responsible for inhibition of nucleotidase activity by pCMPS in chicken eNTPDase2 (the extracellular face of the N-terminal membrane-spanning segment) is different from the location of the
pCMPS-reactive cysteine reside found in this work because cysteine 501 of eNTPDase3 is located deep within the C-terminal membrane-spanning region, slightly closer to the cytoplasmic face than to the extracellular face of the cell membrane.

A diagram summarizing the results and conclusions from this study is presented in Fig. 9. Cysteine 10, located in the N-terminal cytoplasmic tail, is shown to be the site of sulphydryl cross-linking. Cysteine 501, located in the C-terminal membrane-spanning helix, is depicted to be accessible to pCMPS and water. Cysteine 509, located at the interface of the membrane and the C-terminal cytoplasmic tail, is depicted to be accessible to araldehyde cross-linking is depicted as occurring on the extracellular face than to the extracellular face of the cell membrane. Cysteine 501 of eNTPDase3 is located deep within the C-terminal membrane-spanning region, slightly closer to the cytoplasmic face than to the extracellular face of the cell membrane.

Further investigation of the extent and functional significance of this aqueous crevice that has been postulated in this work. Because the transmembrane domains greatly modulate the nucleotidase activity of eNTPDases (17, 29, 30), future understanding of the interactions of the membrane-spanning helices, both within monomers and between monomers in the native tetramer, will be dependent on the delineation of the details of this aqueous crevice that has been postulated in this work. Further investigation of the extent and functional significance of this aqueous crevice in the transmembrane regions of eNTPDase3 and the existence of such an aqueous crevice in other membranous eNTPDases should lead to a better understanding of the structure and function of these enzymes.

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