Inhibition of Human NTPDase 2 by Modification of an Intramembrane Cysteine by *p*-Chloromercuriphenylsulfonate and Oxidative Cross-Linking of the Transmembrane Domains[†]

Wei-Chieh Chiang and Aileen F. Knowles*

Department of Chemistry and Biochemistry, San Diego State University, San Diego, California 92182-1030

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ABSTRACT: Human NTPDase 2 is a cell surface integral membrane glycoprotein that is anchored to the membranes by two transmembrane domains while the bulk of the protein containing the active site faces the extracellular milieu. It contains 10 conserved cysteine residues in the extracellular domain that are involved in disulfide bond formation and one free cysteine residue, C26, which is located in the N-terminal transmembrane domain. The human NTPDase 2 activity is inactivated by membrane perturbation that disrupts interaction of the transmembrane domains and is inhibited by *p*-chloromercuriphenylsulfonate (pCMPS), a sulfhydryl reagent. In this report, we show that C26 is the target of pCMPS modification, since a mutant in which C26 was replaced with a serine was no longer inhibited by pCMPS. Mutants in which cysteine residues are placed in the C-terminal transmembrane domain near the extracellular surface were still modified by pCMPS, but the degree of inhibition of their ATPase activity was lower than that of the wild-type enzyme. Thus, loss of the ATPase activity of human NTPDase 2 in the presence of pCMPS probably results from the disturbance of both transmembrane domain interaction and its active site. Inhibition of human NTPDase 2 activity by pCMPS and membrane perturbation is attenuated when the enzyme is cross-linked by glutaraldehyde. On the other hand, NTPDase 2 dimers formed from oxidative cross-linking of the wild-type enzyme and mutants containing a single cysteine residue in the C-terminal transmembrane domain displayed reduced ATPase activity. A similar reduction in activity was also obtained upon intramolecular disulfide formation in mutants that contain a cysteine residue in each of the two transmembrane domains. These results indicate that the mobility of the transmembrane helices is necessary for maximal catalysis.

Proteins that constitute the NTPDase[1] family are membrane-bound nucleotide hydrolases that are activated by either Mg^{2+} or Ca^{2+} (*1*). They are located on the cell surface and intracellular membranes and are anchored to the membranes by either one or two transmembrane domains, whereas the bulk of the NTPDase protein containing the active site is either in the intracellular lumen or facing the extracellular milieu (*2*). Different members of the NTPDase family have different substrate preferences. NTPDase 2 is primarily an NTPase (*3, 4*), whereas NTPDases 4, 5, and 6 are primarily NDPases (*5–8*), with NTPDases 1, 3, 7, and 8 displaying an NDPase/NTPase ratio ranging from 0.25 to 1 (*9, 10*).

Unlike the membrane-bound ion-motive ATPases, i.e., the P-, V-, and F-type ATPases, the NTPDases have no specific inhibitors. Most of the cell surface NTPDases, i.e., NTPDases 1, 2, 3, and 8, have reduced activity in the presence of detergents (*1*). These results are explained by the effects of the detergents on the intra- and intermolecular interactions of the two transmembrane domains that are located near the N- and C-termini of these proteins, which in turn regulate catalysis in the extracellular domain (*11–13*). Most membrane-bound NTPDases are inhibited by diethyl pyrocarbonate (*14–16*), which reacts with a conserved histidine in apyrase conserved region 1 (ACR1) (*16–18*). 5′-Fluorosulfonyladenosine, an ATP analogue, has also been shown to inactivate human NTPDase 2 (*19*), porcine pancreas NTPDase 1 (*20*), and the *Torpedo* electric organ ATP diphosphohydrolase (*21*). NTPDases that hydrolyze both NTP and NDP, i.e., NTPDases 1, 3, and 8, are inhibited by high concentrations (2−10 mM) of azide (*22–27*). Azide inhibition of the purified chicken ecto-ATP-diphosphohydrolase (NTPDase 8) is of the mixed and uncompetitive types and is most pronounced with MgADP as the substrate, whereas inhibition is markedly weakened with Ca nucleotides as the substrates (*23*).

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* To whom correspondence should be addressed: Department of Chemistry and Biochemistry, San Diego State University, 5500 Campanile Dr., San Diego, CA 92182-1030. Telephone: (619) 594-2065. Fax: (619) 594-4634. E-mail: aknowles@chemistry.sdsu.edu.

[1] Abbreviations: NTPDase, nucleoside triphosphate diphosphohydrolase; ECD, extracellular domain; TMD, transmembrane domain; TMD1, N-terminal transmembrane domain; TMD2, C-terminal transmembrane domain; ACR, apyrase conserved region; NP-40, Nonidet P-40; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid; pCMPS, *p*-chloromercuriphenylsulfonate; pCMB, *p*-chlomercuribenzoate; CuP, copper phenanthroline; ConA, concanavalin A; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DSS, disuccinimidyl suberate; mPEG-maleimide, methoxy poly(ethylene glycol) maleimide.

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A

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chicken gizzard            MARRAAAVLLLLALGCLLGILLLCLGSGDAR
rat brain A              MAGKLVSLVPPLLLAAAGLTGLLLLCVPTQDVR
rat brain B              MAGKLVSLVPPLLLAAAGLTGLLLLCVPTQDVR
mouse hepatoma           MAGKLVSLVPPLLLAAVGLAGLLLLCVPTQDVR
human NTPDase 2       1 MAGKVRSLLPPLLLAAAGLAGLLLLCVPTRDVR 33
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B

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NTPDase 1(h)          MEDTKESNVKTFCSKNILAILGFSSIIAVIALLAVGLTQNK
         (r)          MEDIKDSKVKRFCSKNILIILGFSSVLAVIALLAVGLTHNK
NTPDase 3(h)   MFTVLTRQPCEQAGLKALYRTPTIIALVVLLVSIVVLVSITVIQIHK
         (r)   MFTVMTRQPCEQAGFRALSRTPAIVTLVVLLVSIVVLVTLTLIQIHH
NTPDase 8(h)                  MGLSRKEQVFLALLGASGVSGLTALILLLVEAT
         (m)                  MGLSWKERVFMALLGVAAASGLTMLVLILVKAI
         (ck)                   MEYKGKVVAGLLTATCVFSIIALILSAVDVK
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FIGURE 1: N-Terminal sequences of cell-surface NTPDases. (A) N-Terminal sequences of chicken NTPDase 2 (U74467), rat brain NTPDase 2A (Y11835), rat brain NTPDase 2B (AF129103), mouse NTPDase 2 (AF042811), and human NTPDase 2 (EF495152/AF144748). The N-terminal transmembrane domain (TMD1) is underlined. NTPDase 2 enzymes from different species contain a conserved cysteine residue (bold), which is C26 in human NTPDase 2. (B) N-Terminal sequences of human and rat NTPDase1/CD39 (P49961 and U81295, respectively), human and rat NTPDase 3 (AF034840 and AJ437217, respectively), and human, mouse, and chicken NTPDase 8 (AY903954/AY430414, AY364442, and AF426405, respectively). The N-terminal transmembrane domain (TMD1) is underlined. TMD1 motifs of these proteins do not contain a conserved cysteine residue.

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                          TMD1                    TMD2

WT              SLLPPLLLAAAGLAGLLLLCVPT   FSSWVVLLLLFASALLAALVLLL
C26S            SLLPPLLLAAAGLAGLLLLSVPT   FSSWVVLLLLFASALLAALVLLL
C26A            SLLPPLLLAAAGLAGLLLLAVPT   FSSWVVLLLLFASALLAALVLLL

C26S/S462C      SLLPPLLLAAAGLAGLLLLSVPT   FCSWVVLLLLFASALLAALVLLL
C26S/S463C      SLLPPLLLAAAGLAGLLLLSVPT   FSCWVVLLLLFASALLAALVLLL
C25S/W464C      SLLPPLLLAAAGLAGLLLLSVPT   FSSCVVLLLLFASALLAALVLLL
C26S/V465C      SLLPPLLLAAAGLAGLLLLSVPT   FSSWCVLLLLFASALLAALVLLL
C26S/V466C      SLLPPLLLAAAGLAGLLLLSVPT   FSSWVCLLLLFASALLAALVLLL

S462C           SLLPPLLLAAAGLAGLLLLCVPT   FCSWVVLLLLFASALLAALVLLL
S463C           SLLPPLLLAAAGLAGLLLLCVPT   FSCWVVLLLLFASALLAALVLLL
W464C           SLLPPLLLAAAGLAGLLLLCVPT   FSSCVVLLLLFASALLAALVLLL
V465C           SLLPPLLLAAAGLAGLLLLCVPT   FSSWCVLLLLFASALLAALVLLL
V466C           SLLPPLLLAAAGLAGLLLLCVPT   FSSWVCLLLLFASALLAALVLLL
                                  ↑         ↑    ↑
                                  26        462  466
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FIGURE 2: Amino acid sequences of TMD1 and TMD2 of wild-type and mutant NTPDase 2 enzymes used in this study. The mutated amino acid residues are shown in bold.

NTPDase 2, which displays an ADPase/ATPase ratio of 0.05−0.1, is not inhibited by high concentrations of azide (*15, 28*). Chicken smooth muscle and human tumor NTPDase 2 are inhibited by mercurials, e.g., *p*-chloromercuriphenylsulfonate (pCMPS) or *p*-chlomercuribenzoate (pCMB) (*19, 28, 29*), which react with free cysteine residues. Although cell surface NTPDases contain a variable number of cysteine residues, 10 of these in the extracellular domain are conserved and have been shown to be involved in disulfide bond formation (*30*). In human NTPDase 2, there is only one additional free cysteine residue, C26, located in the N-terminal transmembrane domain (TMD1), and close to the cell surface. This cysteine residue is conserved in TMD1 of NTPDase 2 enzymes of other species (Figure 1A), but not those of NTPDases 1, 3, and 8 (Figure 1B). In this report, we show that C26 in TMD1 of human NTPDase 2 is the target of pCMPS modification. Mutants in which C26 was replaced with serine (C26S) or alanine (C26A) were no longer inhibited by pCMPS, while other characteristics of human NTPDase 2 were not affected. To investigate whether human NTPDase 2 is still inhibited by pCMPS if a free cysteine residue is located in the C-terminal TMD (TMD2), we also generated mutants containing a single free cysteine residue in TMD2 (Figure 2). The level of inhibition of these mutants by pCMPS was reduced when compared to that of wild-type human NTPDase 2.

In a previous report (*4*), we showed that treatment of wild-type human NTPDase 2 with glutaraldehyde or concanavalin A (ConA), which promotes oligomer formation, abolished the time-dependent inactivation of the enzyme by substrate, thus giving rise to apparent stimulation of enzyme activity in a 10 min assay. Glutaraldehyde and ConA cross-linking also attenuated the decrease in activity in the presence of NP-40 and high temperatures, indicating that oligomerized human NTPDase 2 is less susceptible to the inhibitory effect of NP-40, high temperature, and substrates. Since evidence obtained recently in our laboratory indicated that the strength of the interaction of the TMD is an important determinant in enzyme stability and activity (*12, 13*), we generated additional mutants containing two cysteine residues, one in each of the two TMDs, and determined the effect of oxidative cross-linking of these cysteine residues on enzyme activity

(Figure 2). In contrast to the results obtained by cross-linking by glutaraldehyde and ConA, inter- and intramolecular cross-linking of the cysteine residues in the TMD resulted in significant decreases in human NTPDase 2 activity. On the other hand, immobilization of the TMD helices due to formation of intramolecular disulfide bonds abolished inactivation of the residual activity of human NTPDase 2 by detergents and higher temperatures.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), OptiMEM, fetal bovine serum, penicillin/streptomycin solution, trypsin-EDTA, subcloning and library efficiency DH5a cells, and Lipofectamine were purchased from Invitrogen. Newborn calf serum was purchased from Gemini Bio-Products Inc. *Pfu* Turbo DNA polymerase was purchased from Stratagene. DpnI was purchased from New England Biolabs. The DNA miniprep kit was purchased from QIAGEN. SDS−PAGE reagents and the Bio-Rad DC protein assay kit were purchased from Bio-Rad. PVDF membrane and [γ-^{32}P]ATP were purchased from PerkinElmer Life Sciences Inc. Goat anti-rabbit IgG conjugated to alkaline phosphatase was purchased from Promega (Madison, WI). Alkaline phosphatase substrate tablets (NBT/BCIP) were purchased from Roche. Nucleotides, detergents, and all other biochemical reagents were purchased from Sigma Chemical Co. mPEG-maleimide (MW = 5000) was purchased from Laysan Bio Inc. Oligonucleotides used as primers for PCR and sequencing were synthesized at the San Diego State University Microchemical Core Facility or Integrated DNA Technologies. DNA sequencing service was provided by the San Diego State University Microchemical Core Facility. The rabbit polyclonal antibody against the C-terminus (LRQVH-SAKLPSTI-COOH) of human NTPDase 2 was a kind gift from T. Kirley (Department of Pharmacology and Cell Biophysics, University of Cincinnati, Cincinnati, OH).

Site-Directed Mutagenesis. The following human NTPDase 2 mutants were generated for this study. In the C26S and C26A mutants, the single free cysteine, C26, was replaced with serine and alanine, respectively. The C26S/S462C, C26S/S463C, C26S/W464C, C26S/V465C, and C26S/V466C mutants contain a single free cysteine residue in TMD2 and were generated by replacing S462, S463, W464, V465, and V466 with cysteine. The S462C, S463C, W464C, V465C, and V466C mutants contain a cysteine residue in TMD1 (C26) and a cysteine residue in TMD2 at positions 462−466, respectively (Figure 2). The human NTPDase 2 mutant cDNAs were generated by PCR using either wild-type or C26S human NTPDase 2 cDNA in pcDNA3 as the template and appropriate mutagenic primers as previously described (*31*). The following forward mutagenic primers were used: 5′-cctcctactgctgtccgtcccccacccag-3′ for C26S, 5′-cctcctactgctggccgtccccacccgc-3′ for C26A, 5′-ggcacagacttctgctcctgggtcgtcctc-3′ for S462C and C26S/S462C, 5′-cacagacttcagctgctgggtcgtcctcctg-3′ for S463C and C26S/S463C, 5′-gacttcagctcctgcgtcgtcctcctgctgc-3′ for W464C and C26S/W464C, 5′-cttcagctcctggtgcgtcctcctgctgctc-3′ for V465C and C26S/V465C, and 5′-cagctcctgggtctgcctcctgctgctcttc-3′ for V466C and C26S/V466C (codons that give rise to the specified mutation underlined). After transformation in DH5α cells and propagation of the ampicilin-resistant colony, plasmids containing the mutant cDNAs were isolated and sequenced to verify the desired mutation and the absence of unwanted mutation.

Transfection and Membrane Preparation. Transient transfection of HEK293 cells by wild-type and mutant human NTPDase 2 cDNA in pcDNA3 and selection of stable transfectants were performed as described previously (*4, 12, 13, 27*). The plasma membrane-enriched fraction from stably transfected cells was prepared by differential and sucrose gradient centrifugation as described previously (*4, 12, 13, 27*).

Determination of ATPase Activity. The ecto-ATPase activity of the intact transfected cells was determined in a 250 μL reaction mixture containing 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 5 mM [γ-^{32}P]ATP ($\sim 1.5 \times 10^6$ cpm/μmol of ATP). The ATPase activity of membranes prepared from the stably transfected cells was determined in a 250 μL reaction mixture containing 25 mM HEPES (pH 7.5), 4 mM $MgCl_2$, and 1 mM [γ-^{32}P]ATP. The amount of $^{32}P_i$ released was determined as described previously (*19*). To determine the ATPase activity in the presence of pCMPS, membranes were preincubated in the reaction mixture with the indicated concentrations of pCMPS for 5 min at room temperature before the reactions were initiated by the addition of ATP. For glutaraldehyde cross-linking, membranes at a protein concentration of 0.3 mg/mL were incubated in 20 mM MOPS and 5 mM $MgCl_2$ with 10 mM glutaraldehyde. After 20 min at room temperature, the reaction was terminated by the addition of 0.1 volume of 0.25 M lysine. Aliquots of membranes were assayed for ATPase activity in the absence or presence of the indicated concentrations of pCMPS.

Oxidative Cross-Linking. Oxidative cross-linking of cysteine residues of wild-type and mutant NTPDase 2 was carried out using membranes prepared from stably transfected cells. The oxidizing agent, copper phenanthroline (CuP), was prepared by combining cupric sulfate and 1,10-phenanthroline at a 1:3 molar ratio in water. Membranes at a concentration of 0.5 mg/mL were treated with the indicated CuP concentration in 12 mM HEPES at pH 7.5 and 37 °C for 20 min. For samples to be directly applied to the gel, the oxidation reactions were stopped by adding equal volume of 2× nonreducing SDS loading buffer containing 10 mM *N*-ethylmaleimide and 10 mM EDTA. For samples to be used for ATPase assays, reactions with CuP were stopped by 10-fold dilution of the mixtures with buffer containing 25 mM HEPES (pH 7.5) and 2 mM EDTA. For samples to be further treated with mPEG-maleimide (MW = 5000), the cross-linking reactions were stopped by adding 0.5 volume of 20 mM Tris-HCl (pH 7.5) containing 20 mM EDTA and 1% SDS. mPEG-maleimide was then added to a final concentration of 3 mM, and the mixtures were incubated at room temperature for 20 min. The reactions were stopped by the addition of an equal volume of 2× nonreducing SDS loading buffer.

SDS−PAGE and Western Blot Analysis. SDS−PAGE was performed using a 7.5% polyacrylamide gel in a MINI-PROTEAN II apparatus (Bio-Rad). For Western blot analysis, the antibody against the C-terminus of human NTPDase 2 (5000-fold dilution) was used as the primary antibody and goat anti-rabbit IgG conjugated to alkaline phosphatase (5000-fold dilution) was used as the secondary antibody. The

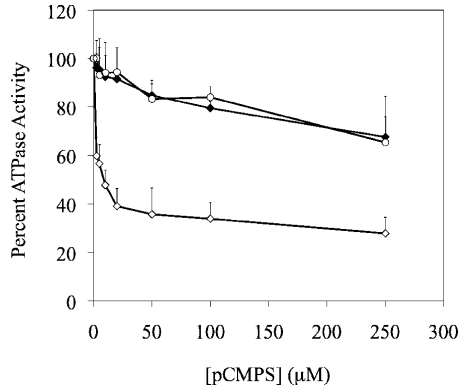

FIGURE 3: Effect of pCMPS on ATPase activity. Membranes containing wild-type human NTPDase 2 were pretreated with (◆) or without (◇) 10 mM glutaraldehyde or C26S mutant NTPDase 2 without glutaraldehyde (○) as described in Materials and Methods. Aliquots of membranes were assayed for ATPase activity in the absence or presence of the indicated concentrations of pCMPS. The 100% activities for wild-type human NTPDase 2 with and without glutaraldehyde treatment and the C26S mutant were 18.0 ± 1.3, 23.9 ± 1.6, and 19.9 ± 0.1 μmol min^{-1} mg^{-1}, respectively.

immunoreactive proteins were detected using an alkaline phosphatase substrate (NBT/BCIP) solution.

RESULTS

Inhibition of Expressed Human NTPDase 2 by pCMPS. We previously reported that pCMPS inhibited the plasma membrane ATPase of the human small cell lung carcinoma xenograft, an NTPDase 2 (*19*), and the ecto-ATPase activity of intact lung tumor cells (*28*). Inhibition of ATPase activity by mercurials was also observed in plasma membranes prepared from chicken gizzard smooth muscle (*29*), a tissue abundant in ecto-ATPase and NTPDase 2 (*32*). The sensitivity to pCMPS inhibition was retained in the ATPase in membranes prepared from HEK293 cells stably transfected with the human NTPDase 2 cDNA (Figure 3, bottom curve). Approximately 40% inhibition was obtained at a pCMPS concentration of 2.5 μM. Maximal inhibition, ~70%, was obtained at a pCMPS concentration of 0.25 mM.

The Target of pCMPS Modification Is Cysteine 26. Wild-type human NTPDase 2 contains the same 10 conserved cysteine residues, i.e., C75, C99, C242, C265, C284, C310, C323, C328, C377, and C409, in the extracellular domain as other cell surface NTPDases. These conserved cysteine residues have been shown to be involved in disulfide bond formation in human NTPDase 3 (*30*) and most likely serve the same function in human NTPDase 2. Human NTPDase 2 contains an additional free cysteine residue, C26, located in TMD1 (Figure 1A), which is the most likely target of pCMPS. If this is the case, a mutant protein in which C26 is replaced with other amino acids should become resistant to pCMPS inhibition. Two mutants, C26A and C26S, which lack the free cysteine, were generated and gave very similar results. Only those results obtained with C26S are summarized here. Substitution of C26 with serine had a minimal effect on either protein expression (Figure 4, lane 2) or activity (Table 1). However, the level of pCMPS inhibition was markedly reduced [Figure 3, top curve (○)]. The conclusion that C26 is the target of pCMPS modification is further supported by the results of oxidative cross-linking. Wild-type human NTPDase 2 is a 66 kDa protein (Figure 5, lane 1). Treatment of the membranes with pCMPS did not alter protein mobility on SDS−PAGE (Figure 5, lane 2). Upon oxidative cross-linking with Cu phenanthroline, the majority of the wild-type 66 kDa monomer was converted to a dimer due to formation of a disulfide bond between the C26 residues of the two monomers (Figure 5, lane 3). Dimer

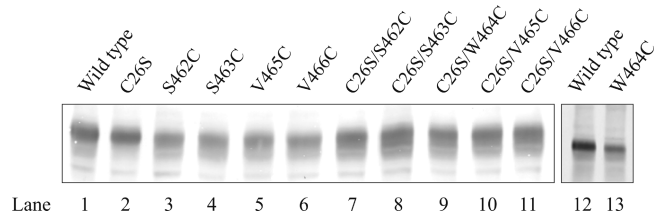

FIGURE 4: Protein expression of wild-type and mutant human NTPDase 2 in HEK cells. Cell lysates (20 μg of protein) of HEK293 cells transiently transfected with wild-type and mutant human NTPDase 2 cDNA in pcDNA3 were subjected to SDS−PAGE and Western blot analysis. Protein expression of the W464C mutant was assessed in a separate experiment.

Table 1: ATPase Activities of HEK Cells Transfected with Wild-Type and Cysteine Mutant Human NTPDase 2 cDNAs[a]

human NTPDase 2 cDNA	percentage of wild-type ATPase activity	human NTPDase 2 cDNA	percentage of wild-type ATPase activity
wild type	100	C26S/V466C	97.3 ± 3.3
C26S	88.7 ± 8.4	S462C	58.1 ± 2.1
C26S/S462C	86.6 ± 9.1	S463C	55.4 ± 8.4
C26S/S463C	73.5 ± 9.7	W464C	2.4 ± 0.04
C26S/W464C	4.9 ± 1.1	V465C	60.1 ± 8.2
C26S/V465C	80.0 ± 9.0	V466C	63.9 ± 9.2

[a] HEK293 cells were transfected with wild-type and mutant human NTPDase 2 in pcDNA3 as described in Materials and Methods. The ATPase activity of the intact cells was determined 48 h after transfection. The ATPase activity of the cells transfected with wild-type human NTPDase 2 cDNA ranged from 0.8 to 4.5 μmol min^{-1} mg^{-1}. Values are reported as a percentage of the wild-type ATPase activity and the averages of three separate transfection experiments ± standard deviations.

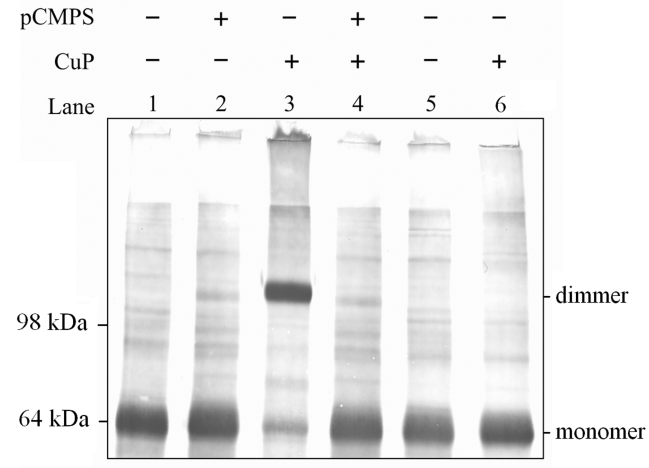

FIGURE 5: Oxidative cross-linking of wild-type and C26S mutant human NTPDase 2 protein without and with covalent modification by pCMPS. Membranes (10 μg) containing wild-type and mutant human NTPDase 2 were incubated for 5 min without and with 0.5 mM pCMPS in a volume of 18 μL at room temperature, after which 2 μL of 5 mM CuP was added to a concentration of 0.5 mM to the indicated samples and incubated at 37 °C for 20 min. Aliquots of the membranes (7.5 μg) were subjected to SDS−PAGE and Western blot analysis: lanes 1−4, membranes containing wild-type human NTPDase 2; lanes 5 and 6, membranes containing C26S mutant NTPDase 2.

formation was not detected (Figure 5, lane 4) if human NTPDase 2 was first treated with pCMPS, indicating that C26 has reacted with pCMPS and oxidative cross-linking by CuP could not occur. In contrast, the C26S mutant protein (Figure 5, lane 5), which lacks a free cysteine residue, did not form a dimer upon oxidative cross-linking (Figure 5, lane 6). These results provided conclusive evidence that formation of a dimer upon oxidative cross-linking requires the presence of free C26. Dimer formation was abolished if C26 was either substituted with serine or modified by pCMPS.

The Level of Inhibition by pCMPS Is Reduced if the Free Cysteine Residue Is Located in TMD2. We then set out to determine if pCMPS will inhibit the ATPase activity of human NTPDase 2 by reacting with a cysteine residue in TMD2 that is located in a position similar to that occupied by C26 in TMD1 near the exterior of the bilayer. Different programs predicting transmembrane topology were used to define the TMD2 region. Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) predict that TMD2 begins with F461, thus placing S462 and S463 within TMD2, whereas TopPred (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) and DAS (http://www.sbc.su.se/~miklos/DAS/) designate S463 as the first amino acid of TMD2 (see Figure 2). Five mutants, C26S/S462C, C26S/S463C, C26S/W464C, C26S/V465C, and C26S/V466C, were generated to cover both possibilities. These mutant cDNAs were generated by PCR using the cDNA encoding the C26S mutant as the template. The resultant mutant proteins contain a single free cysteine residue in positions 462−466 of the human NTPDase 2 polypeptide (Figure 2).

The ATPase activity of HEK cells transiently transfected with these mutant cDNAs ranged from 75 to 100% of that of the cells transfected with wild-type human NTPDase 2 cDNA, except for C26S/W464C, which lost ~95% of the activity (Table 1). Protein expression of all five mutants, including that of C26S/W464C, was similar to that of the wild-type protein (Figure 4, lanes 7−11).

Membranes were prepared from HEK cells stably transfected with the C26S/S462C, C26S/S463C, C26S/V465C, and C26S/V466C mutant cDNAs and used for ATPase assays in the absence and presence of 0.1 mM pCMPS. Although all four mutant proteins contain a free cysteine, the degree of inhibition of the ATPase activities by pCMPS was greatly reduced compared to that of the wild-type enzyme containing C26 (Table 2). The ATPase activity of the C26S/S462C and C26S/S453C mutant proteins was inhibited by 24 and 14%, respectively, while the ATPase activity of the C26S/V465C and C26S/V466C mutant proteins was inhibited by ~40%.

The significantly attenuated inhibition of the ATPase activity of the mutants containing a single cysteine residue in TMD2 by pCMPS was not due to a failure of the reaction of the mercurial with the introduced cysteine residues. Similar to the wild-type enzyme (Figure 5, lanes 3 and 4), the mutant proteins were able to form dimers when subjected to oxidative cross-linking by CuP (Figure 6, lanes 1, 3, 5, and 7), albeit to different extents. However, no dimer formation was obtained with three of the mutant proteins if the membranes were previously incubated with pCMPS (Figure 6, lanes 2, 4, and 8). The level of dimer formation

Table 2: Inhibition of Wild-Type and Mutant Human NTPDase 2 ATPase Activity by pCMPS[a]

	percent inhibition (by 0.1 mM pCMPS)		percent inhibition (by 0.1 mM pCMPS)
wild type	77.4 ± 2.9	C26S/V466C	37.7 ± 5.6
C26S	7.6 ± 5.1	S462C	80.7 ± 1.1
C26S/S462C	24.2 ± 1.4	S463C	90.7 ± 0.7
C26S/S463C	14.4 ± 3.0	V465C	91.5 ± 1.4
C26S/V465C	39.9 ± 3.6	V466C	82.8 ± 1.2

[a] Membranes used for ATPase assays were prepared from the stably transfected cells as described in Materials and Methods. The ATPase activity of the membranes containing wild-type and mutant human NTPDase 2 ranged from 25 to 50 μmol min^{-1} mg^{-1}. Values reported are the averages of three separate ATPase assays ± standard deviations. Percent inhibition was calculated using ATPase activity of the wild type and different mutants obtained in the absence of 0.1 mM pCMPS as 100%.

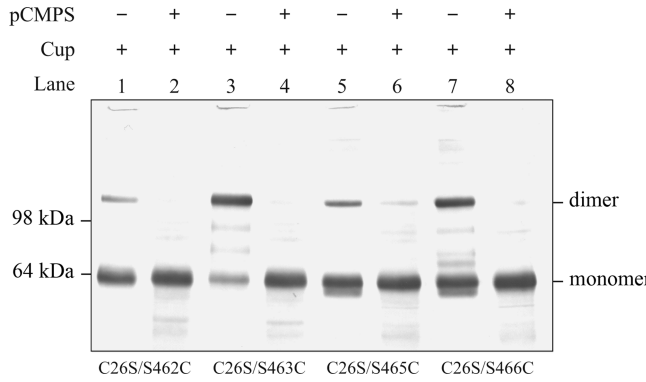

FIGURE 6: Oxidative cross-linking of human NTPDase 2 mutant proteins containing a single cysteine residue in TMD2 without and with covalent modification by pCMPS. Membranes containing C26S/S462C (lanes 1 and 2), C26S/S463C (lanes 3 and 4), C26S/V465C (lanes 5 and 6), and C26S/V466C (lanes 7 and 8) mutant NTPDase 2 were incubated without and with 0.5 mM pCMPS as described in the legend of Figure 5, after which 2 μL of CuP was added to a concentration of 0.5 mM and incubated for 20 min at 37 °C. Aliquots of the membranes (7.5 μg) were subjected to SDS−PAGE and Western blot analysis.

of C26S/V465C was markedly reduced if the membranes were pretreated with pCMPS (Figure 6, lane 6).

It is interesting to note that the largest amount of dimer was obtained with the C26S/S463C mutant protein. The extent of dimer formation of this mutant protein (Figure 6, lane 3) was comparable to that of the wild-type enzyme (Figure 5, lane 3). The facile cross-linking of cysteine residues at positions 26 and 463 was further supported by the observation that a significant amount of dimer formation was obtained with the wild-type enzyme and the C26S/S463C mutant protein only when the reaction with CuP was carried out at 4 °C (data not shown).

Intramolecular Cross-Linking of Human NTPDase 2 Mutants That Contain One Cysteine Residue Each in TMD1 and TMD2. We showed previously that treatment of wild-type human NTPDase 2 with glutaraldehyde, which promotes oligomer formation, abolished the inhibitory effect of NP-40, high temperature, and substrate (*4*). Figure 3 (top curve, ◆) shows that the inhibition of the ATPase activity of glutaraldehyde-treated membranes with 50 μM pCMPS was negligible when ~60% inhibition of the ATPase activity was obtained in untreated membranes. These results suggest that oligomers of human NTPDase 2 are less susceptible to

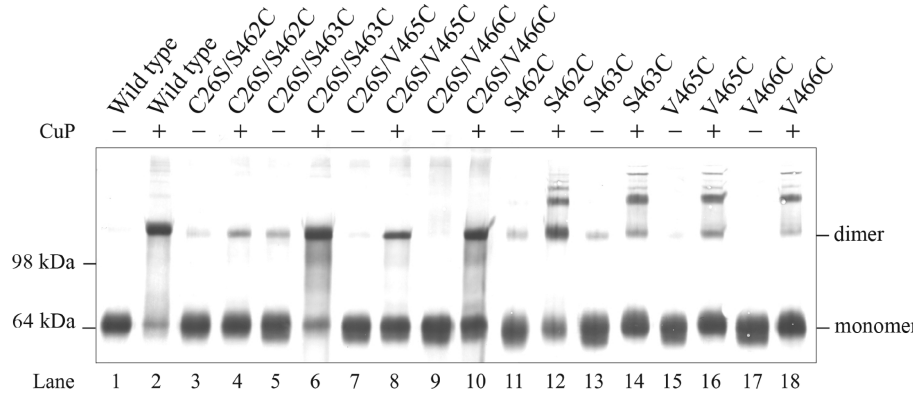

FIGURE 7: Oxidative cross-linking of wild-type human NTPDase 2 and mutant proteins containing one or two cysteine residues. Membranes (10 μg) containing wild-type and mutant NTPDase 2 were incubated without or with 0.5 mM CuP in a 20 μL solution containing 12 mM HEPES buffer (pH 7.5) for 20 min at 37 °C, after which 20 μL of 2× SDS gel sample buffer was added. The entire samples were used for SDS−PAGE and Western blot analysis.

pCMPS inhibition. However, because of the large number of lysine and other amino acid residues that can react with glutaraldehyde in the human NTPDase 2 protein, intramolecular cross-linking within an NTPDase 2 monomer may also occur, and the possibility that some of the effects of glutaraldehyde are due to intramolecular cross-linking cannot be ruled out. This possibility is of particular interest since recent evidence suggests that the strength of the interhelical interaction of TMD1 and TMD2 within the monomer of the NTPDases is more important in regulating catalysis than the intermolecular interaction of TMD between two different NTPDase 2 monomers (*13*). Oxidative cross-linking between two cysteine residues in TMD1 and TMD2 would provide one means of strengthening intramolecular TMD interaction. Thus, we generated mutants containing one cysteine residue in TMD1 (C26) and a second cysteine in TMD2 in the proximity of C26, i.e., S462C, S463C, W464C, V465C, and V466C (Figure 2).

The ATPase activity of HEK293 cells transiently transfected with these mutant cDNAs was approximately 60% of that of cells transfected with wild-type human NTPDase 2 cDNA, except for W464C, which lost ~98% of the activity (Table 1). The level of protein expression of these mutants was also lower than that of the wild-type enzyme (Figure 4, lanes 3−6 and 13). Nevertheless, the ATPase activity of membranes obtained from HEK cells stably transfected with the four active mutants was 80−90% inhibited by 0.1 mM pCMPS (Table 2). This was expected since these four mutants retain the original free cysteine residue, C26.

Upon CuP treatment, three of the mutants with a cysteine residue each in TMD1 and TMD2, S463C, V465C, and V466C, formed fewer dimers than C26S/S463C, C26S/V465C, and C26S/V466C, which contain only one cysteine residue in TMD2 (compare lane 14 with lane 6, lane 16 with lane 8, and lane 18 with lane 10 in Figure 7). This was most noticeable with the C26S/S463C and S463C mutant pair (Figure 7, lanes 6 and 14).

S462C was an exception in that it formed more dimers than C26S/S462C after CuP treatment (compare lane 12 and lane 4 in Figure 7). It appears that the presence of C26 in the S462C mutant promotes formation of a dimer between C26 and C462 of different monomers, suggesting that these two residues are at the interface between two different monomers.

Higher-order oligomers were consistently obtained in mutant proteins containing two free cysteine residues after oxidative cross-linking, although they account for only a small amount of the total protein. The majority of the S463C, V465C, and V466C mutant proteins remained as monomers after CuP treatment (Figure 7, lanes 14, 16, and 18). These results suggest that intramolecular cross-linking was generally favored over intermolecular cross-linking when these mutants were treated with CuP. While more dimer formation was observed in the S462C mutant than in the corresponding mutant containing a single cysteine residue, C26S/S462C, after oxidative cross-linking, intramolecular cross-linking in the S462C mutant also occurred and will be described below.

Intramolecular cross-linking of the S462C, S463C, V465C, and V466C mutants was further demonstrated when membranes without and with oxidative cross-linking were treated by mPEG-maleimide, which reacts with free cysteine residues and increases the molecular mass of the target proteins. Without CuP treatment, the reaction of the two free cysteine residues in these mutants with mPEG-maleimide was observed by the appearance of immunoreactive protein bands of higher molecular mass due to the added mass of one or two mPEG-maleimide molecules (5 or 10 kDa, respectively). With CuP treatment, the amounts of monomers remained the same without and with subsequent reactions with mPEG-maleimide (see the Supporting Information). These results indicate that intramolecular disulfide bonds have formed between the two cysteine residues in TMD1 and TMD2 and there is no free cysteine available to react with mPEG-maleimide.

Effect of Inter- and Intramolecular Oxidative Cross-Linking on ATPase Activity. The ATPase activity of wild-type human NTPDase 2 and the mutant proteins without and with CuP oxidative cross-linking was compared. Of the proteins containing one cysteine residue, the wild-type enzyme and the C26S/S463C mutant enzyme suffered an ~70% loss of activity after cross-linking (Table 3). The reduction of activity correlated with the extent of intermolecular cross-linking since the largest amounts of dimer were obtained in these two proteins after CuP treatment (Figure 7). The activity loss of the C26S/S462C, C26S/V465C, and C26S/V466C mutants, which formed less dimer, was 40−60% after CuP treatment.

Table 3: Effect of CuP Oxidative Cross-Linking on the ATPase Activity of Wild-Type Human NTPDase 2 and Its Mutants[a]

	percent activity		percent activity
wild type	26.2 ± 6.5	C26S/V466C	40.5±2.6
C26S	87.7 ± 11.3	S462C	26.4±9.5
C26S/S462C	60.0 ± 3.2	S463C	17.5±7.5
C26S/S463C	28.0 ± 3.8	V465C	28.6±2.7
C26S/V465C	58.2 ± 10.2	V466C	39.3±4.1

[a] Membranes were preincubated with or without CuP, and the ATPase activity was determined as described in Materials and Methods. The ATPase activity of the membranes ranged from 13 to 18 μmol min^{-1} mg^{-1}. Percent activity was calculated using the ATPase activity of the wild type and mutants obtained without CuP cross-linking as 100%. Values reported are the averages of three separate experiments ± standard deviations.

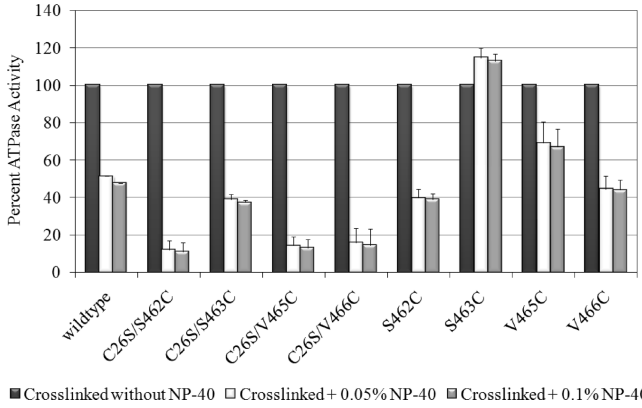

FIGURE 8: Effect of inter- and intramolecular cross-linking on the NP-40 inactivation of ATPase activity of wild-type and mutant human NTPDase 2. Membranes (10 μg) containing C26S/S462C, C26S/S463C, C26S/V465C, C26S/V466C, S462C, S463C, V465C, or V466C mutant NTPDase 2 were incubated for 20 min without or with 0.5 mM CuP in a 20 μL reaction mixture at 37 °C for 20 min. Aliquots of the membranes were assayed for ATPase activity in the absence or presence of the indicated concentrations of NP-40. The ATPase activity of the cross-linked wild type and mutants was used as 100%. Values reported are the average of three separate experiments ± standard deviations.

The ATPase activity of the S462C, S463C, V465C, and V466C mutant proteins that contain a cysteine residue each in TMD1 and TMD2 was reduced to 20−40% after CuP treatment, with the S463 mutant showing the greatest loss of activity (Table 3). Taken together, these data and the Western blot analysis shown in Figure 7 indicate that the loss of ATPase activity of these mutants was a consequence of both intramolecular cross-linking and oligomer formation after CuP treatment. In conclusion, both inter- and intramolecular cross-linking of the TMDs caused human NTPDase 2 to suffer a marked loss of enzyme activity.

The Level of Inhibition of ATPase Activity by NP-40 Is Reduced after Intramolecular Oxidative Cross-Linking of the TMDs. Similar to that of wild-type human NTPDase 2, the activity of all the mutants generated in this study was inhibited 90% by 0.1% NP-40. As described above, CuP treatment reduced the ATPase activity of mutant NTPDase 2 to a variable extent. However, the residual activity of oxidatively cross-linked wild-type human NTPDase 2 and C26S/S463C, which formed the most dimers, was inhibited by NP-40 by only 50−60% (Figure 8). In contrast, the residual activity of the C26S/S462C, C26S/V465C, and C26S/V466C mutants, which formed less dimer, was still inhibited 90% by NP-40. Thus, the reduced level of inhibition by NP-40 correlated with the amount of dimer formed.

All of the mutants containing one cysteine residue each in TMD1 and TMD2 exhibited a decreased level of inhibition by NP-40 after oxidative cross-linking. The S463C mutant was completely resistant to inhibition by NP-40 after intramolecular cross-linking, while the level of inhibition of the cross-linked S462C, V465C, and V466C mutant enzymes by 0.1% NP-40 was reduced to 40−60%. These results suggest that both inter- and intramolecular oxidative cross-linking of the TMDs increased the degree of TMD interaction and reduced or abolished the inhibitory effect of NP-40.

Inter- and Intramolecular Oxidative Cross-Linking of TMDs Abolishes Inhibition of ATPase Activity by Higher Temperatures. The effect of temperature on the NTPDase 2 mutants containing one cysteine in TMD2, or with a cysteine residue each in TMD1 and TMD2, was similar to that of the wild-type enzyme in that ATPase activity declines at higher temperatures (*4*). The effect of high temperature was attributed to the disruption of TMD interaction due to increased membrane fluidity, which in turn destabilizes the active site. This is illustrated by the data obtained with the C26S/S463C and S463C mutants. The ATPase activity of these mutant enzymes at 60 °C was usually ∼30% of the activity obtained at 37 °C (Figure 9A,B). After CuP treatment, the residual ATPase activity of the C26S/S463C mutant increased, rather than diminished, at high temperatures up to 61 °C (Figure 9C), so the ratio of the ATPase activity obtained at 61 °C to that at 37 °C was ∼1.5. This was also seen with wild-type human NTPDase 2, the activity of which increased with temperature after CuP treatment (data not shown). In contrast, the activity of the C26S/S462C, C26S/V465C, and C26S/V466C mutants, which remained mostly as monomers after CuP treatment, decreased with temperature with or without CuP treatment (data not shown).

The S462C, S463C, V465C, and V466C mutants, which contain one cysteine residue each in TMD1 and TMD2, were insensitive to the effect of temperature following CuP treatment. Data obtained with S463C are shown in Figure 9D. After CuP cross-linking, the ATPase activity of the S463C mutant increased with temperature and the ratio of the ATPase activity obtained at 61 °C to that at 37 °C was ∼3.5.

In summary, our data indicate that the inhibitory effect of high temperatures on ATPase activity can be abolished by CuP treatment, when significant inter- and intramolecular oxidative cross-linking of the human NTPDase 2 protein occurs.

DISCUSSION

Inhibition of a cell surface ATPase by *p*-chlomercuribenzoate (pCMB) was first reported by Karasaki (*33*). This and subsequent studies showed that (i) a cell surface ATPase activity in the normal rat liver was localized at the bile canaliculi, (ii) the cell surface ATPase activity in *N*,*N*′-dimethylaminoazobenzene-induced rat hepatoma increased and was distributed over the entire surface of the hepatoma cells, and (iii) cytochemical staining of the cell surface ATPase activity in the rat hepatoma cells was abolished by 10 mM pCMB whereas ATPase staining in normal rat hepatocyte cells was not affected by pCMB (*33, 34*). The

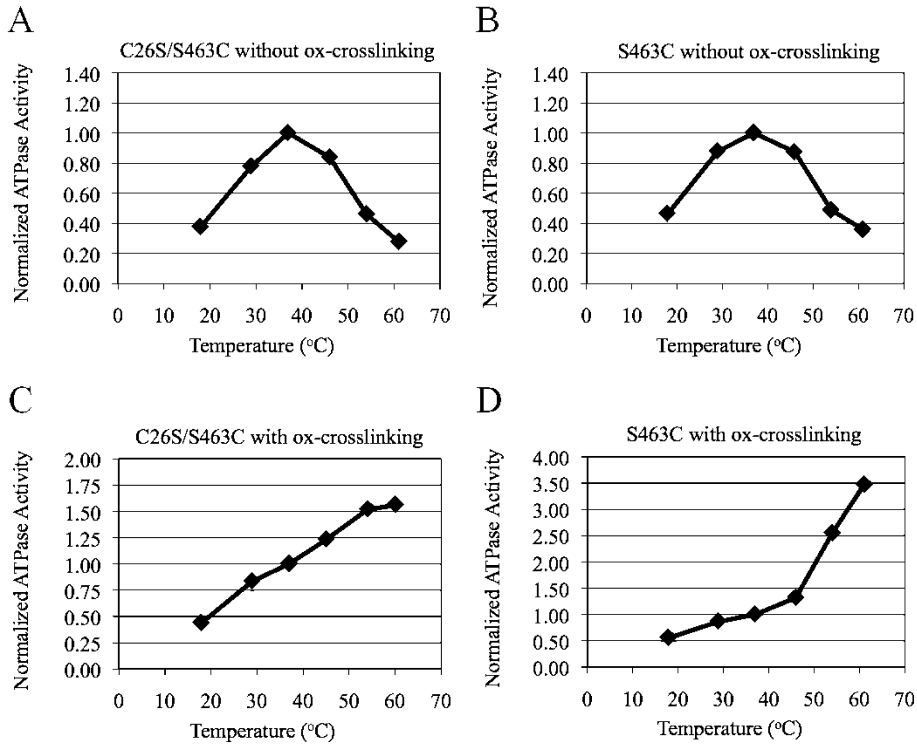

FIGURE 9: Effect of inter- or intramolecular cross-linking on the effect of temperature on the ATPase activity of the C26S/S463C and S463C mutants. Membranes (10 μg) containing S463C or C26S/S463C mutant NTPDase 2 were incubated for 20 min without or with 0.5 mM CuP in a 20 μL reaction mixture at 37 °C for 20 min. Aliquots of the membranes were assayed for ATPase activity in a 1 min reaction at the indicated temperatures. Data were normalized using the ATPase activity obtained at 37 °C as 1.

differential effects of pCMB suggested that the ATPase in the normal rat liver differs from that of the rat hepatoma. Previous studies of ecto-ATPases of a human hepatoma cell line and three small cell lung carcinoma cell lines showed that the major ecto-ATPase in these tumor cells was also inhibited by the mercurial pCMPS (*28, 35, 36*). This ecto-ATPase, an NTPDase 2, has been cloned from the small cell lung carcinoma and was inhibited by pCMPS when expressed in HeLa and HEK293 cells (*4*). Although mercurials, such as pCMPS and pCMB, are not specific ATPase inhibitors, they are the only reagents known to discriminate between the mercurial-sensitive NTPDase 2 and the other cell surface NTPDases that are not inhibited or are inhibited by only high concentrations of pCMPS (*15, 33, 35, 37, 38*).

In this report, we showed that the target of pCMPS modification is C26 in TMD1 of human NTPDase 2. A mutant NTPDase 2, in which C26 was replaced with serine, was no longer inhibited by pCMPS. In addition, the wild-type enzyme, which contains C26, would not form dimers upon oxidative cross-linking if it was first treated with pCMPS. We hypothesize that, upon reacting with C26, the bulky hydrophilic side group of pCMPS causes disruption of the TMD interaction and results in inhibition of catalysis at the active site.

If this hypothesis were correct, human NTPDase 2 should also be inhibited by pCMPS if the free cysteine is situated in TMD2. To investigate this possibility, we generated five mutants, C26S/S462C, C26S/S463C, C26S/W464C, C26S/V465C, and C26S/V466C, which contain only a single free cysteine residue in positions 462−466 located in TMD2 and near the exterior of the bilayer. Of the five mutants, C26S/W464C displayed only 5% of the wild-type human NTPDase 2 activity; however, its activity was partially rescued after cross-linking by ConA (data not shown), indicating that W464 is critical for protein stability and ATPase activity. The other four mutants exhibited 75−100% of the wild-type human NTPDase 2 activity.

Interestingly, the C26S/S462C, C26S/S463C, C26S/V465C, and C26S/V466C mutants exhibited reduced levels of pCMPS inhibition compared to wild-type human NTPDase 2. This was not due to a failure of reaction of the mercurial with the introduced cysteine residues, since CuP-induced dimer formation was also abolished or reduced if these mutants were pretreated with pCMPS (Figure 6). We conclude that while the reactivity of the cysteine residue at these positions with pCMPS is the same as that of C26, the reduced inhibitory effect of pCMPS of these mutants may be ascribed to the greater distance of C462−C466 from ACR1, proposed to contain a phosphate 1 motif of the human NTPDase 2 active site (*30*). The shorter distance between the bulky side group of pCMPS and C26 of ACR1 may also alter the conformation of the active site in addition to disrupting the interaction of the TMDs.

While using oxidative cross-linking with CuP to ascertain the reaction of the cysteine residues with pCMPS, we noticed that oxidative cross-linking per se caused reduction of the ATPase activity of human NTPDase 2 and its mutants. The level of reduction of ATPase activity correlated with the amount of dimer formed. The greatest reduction in ATPase activity was observed in CuP-treated wild-type human NTPDase 2 and the C26S/S463C mutant, which formed dimers most readily. The reduction of ATPase activity after CuP treatment was smaller in magnitude for the C26S/S462C, C26S/V465C, and C26S/V466C mutants. These results indicate that the cysteine residues at positions

26 and 463 are more susceptible to oxidative cross-linking than the cysteine residues at the other positions.

The effect of oxidative cross-linking of cysteine residues in the TMD on ATPase activity was strikingly different from the effect of cross-linking by DSS, glutaraldehyde, or ConA. While these reagents all promote dimer or oligomer formation, the major sites of cross-linking by DSS, glutaraldehyde, and ConA are in the extracellular domain of human NTPDase 2 because of their reactivity with lysine residues (DSS and glutaraldehyde) and binding to glycans (ConA). The resultant dimers and oligomers displayed an apparent increase in ATPase activity, because they were no longer susceptible to inactivation by substrate; i.e., they did not exhibit a decline of activity with reaction time (*4*). Cross-linking of human NTPDase 2 by glutaraldehyde and ConA also attenuated the decrease in ATPase activity in the presence of NP-40 and high temperatures (*4*), suggesting that cross-linking of the extracellular domain of NTPDase 2 by these reagents also promotes TMD interaction. On the other hand, CuP cross-linking of wild-type human NTPDase 2 and the mutants containing only one free cysteine residue in TMD2 is restricted to the TMD and gave rise to mostly dimers. The loss of ATPase activity of such dimers can only be attributed to the negative effect of reduced TMD mobility on catalysis.

Despite the loss of ATPase activity due to oxidative cross-linking, inhibition of the residual activity by NP-40 and high temperatures was attenuated in these dimers. This was seen most clearly with wild-type human NTPDase 2 and the C26S/S463C mutant, which formed most dimers. On the other hand, the activity of the C26S/S462C, C26S/V465C, and C26S/V466C mutants, which remained mostly as monomers after CuP treatment, was still decreased by NP-40 and high temperatures. These results indicate that while intermolecular TMD cross-linking impairs enzyme activity, such dimers become insensitive to membrane perturbation by detergents and high temperatures.

Since our recent studies indicated that the strength of the intramolecular interaction of the TMDs of the NTPDases is important in the regulation of their responses to membrane perturbation (*12, 13*), we further investigated the effect of formation of an intramolecular covalent disulfide bond between TMD1 and TMD2. Mutants S462C, S463C, W464C, V465C, and V466C, which contain C26 in TMD1 and a cysteine residue in TMD2, were utilized. Oxidative cross-linking of these mutants by CuP resulted in some oligomer formation; however, there was a general reduction in the level of dimer formation, except for S462C, indicating that intramolecular cross-linking is favored. Compared to the activities of the untreated membranes, the activity of the CuP-treated mutants was reduced to 20−40% (Table 3), further supporting the conclusion that reduced TMD mobility limits the dynamics of the active site in achieving maximal activity. However, the extent of activity inhibition of the different mutants after oxidative cross-linking varied, indicating that intramolecular cross-linking of C26 and cysteine residues at different positions in TMD2 may change the conformation of the active site differently.

Similar to the results obtained with cross-linked dimers, the residual activity of the intramolecularly cross-linked monomers was less sensitive to inhibition by NP-40. The inhibitory effect of NP-40 on the activity of the oxidatively cross-linked S462C, V465C, and V466C mutants was reduced to 30−60%, while that of the oxidatively cross-linked S463C mutant was completely abolished (Figure 8). Furthermore, unlike wild-type human NTPDase 2 or mutants without CuP treatment, which had decreased ATPase activity at temperatures higher than 37 °C, the activity of the intramolecularly cross-linked mutants at 61 °C was ~3.5-fold greater than that at 37 °C (Figure 9D). These results suggest that locking the TMDs causes the mutant human NTPDase 2 to be less sensitive to the inhibitory effect of membrane perturbation by NP-40 and high temperatures.

Since our previous studies showed that a weaker interaction of the TMDs of the human NTPDase 2 contributed to its susceptibility to membrane perturbation, the observation that ATPase activity of human NTPDase 2 was reduced upon strengthening TMD interaction by oxidative cross-linking was unexpected. However, our finding is similar to that obtained with rat NTPDase 1/CD39 (*39*). Using various engineered single-cysteine and double-cysteine substituted mutant rat NTPDase 1 enzymes, Grinthal and Guidotti showed that inter- and intra-TMD disulfide bond formation occurred most readily when the cysteine residues introduced in the two TMDs were in the region near the cell surface and were associated with reduction of ATPase activity. Furthermore, the TMDs of rat NTPDase 1 exhibited a high degree of rotational mobility. While primary interfaces in TMD1 and TMD2 could be demonstrated when CuP treatment was conducted at 4 °C, cross-linking was complete at 37 °C for all the rat NTPDase 1 mutants tested in which the cysteine residue occupied positions as many as six amino acid residues away from the cell surface. In spite of the similar effect of TMD cross-linking on ATPase activity, our results show that the rotational mobility of the TMDs of human NTPDase 2 is more limited than that of rat NTPDase 1, since dimer formation was most easily demonstrated with the enzyme containing a single cysteine residue at either position 26 (the wild-type enzyme) or position 463 (the C26S/S463C mutant). Additionally, intramolecular cross-linking of the double cysteine mutants of rat NTPDase 1 resulted exclusively in monomers, which was only possible if disulfide bond formation occurred regardless of the helix faces of the cysteine residues, further supporting the conclusion that TMDs of rat NTPDase 1 are highly mobile. On the other hand, intramolecular cross-linking in human NTPDase 2 with double cysteine residues was most clearly seen with the S463C mutant, whereas the level of dimer formation actually increased in the S462C mutant probably due to the presence of C26. Thus, C26 in TMD1 and S462 in TMD2 probably define an interface between the monomers. In spite of the different TMD mobilities in the two NTPDases, human NTPDase 2 is similar to rat NTPDase 1 in that (i) its monomer is in rapid equilibrium with dimers and oligomers and a defined quaternary structure probably does not exist for the enzyme and (ii) the mobility of the TMD is necessary for maximal catalysis.

In summary, three major findings emerged from this study. First, the cysteine residue at position 26 was the target of pCMPS modification, which resulted in the loss of ATPase activity of human NTPDase 2 by disturbing the TMD interaction of human NTPDase 2 as well as affecting the conformation of the active site. This result differs from that obtained with human NTPDase 3, in which pCMPS modi-

fication of C501 located in TMD2 near the cytoplasmic side caused inhibition by interfering with monomer−monomer interactions in the native tetrameric quaternary structure of human NTPDase 3 (*38*). Second, intra- and intermolecular oxidative cross-linking was accompanied by reduction of ATPase activity, suggesting that the mobility of the TMDs is essential for enzyme function. Third, when the TMDs cross-linked, human NTPDase 2 became resistant to inactivation caused by membrane perturbation. Future investigations addressing communication between TMDs and the active site when the TMD is modified by pCMPS or when the TMD interaction is disturbed by membrane perturbation should yield greater insight into the mechanism by which TMD interaction regulates the catalytic activity of NTPDases.

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SUPPORTING INFORMATION AVAILABLE

Demonstration of intramolecular cross-linking of the S462C, S463C, V465C, and V466C mutants using mPEG-maleimide treatment. This material is available free of charge via the Internet at http://pubs.acs.org.

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