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ORIGINAL ARTICLE

Proline residues link the active site to transmembrane domain movements in human nucleoside triphosphate diphosphohydrolase 3 (NTPDase3)

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Abstract The active sites of the membrane-bound nucleoside triphosphate diphosphohydrolases (NTPDases) regulate and are regulated by coordinated and spatially distant movements of their transmembrane helices, modulating enzyme activity, and substrate specificity. Using site-directed mutagenesis, the roles of the conserved proline residues (N-terminal: P52 and P53; C-terminal: P472, P476, P481, P484, and P485) of human NTPDase3, located in the "linker regions" that connect the N- and C-terminal transmembrane helices with the extracellular active site, were examined. Single cysteine substitutions were strategically placed in the transmembrane domain (N-terminal helix: V42C; C-terminal helix: G489C) to serve as cross-linking "sensors" of helical interactions. These "sensor" background mutant proteins (V42C and G489C NTPDase3) are enzymatically active and are cross-linked by copper phenanthroline less efficiently in the presence of adenosine triphosphate (ATP). Proline to alanine substitutions at P53, P481, P484, and P485 in the V42C background, as well as P53, P481, and P484 in the G489C background, exhibited decreased nucleotidase activities. More importantly, alanine substitutions at P53 and P481 in the V42C background and P481 in the G489C background no longer exhibited the ATP-induced decrease in transmembrane cross-linking efficiency. Interestingly, the P485A mutation abolished oxidative cross-linking at G489C both in the presence and absence of ATP. Taken together, these results suggest a role for proline residues 53 and 481 in the linker regions of human NTPDase3 for coupling nucleotide binding at the enzyme active site to movements and/or rearrangements of the transmembrane helices necessary for optimal nucleotide hydrolysis.

Keywords Ecto-nucleotidase · NTPDase3 · Linker region · Conserved proline residues · Site-directed mutagenesis · Transmembrane cross-linking

Abbreviations

NTPDase3	Nucleoside triphosphate diphosphohydrolase 3
TM	Transmembrane
TMD	Transmembrane domain
CuPhen	Copper phenanthroline
NEM	*N*-ethylmaleimide
MOPS	3-(*N*-Morpholino)-propanesulfonic acid

Introduction

The nucleoside triphosphate diphosphohydrolases (NTPDases) are a family of nucleotidases, some of which regulate purinergic signaling by divalent cation-dependent hydrolysis of nucleotides acting as agonists at purinergic receptors. In humans, there are six membrane-bound enzymes (NTPDases 1–4, 7, and 8), whose carboxy- and amino-terminal ends are each anchored to the membrane [1, 2], and two enzymes that lack a carboxy-terminal transmembrane (TM) helix (NTPDase5 and NTPDase6), which can be secreted as soluble enzymes after cleavage of their respective N-terminal signal sequences [3, 4]. Due to the scarcity of specific inhibitors and genetically modified animals, the functions of most individual NTPDases are poorly understood and still under investigation. However,

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the NTPDases are implicated to play a role in many biological and physiological processes, including secretion [5], cell adhesion [6], cancer and malignant transformation [7, 8], adenosine recycling, and platelet aggregation [9].

Membrane-bound NTPDase3 is expressed on the cell surface and has a large extracellular domain, N- and C-terminal "linker regions," and a transmembrane domain (TMD) consisting of an N- and a C-terminal TM helix [10]. The extracellular domain contains five disulfide bonds that are conserved among all cell surface NTPDases, which when disrupted in NTPDase3 yield five distinct biochemical phenotypes [2]. There are also five extracellular apyrase conserved regions (ACRs) shared among all members of the NTPDase family [11]. Based on sequence homology of the NTPDases with the actin/heat shock protein/sugar kinase superfamily of proteins [12], ACR1 and ACR4 are recognized as phosphate-binding domains of the extracellular active site. The roles and importance of the ACRs and specific amino acids contained in those regions were confirmed and refined by the recent publication of crystal structures of the extracellular portion of rat NTPDase2, both with and without a co-crystallized non-hydrolyzable nucleotide analogue [13].

The NTPDase3 "linker regions" (N-terminal: ^{46}HKQEVLPPGLK^{56}; C-terminal: ^{472}PAESPLIRLPIEPP^{485}) are stretches of residues in the amino acid sequence between the extracellular ends of the N- and C-terminal TM helices and ACR 1 and ACR5 in the extracellular lobes (location of the enzyme active site). These linker regions contain several conserved proline residues (N-terminal: P52 and P53; C-terminal: P472, P476, P481, P484, and P485—see Fig. 1a). Proline is structurally unique among the 20 protein-forming amino acids since its side-chain binds to the backbone amide position resulting in a distinctive cyclic structure. This

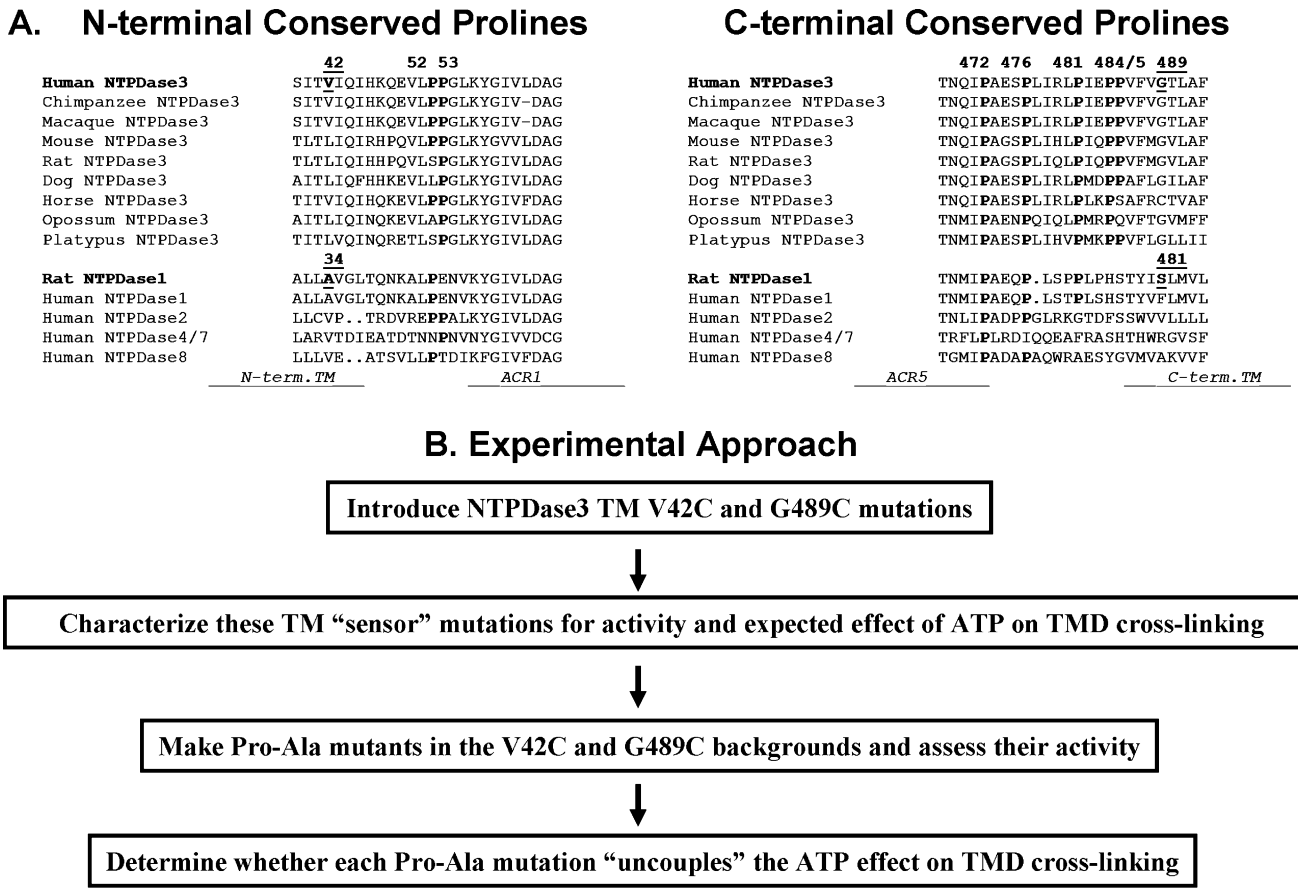

Fig. 1 Rationale and experimental approach. **a** Multiple sequence alignments for portions of NTPDase sequences between the N- and C-terminal transmembrane (TM) helices and the extracellular domain containing the active site. Conserved proline residues found in human nucleoside triphosphate diphosphohydrolase 3 (NTPDase3) are *bolded and numbered*, and the corresponding prolines in other NTPDases are *bolded*. The cysteine substitutions of rat NTPDase1/CD39 made in a previous study (A34C and S481C, [23]) are *bolded and underlined*, and guided the choice of the cysteine substitutions used in this work (V42C and G489C), which are *bolded and underlined* in the human NTPase3 sequence (*top line of each alignment*). To give a perspective as to the location of these conserved proline residues in the overall structure of NTPDase3, the locations of the end of the N-terminal TM helix, the beginning of the ACR1 region (N-terminal), the end of ACR5 region (C-terminal), and the beginning of the C-terminal TM helix are indicated by *lines and italic labeling under the alignments*. **b** A flow chart of the experimental approach utilized

replaces the amide proton with a $–CH_2$ group, limiting proline to act only as a hydrogen bond acceptor. The bulkiness of the $N–CH_2$ group places restrictions on the conformation of the residue preceding proline [14], thereby destabilizing α-helix secondary structures [15, 16]. The elimination of the hydrogen bond donor, together with the bulkiness of the side-chain, produces the well-known "helix-breaker" (and β-sheet breaker) secondary structure effect of proline residues. In addition, the ring structure of proline also locks the available backbone ø dihedral angle at approximately 65°, resulting in a conformational rigidity unique to proline [17, 18]. Thus, proline residues confer unique structural features and rigidity needed for some conformational changes in proteins.

Previous studies have demonstrated that interactions between the extracellular and TM domains exist in NTPDases. One of the earliest hints of this phenomenon was the loss of activity caused by detergents during enzyme purification. Almost every detergent traditionally used to solubilize membrane-bound enzymes inhibits the enzymatic activity of many NTPDases [9]. Later, it was shown that both TM helices are required for full enzymatic activity, and the nucleotide specificity of the NTPDases is altered by the lack of, or manipulation of, either TM helix, further suggesting interplay between the active site and the TMD [19–22]. Grinthal and Guidotti expanded this work by demonstrating that the TM helices of rat CD39/NTPDase1 undergo dynamic motions that reflect their functional relationship with the enzyme active site upon substrate binding [23]. Specifically, in the absence of substrate (adenosine triphosphate (ATP) or adenosine diphosphate (ADP)), oxidative cross-linking of single cysteine residues substituted into the TM helices of rat CD39/NTPDase1 was found to be efficient, if the residues were located near the extracellular surface of either the N- or C-terminal TM helices. In addition, Grinthal and Guidotti [23] observed that ATP greatly attenuated dimer formation via oxidation of cysteine mutations of alanine 34 (corresponding to valine 42 in human NTPDase3) on the N-terminal TM helix and serine 481 (corresponding to glycine 489 in human NTPDase3) on the C-terminal TM helix (see Fig. 1a). Therefore, V42 and G489 in human NTPDase3 were chosen for mutation to cysteine to serve as TMD "sensor" residues to monitor the effect of ATP binding in the extracellular domain on changes in the cross-linking efficiency of the TM helices.

In the present study, we investigated the hypothesis that the conserved, linker region proline residues provide the rigidity necessary to facilitate the structural linkage and/or "coupling" of the presumed movements of the active site lobes (active site lobe movements presumed by analogy to other members of the actin superfamily of nucleotide-handling enzymes [12]) to movements of the TM helices. Site-directed mutagenesis was utilized to introduce cysteine residues at strategic locations (V42C and G498C mutations) near the extracellular ends of the N- and C-terminal TM helices that were used as "sensors" to determine differences in TM helix interactions assessed by changes in oxidative cross-linking efficiency in the presence and absence of substrate. Then, the conserved proline residues in the linker regions were singly mutated to alanine, and the resultant mutants were assessed for enzymatic activity and TM cross-linking efficiency in the presence and absence of substrate. Our data demonstrates that alanine substitution of proline 53 and 481 results in a decrease in enzymatic activity and abolishment of the ATP-induced decrease in TM cross-linking efficiency. This result suggests that P53 and P481 are involved in the structural framework used to link events and movements that occur in the enzyme active site with movements of the TM helices.

Materials and methods

Site-directed mutagenesis of NTPDase3

Mutagenesis of human NTPDase3 in the pcDNA3 vector was performed using a QuikChange site-directed mutagenesis kit (Stratagene) as previously described [24]. The University of Cincinnati DNA Core Facility produced the synthetic oligonucleotide primers needed to engineer each mutant. The sense primers used for mutagenesis are as follows:

V42C, 5′-GGTACTTGTGAGTATCACT**TGC**ATC CAGATCCACAAGCAAG-3′;
G489C, 5′-CCACCTGTCTTTGTG**TGC**ACC CTCGCTTTC-3′;
P52A, 5′-CACAAGCAAGAGGTCCTC**GCT**CCAG GACTGAAGTATG-3′;
P53A, 5′-CAAGAGGTCCTCCCT**GCA**GGACT GAAGTATGC-3′;
P472A, 5′-CTGACCAACCAGATC**GCA**GCT GAAAGCCCTC-3′;
P476A, 5′-GATCCCAGCTGAAAGC**GCT**CT GATCCGTCTGC-3′;
P481A, 5′-CCTCTGATCCGTCTG**GCC**ATAGAAC CACCTG-3′;
P484A, 5′-CGTCTGCCCATAGAA**GCA**CC TGTCTTTGTGGG-3′;
P485A, 5′-CTGCCCATAGAACCA**GCT**GT CTTTGTGGGCAC-3′.

The altered codons are bolded and underlined (the complimentary anti-sense oligonucleotides, also needed for mutagenesis, are not shown). The mutants were made in the wild-type-like "free sulfhydryl-less" C10S/C501S/

C509S NTPDase3 background to eliminate all other free sulfhydryls [25]. The resulting cDNA constructs were sequenced by the University of Cincinnati DNA Core Facility to verify the presence of the desired mutation and the absence of any unwanted changes.

Transient transfection and preparation of COS-1 cell membranes

COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine serum and a 2% mixture of antibiotics and antimycotics (Invitrogen). The cells were transfected with 4 μg plasmid DNA per 100 mm cell culture plate using Lipofectamine and PLUS reagents (Invitrogen) as previously described [26]. Cells were transfected with 4 μg empty pcDNA3 vector as a control. The COS-1 cells were harvested ~48 h post-transfection. The crude cell membrane preparations were obtained as previously described [26].

Protein assay

Protein concentrations were determined using the Bio-Rad protein assay reagent with the modifications of Stoscheck [27]. Bovine serum albumin was used as a standard.

Nucleotidase assay

Nucleotidase activities were determined by measuring the amount of inorganic phosphate (P_i) released from nucleotide substrates (Sigma) at 37°C using modifications of the technique Fiske and Subbarow [28] as previously described [24]. Either 5 mM $CaCl_2$ or 5 mM $MgCl_2$ buffer (both in 20 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), pH 7.1 buffer) was used for cation-dependent nucleotidase activity. The reactions were initiated by the addition of nucleotide to a final concentration of 2.5 mM in the 0.3-ml assay solutions. Hydrolysis was allowed to proceed for 30 min or 1 h, depending on the substrate used. The activities were corrected for pcDNA3 vector/COS-1 cell background (membranes from COS-1 cells transfected with an empty vector), as well as differences in expression levels as determined by quantitative Western blotting of each sample (i.e., the activities determined in micromoles per milligram COS protein/hour were divided by the relative NTPDase3 expression level of each mutant (see Table 1), and this corrected activity is used for comparison throughout this study).

SDS-PAGE and Western blotting

Pre-cast 10-well or 15-well 4–15% gradient mini-gels (Bio-Rad) were used to resolve aliquots of crude membrane proteins (0.5–2 μg, depending on the sample and the experimental purpose), usually after incubating for 10 min at 60°C in non-reducing sodium dodecyl sulfate (SDS) loading buffer. Following SDS-polyacrylamide gel electrophoresis (PAGE), the proteins were electrotransferred to Immun-Blot polyvinylidene fluoride (PVDF) membrane (Bio-Rad) for 3 h at 33 V in cold 10 mM CAPS/NaOH, pH 11. After transfer, the PVDF membrane was incubated for 1 h in blocking solution (5% nonfat dry milk in Tris-buffered saline (TBS)) at room temperature (22°C). Next, the PVDF membrane was incubated overnight at room temperature in blocking solution containing 0.02% sodium azide and a 1:5,000 dilution of rabbit polyclonal primary antisera of KLH1, generated against the cytoplasmic C-terminal peptide (amino acid residues 515–529) of human NTPDase3 [29]. After washing the blot in a TBS 0.05% Tween 20 solution, a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce) was applied at a 1:4,000 dilution for 1 h at room temperature followed by washing and application of Pierce SuperSignal West Dura Extended Duration Substrate to the PVDF membrane for 5 min to detect immunoreactivity by chemiluminescence. Chemiluminescence was recorded and quantified using a FluorChem IS-8800 system (Alpha Innotech). Quantification of cross-linking efficiency was calculated by dividing the NTPDase3 monomer band densities in copper phenanthroline (CuPhen)-treated samples by the monomer band densities in non-cross-linked control samples from the same mutant processed in parallel.

Oxidative cross-linking in the presence or absence of substrate

CuPhen was the oxidative cross-linker used and was prepared fresh each day, just prior to use, by combining cupric sulfate and 1,10-phenanthroline (Sigma) at a 1:3 ratio in 20% ethanol [30]. Cysteine-substituted protein from COS-1 cell crude membranes was diluted in 50 mM Tris–HCl, pH 7.5 to a final concentration of 0.1 mg/ml and oxidized with a final concentration of 5 mM CuPhen in the absence or presence of a final concentration of 5 mM ATP (Sigma), added just before the CuPhen, at 37°C for 5 min in a total volume of 25 μl. The reaction was stopped by adding an equal volume of non-reducing SDS loading buffer containing 20 mM *N*-ethylmaleimide (NEM) and 20 mM EDTA. The samples were then loaded on a gel for SDS-PAGE and Western blotting as described above.

Measurement of enzyme activity after cross-linking

CuPhen reactions were stopped by adding an equal volume of 50 mM Tris–HCl, pH 7.5 containing 20 mM NEM and 20 mM EDTA. The samples were incubated at room

Table 1 Expression levels and nucleotidase activities of human nucleoside triphosphate diphosphohydrolase 3 (NTPDase3) mutants used in this study

Mutant	Relative expression level	Normalized Ca^{+2}-ATPase	Normalized Ca^{+2}-ADPase	Normalized Mg^{+2}-ATPase	Normalized Mg^{+2}-ADPase
NTPDase3 cysteine sensor V42 and G489 mutants in C10S/C501S/C509S background					
C10S/C501S/C509S (background)	1	178±2 (*100%*)	89±2	96±3	49±2
V42C	0.90±0.07	188±4[a] (*106%*[a])	89±1	92±3	45±2
G489C	1.10±0.11	150±4[b] (*84%*[b])	75±4[b]	84±3[b]	43±1[b]
NTPDase3 proline residue to alanine mutants in V42C background					
V42C (background)	1	362±5 (*100%*)	177±7	229±11	115±4
P52A	0.80±0.17	362±17 (*100%*)	174±7	220±4	110±6
P53A	0.65±0.12[b]	283±29[b] (*78%*[b])	136±11[b]	196±6[b]	98±7[b]
P472A	0.56±0.05[b]	415±23 (*115%*)	213±12	270±21	130±10
P476A	0.58±0.13[b]	336±26 (*93%*)	168±10	224±21	113±2
P481A	0.65±0.02[b]	212±11[b] (*59%*[b])	106±6[b]	149±8[b]	75±7[b]
P484A	0.51±0.02[b]	311±5[b] (*86%*[b])	156±4[b]	127±6[b]	64±3[b]
P485A	0.63±0.13[b]	298±12[b] (*82%*[b])	149±4[b]	228±19	115±3
NTPDase3 proline residue to alanine mutants in G489C background					
G489C (background)	1	244±4 (*100%*)	126±6	144±6	72±2
P52A	0.88±0.07	251±4 (*103%*)	114±4	157±4	79±2
P53A	0.71±0.14[b]	166±7[b] (*68%*[b])	80±2[b]	75±3[b]	38±3[b]
P472A	1.05±0.18	337±24[a] (*138%*[a])	169±9[a]	192±7[a]	96±4[a]
P476A	1.02±0.08	257±7 (*105%*)	125±3	159±4	75±6
P481A	0.51±0.06[b]	125±7[b] (*51%*[b])	63±2[b]	9±1[b]	4±1[b]
P484A	1.05±0.08	204±7[b] (*84%*[b])	105±3[b]	112±2[b]	58±4[b]
P485A	1.13±0.15	258±3[a] (*106%*[a])	131±4	161±8	85±6[a]

Activities are expressed in units of micromoles per milligram per hour after normalization for relative NTPDase3 protein expression levels by dividing the activities in micromoles per milligram COS protein/hour by the relative expression level for each mutant. The numbers in italics and parentheses are the percentage of the mean Ca-ATPase activities compared to their respective NTPDase3 background sensor mutants. Note that different sets of experiments (NTPDase3 cysteine sensor V42 and G489 mutants in C10S/C501S/C509S background, NTPDase3 proline residue to alanine mutants in V42C background, and NTPDase3 proline residue to alanine mutants in G489C background) were transfected at different times. The transfection efficiencies between sets of experiments are variable due to many factors such as slight differences in COS cell confluency at the time of transfection, leading to variations in the absolute nucleotidase specific activities seen in the three background mutants

[a] Statistically increased compared to the corresponding background NTPDase3

[b] Statistically decreased compared to the corresponding background NTPDase3

temperature for 30 min and then centrifuged for 1 min in an Eppendorf tabletop centrifuge at 13,500*g*. The supernatant was removed, and the pellet was re-suspended in 100 μl of tissue homogenization buffer (250 mM sucrose, 2 mM EDTA, 30 mM MOPS-NaOH, pH 7.4). The entire 100-μl sample was used for the Ca-ATPase assay as described above.

Results

Rationale and experimental approach

Multiple sequence alignments of the N- and C-terminal "linker regions" of several known NTPDase3 sequences with other membrane-bound NTPDases (rat NTPDase1 and human NTPDase 1, 2, 4/7, and 8) revealed complete conservation of P472, a high degree of conservation of P53, P476, and P481 (completely conserved in the NTPDase3 enzymes) and a lesser degree of conservation of P52, P484, and P485 (Fig. 1a).

Cysteine residues (V42C and G489C) were introduced in the "free sulfhydryl-less" C10S/C501S/C509S NTPDase3 background (C10S/C501S/C509S, which has very similar enzymatic properties to WT NTPDase3 [25]) for subsequent site-specific, oxidative, intermolecular cross-linking of the TM helices of human NTPDase3. These amino acids correspond to those of rat CD39/NTPDase1, which were shown to be excellent "sensors" of ATP effects on TM helix cross-linking [23]. Thus, V42 (corresponding to A34 in rat NTPDase1) and G489 (corresponding to S481 in rat NTPDase1) in human NTPDase3 were singly mutated to cysteine (see Fig. 1a). These mutants were used as

background/control enzymes and TMD-specific cross-linking efficiency "sensors" for subsequent proline to alanine mutations used to test our hypothesis (see Fig. 1b).

Characterization of the TM "sensor" mutations (V42C and G489C NTPDase3)

The V42C and G489C mutants were constructed in the C10S/C501S/C509S NTPDase3 background to ensure the introduced cysteine is the only free sulfhydryl available for specific cross-linking at either the N- or C-terminal TM helix. The relative expression level and nucleotidase activities of the V42C and G489C mutants were similar to the C10S/C501S/C509S NTPDase3 control, with only slight but statistically significant decreases in nucleotidase activities for G489C (Table 1, NTPDase3 cysteine sensor V42 and G489 mutants in C10S/C501S/C509S background). CuPhen cross-linking of the TM helices via V42C or G489C caused a significant decrease in Ca-ATPase enzymatic activity (Fig. 2). However, the decrease in Ca-ATPase activity was attenuated in the presence of 5 mM ATP (Fig. 2). The cross-linking efficiency of V42C and G489C in the presence and absence of various nucleotides (ATP, ADP, adenosine monophosphate (AMP), and adenosine 5'-(β,γ-imido)triphosphate (AMP-PNP) all at 5 mM) and 5 mM inorganic phosphate (Pi) was then assessed, to determine the nucleotide specificity of the modulation of CuPhen TM cross-linking at these introduced cysteine "sensors," as well as the utility of these background sensor mutations for the subsequent proline residue mutations. In the presence of 5 mM CuPhen, both V42C (Fig. 3a) and G489C (Fig. 3b) mutants are efficiently

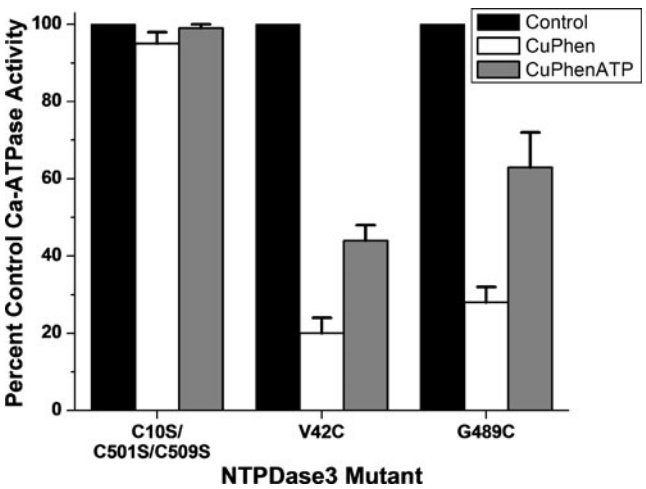

Fig. 2 Adenosine triphosphate (ATP) attenuates the copper phenanthroline (CuPhen)-induced decrease in nucleoside triphosphate diphosphohydrolase 3 (NTPDase3) Ca-ATPase activity. Nucleotidase activities were assayed as described in "Materials and methods." Values represent the mean percent activity relative to the control samples ± standard deviation from three separate experiments

cross-linked, forming dimers (it should be noted that the antibody reacts much stronger with dimeric than monomeric NTPDase3, and thus only monomer bands were used in the calculation of the cross-linking efficiency [31]). When 5 mM ATP was present with CuPhen, the cross-linking efficiency of both V42C and G489C mutants decreased (the percent monomer remaining increased), mimicking the results obtained using cysteine substitutions at the same positions in rat CD39/NTPDase1 [23]. Also consistent with those previous NTPDase1 results, the cross-linking efficiency of V42C and G489C was attenuated by the addition of 5 mM ADP and AMP-PNP (Fig. 3a, b), but not by 5 mM AMP or P_i. Lastly, in the presence of Mg^{+2} or Ca^{+2}, the decrease in CuPhen cross-linking efficiency caused by 5 mM ATP is attenuated in a divalent cation concentration-dependent manner for human NTPDase3 (data not shown), similar to what was observed earlier for NTPDase1 [23].

This data validates the use of V42C and G489C NTPDase3 mutants as "sensors" for active site-dependent changes of the TM helices in NTPDase3 and allows testing of the hypothesis that the linkage between the active site and the TM helices is dependent on the presence of one or more of the conserved proline residues located in the "linker regions" of NTPDase3, denoted in Fig. 1a.

Characterization of proline to alanine mutants in the V42C or G489C NTPDase3 background

To determine if the proline residues play a role in "coupling" ATP binding at the enzyme active site to movements of the TM helices, site-directed mutagenesis was used to singly substitute the conserved proline residues in the "linker regions" to alanine in both the V42C and G489C NTPDase3 backgrounds. In the V42C background, proline to alanine substitution at all the positions except 52 resulted in a significant decrease in relative expression level as compared to the V42C background control (Table 1, NTPDase3 proline residue to alanine mutants in V42C background). Proline to alanine substitutions at positions 53 and 481 resulted in substantial decreases in Ca-ATPase activity as compared to the V42C background control (Table 1, NTPDase3 proline residue to alanine mutants in V42C background). In addition, proline to alanine substitutions at positions 484 and 485 resulted in smaller but significant decreases in Ca-ATPase activity as compared to the V42C background control. In the G489C background, proline to alanine substitution at positions 53 and 481 resulted in significant decreases in relative expression levels as compared to the G489C background control (Table 1, NTPDase3 proline residue to alanine mutants in G489C background). Proline to alanine substitutions at positions 53, 481 and, to a lesser extent, 484, resulted in substantial decreases in Ca-ATPase activity, compared to the G489C

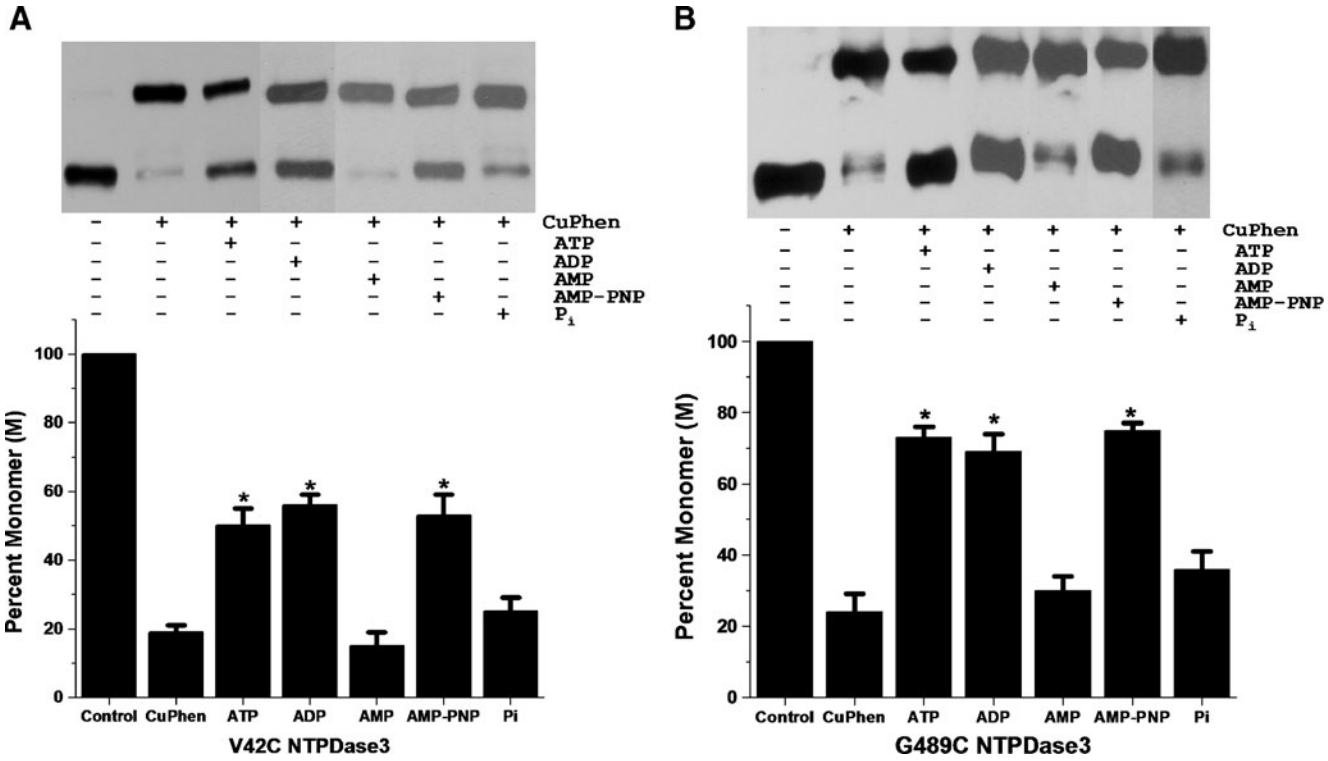

Fig. 3 Adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine 5'-(β,γ-imido)triphosphate (AMP-PNP) binding at the extracellular domain decreases the copper phenanthroline (CuPhen) cross-linking efficiency of V42C and G489C "sensor" background nucleoside triphosphate diphosphohydrolase 3 (NTPDase3) mutants. CuPhen cross-linking was performed, and the amount of monomer remaining as compared to the untreated control was quantified as described in "Materials and methods." **a** V42C NTPDase3 mutant results. **b** G489C NTPDase3 mutant results. Above each mutant percent monomer data bar is a representative Western blot. The *bar graph* on the bottom of each panel is the quantification of three such Western experiments, expressed as the mean percent of untreated monomer remaining ± standard deviation. *Asterisks* represent statistical significance ($p \leq 0.05$) between mutant proteins oxidized with CuPhen in the presence versus the absence of nucleotide or Pi

background control (Table 1, NTPDase3 proline residue to alanine mutants in G489C background). Other nucleotidase activities were also measured for these mutants, and the changes in Ca-ADPase, Mg-ATPase, and Mg-ADPase activities were similar to the results for Ca-ATPase activities (see Table 1).

Each mutant was also oxidized with 5 mM CuPhen in the presence and absence of 5 mM ATP to assess CuPhen cross-linking efficiency (measured by quantifying the monomer band remaining relative to the untreated control). In the V42C background, the ATP-induced decrease in CuPhen cross-linking efficiency was abolished by proline to alanine substitution at positions 53 and 481 (Figs. 4a and 5a). Proline to alanine substitution at position 481 abolished the ATP-induced decrease in CuPhen cross-linking efficiency in the G489C background (Figs. 4b and 5b). Interestingly, alanine substitution at position 485 in the G489C background resulted in an inability of the mutant to be cross-linked at G489C by CuPhen, both in the presence or absence of ATP (Figs. 4b and 5b).

All the results are visually summarized in the cartoon representation of NTPDase3 monomers shown in Fig. 6. Mutations which abolish the ability of substrate (ATP) to diminish the cross-linking efficiency of the introduced TM helix cysteine residues ("uncoupling mutations") are indicated by boxes around the mutations. Mutations exhibiting statistically significant decreases in nucleotidase activities are shown in bold type and include the P53A and P481A mutants in both sensor backgrounds. Taken together, this data suggests that a few specific proline residues in the linker region (i.e., P53 and P481) facilitate the linkage between binding of substrate at the enzyme active site to movements or reorientations of the TM helices that are necessary to achieve full enzymatic activity of NTPDase3.

Discussion

Proteins often undergo domain rearrangements that involve substrate or ligand binding in one area of a protein resulting in the movement of another part(s) of the protein [32]. These dynamic motions of domain rearrangement are necessary for optimal function of these proteins. Specifically, interplay between the extracellular domain and TMD

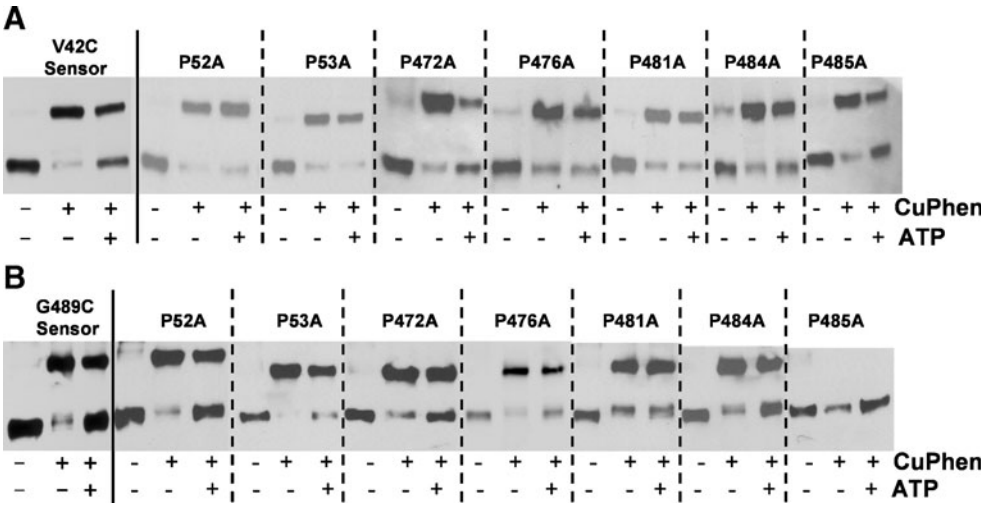

Fig. 4 Copper phenanthroline (CuPhen) cross-linking efficiency of proline to alanine mutants in the presence and absence of 5 mM adenosine triphosphate (ATP). Cross-linking was performed, and ATP was added before 5 mM CuPhen, as described in "Materials and methods." **a** V42C proline to alanine mutants. **b** G489C proline to alanine mutants

of the cell surface NTPDases govern not only enzymatic activity but also substrate specificities [33]. Thus, NTPDase activities and substrate specificities are modulated not only by mutation of amino acids in the active site [10, 34], post-translational modifications such as glycosylation [35, 36] and N-acetylation [37] in the extracellular domain, but also by removal of the TM helices and perturbations of TM helical interactions [19, 38–41] and modification of TMD cysteine by p-chloromercuriphenylsulfonate ([25, 42]).

The hypothesis tested in the present study is that one or more of the conserved proline residues found in the "linker regions" of human NTPDase3 mediate the phenomenon of substrate binding at the active site (located between the two lobes of the extracellular domain) causing movements and/or rearrangement of the TM helices to facilitate optimum nucleotide hydrolysis. When proline to alanine mutations were added to the "sensor" background/control NTPDase3 enzymes (V42C and G489C), alanine substitution at positions P53 and P481 in V42C caused a substantial decrease in activity, while a significant but smaller decrease in activity was noted for P484 and P485 (Table 1, NTPDase3 proline residue to alanine mutants in V42C

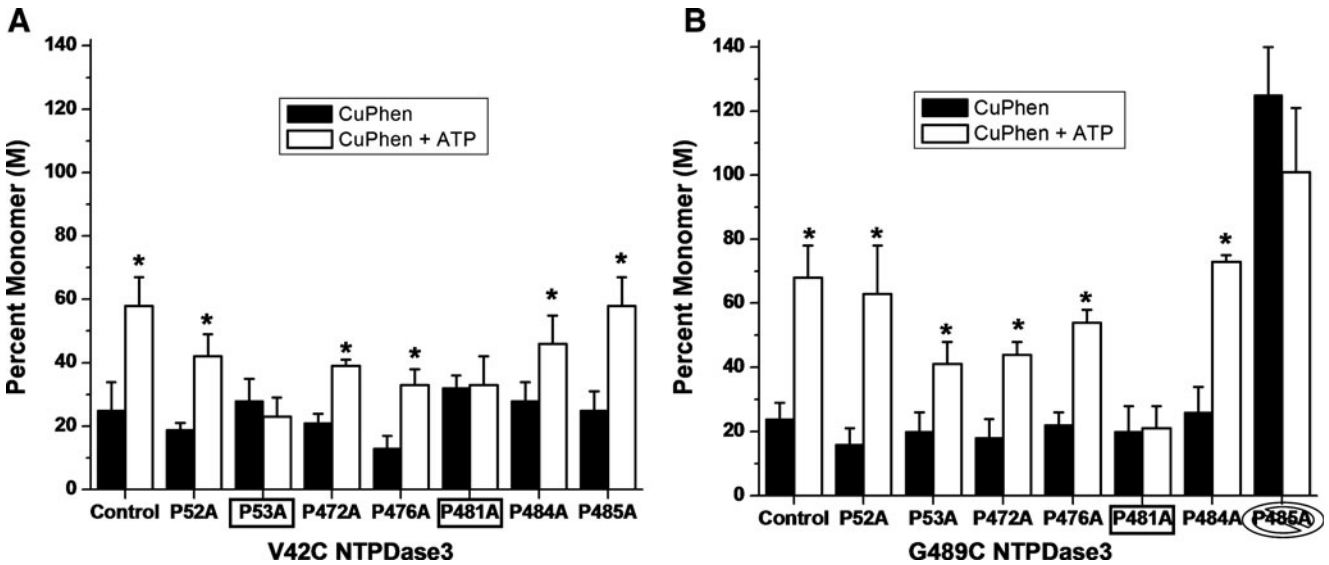

Fig. 5 Specific proline to alanine substitutions abolish the adenosine triphosphate (ATP)-induced decrease in copper phenanthroline (CuPhen) cross-linking efficiency. Cross-linking was performed, and the efficiency of cross-linking was quantified by comparing the amount of monomer remaining to the monomer in the untreated control, as described in "Materials and methods." **a** V42C proline to alanine mutants. **b** G489C proline to alanine mutants. Values represent the mean percent of monomer remaining after CuPhen ± standard deviation from three separate experiments (a single representative Western blot experiment for each mutant is shown in Fig. 4). *Asterisks* represent statistically significant differences between each proline to alanine mutant in the presence versus the absence of ATP ($p \leq 0.05$). The *boxes* around the mutations used to label the *x*-axis denote mutants that abolish the ATP-induced decrease in CuPhen cross-linking. The P485A mutation in the G489C background (**b**) abolishes transmembrane CuPhen cross-linking both in the absence and presence of ATP and is annotated by a *strikethrough ellipse*

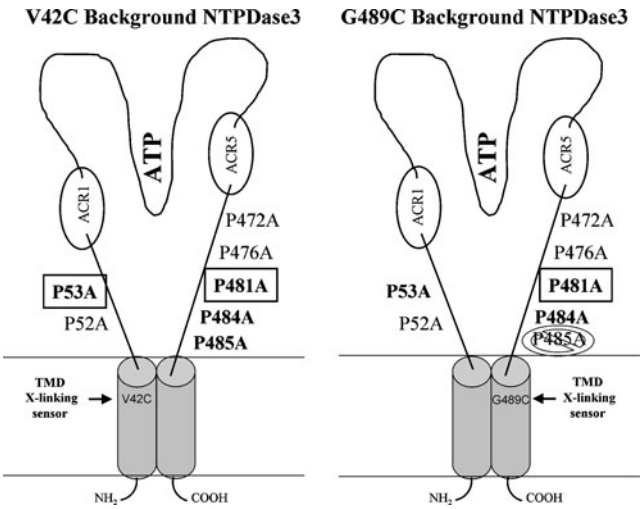

Fig. 6 Summary of the proline to alanine nucleoside triphosphate diphosphohydrolase 3 (NTPDase3) mutant data. Shown are cartoon representations of two monomers of human NTPDase3. *On the left*, the results for the proline substitutions in the V42C N-terminal transmembrane (TM) cysteine substitution background are schematically represented. *On the right*, the results for the proline substitutions in the G489C C-terminal TM cysteine substitution background are schematically represented. The N- and C-terminal TM helices are represented by *gray cylinders*, the cell membrane by *thin horizontal lines*, and the "linker regions" containing the conserved and mutated proline residues are indicated by *straight lines* connecting the TM helices with ACR1 and ACR5 regions (indicated by *ellipses*). The majority of the extracellular portion of NTPDase3, consisting of two lobes, is represented by a *free-form curve*, and forms the active site crevice, shown with substrate (adenosine triphosphate (ATP)) present. Proline to alanine mutations which decrease nucleotidase activities (normalized for expression levels) are indicated by *bold type*. Those mutations which abolish the ability of substrate (ATP) to diminish the cross-linking efficiency of the introduced TM helix cysteine residues ("uncoupling mutations") are indicated by *boxes* drawn around the mutations. The P485A mutation in the G489C background is fully active but abolishes TM copper phenanthroline cross-linking both in the absence and presence of ATP and is annotated by a *strikethrough ellipse*

background). Alanine substitution at positions P53 and P481 in G489C resulted in a substantial decrease in activity, while the P484A mutant exhibited a smaller decrease in activity (Table 1, NTPDase3 proline residue to alanine mutants in G489C background). The reduction in activity of the proline to alanine mutants (30–50%) is a little less than that seen upon deglycosylation (where a 50–60% decrease in Mg-ATPase activity was previously observed for NTPDase3 [35]). The decrease in enzyme activity of the proline to alanine mutants is slightly larger than that caused by the detergent Triton X-100 when the activity of NTPDase3 is assayed in the presence of Ca^{+2} (30–50% versus 20–40%), but this 30–50% decrease in enzyme activity of the proline to alanine mutants is smaller than the decrease observed in Mg-nucleotidase activities after Triton X-100 treatment, (70–90% [31]). Interestingly, alanine substitution at P472 increased nucleotidase activities, but these increases were smaller and not statistically significant in the V42C sensor background (Table 1, NTPDase3 proline residue to alanine mutants in V42C background and NTPDase3 proline residue to alanine mutants in G489C background). Taken together, this data suggests P53, P481, and P484, which are completely conserved in NTPDase3, but not in other NTPDases (see Fig. 1a), are needed for optimal enzymatic activity of NTPDase3.

Assessment of the proline to alanine mutations for possible "uncoupling" effects (abolishment of the ATP-dependent decrease in TM cross-linking efficiency) revealed that mutations at positions P53 and P481 in the V42C background were indeed capable of uncoupling ATP binding at the active site to reorientations or movements of the TM helices as assessed by CuPhen cross-linking (Figs. 4a and 5a). In the G489C background, proline to alanine substitution at position P481 also caused this effect (Figs. 4b and 5b). Only alanine substitutions at positions P53 and P481 resulted in both decreases in nucleotidase activities and abolishment of the decreased cross-linking efficiency of the TM helices in the presence of ATP. Surprisingly, alanine substitution at position P485 abolished CuPhen-induced cross-linking of G489C both in the presence and absence of ATP, although this was not true for cross-linking of V42C after alanine substitution at P485 (Figs. 4 and 5). This suggests that the placement of the cysteine residue used as the cross-linking TM sensor is important for the ability to measure the effects of the proline mutations, and that such proline to alanine mutations may affect the TM helix closer to the mutation more than the TM helix on the other end of the protein. Thus, alanine substitution at P485, which is only four amino acids away from the G489C mutation used as a sensor in the C-terminal helix, changes the orientation or freedom of movement of the C-terminal TM helix of NTPDase3 in a way that inhibits intermolecular, CuPhen-induced cross-linking at G489C, but not cross-linking at V42C, which is located in the N-terminal TM helix.

The current study is the first to demonstrate the importance of specific, conserved proline residues in the "linker regions" of NTPDase3 that "couple" the extracellular domain (site of nucleotide binding) to the dynamic motions and interactions of the TM helices, a linkage which is functionally important for optimal nucleotide hydrolysis. Analogous to results obtained using rat CD39/NTPDase1 [23], cysteine mutations in both TM helices near the extracellular surface (V42C and G489C in NTPDase3) proved to be useful TM cross-linking "sensors." Both V42C and G489C NTPDase3 mutants were enzymatically active (Table 1) and efficiently cross-linked in the presence of CuPhen (Fig. 3). In the presence of ATP (as well as ADP and AMP-PNP), the CuPhen-induced cross-linking efficiency of the V42C and G489C NTPDase3 mutants was

attenuated, resulting in an increase in the amount of monomer remaining (Fig. 3). Disulfide cross-linking of V42C and G489C via CuPhen also inhibited the nucleotidase activity of the mutants (Fig. 2), as was also observed with CD39/NTPDase1, suggesting the need for TM helix rearrangements and/or movements for full enzymatic activity. Not only does 5 mM ATP attenuate TM cross-linking (Fig. 3) but it also attenuates the CuPhen-induced decrease in enzyme activity (see Fig. 2). Thus, TM helix rearrangements and/or movements may be important for full activity of all cell surface NTPDases.

As defined by the polar TM interactions described in a recent study detailing the spatial organization and interactions of the TM helices of human NTPDase3 [31], efficient intermolecular cross-linking between monomers within an NTPDase3 dimer is not expected for either V42C or G489C, since that TM helix interaction model does not place either of these residues in close proximity to the same residue in the other monomer comprising the dimer [31]. This suggests that similar to rat NTPDase1 [23], NTPDase3 TM helices must have considerable rotational freedom near the extracellular surface of the membrane, and that this rotational freedom is substantially decreased in the presence of ATP, resulting in the nucleotide-induced decrease in CuPhen cross-linking efficiency.

In summary, consistent with our hypothesis, some proline to alanine mutants exhibit decreases in enzymatic activities, uncoupling of the interactions between the active site and TMD, or both. Importantly, other proline to alanine mutants also located in the linker region had little, if any, effect, demonstrating that the results obtained are not simply nonspecific artifacts, in the sense that any proline to alanine substitution in this area of the protein would result in decreases in activity and changes in ATP-induced cross-linking efficiencies as a result of adverse effects on protein folding. These conserved proline residues in the linker regions of NTPDase3 constitute proline-rich regions that mediate interactions between the extracellular and TM domains, suggesting the possible existence of polyproline II (PPII) helices. Typically, PPII helices are shorter than five amino acids, contain multiple proline residues, and often also contain positively charged amino acids [43–45]. These characteristics are consistent with the amino acid sequence of the linker regions of NTPDase3, as well as with the importance of the P53 and P481 residues identified in this study (N-terminal: QEVL**PP^{53}GLK**; C-terminal: PAESPLIRL**P^{481}IEPP**). Interestingly, the X-ray structure of the soluble portion of rat NTPDase2 does contain parts of these linker regions, including residues corresponding to some of these proline residues (N-terminal: ^{53}P; C-terminal: ^{472}P and ^{476}P, [13]). In the crystal structure, there is a short left-handed 3_{10} helix in the C-terminal linker region, suggesting the possibility of polyproline II helix in this linker region of NTPDase3.

Taken together, the data presented in this study suggest that the interplay between the extracellular active site and the TMD, as determined by reduction of oxidative cross-linking in the presence of substrates and previously observed for NTPDase1, may be generalized to all cell surface NTPDases. Also, conserved prolines in the linker regions connecting the N- and C-terminal helices may couple these domain interactions for other NTPDases. However, it should be noted that the most conserved proline residue in these linker regions (P472 in NTPDase3), as well as a residue conserved in the cell surface NTPDases (P52 in NTPDase3), are not essential for NTPDase3 optimal activity and, when mutated, do not abolish the ability of ATP to modulate NTPDase3 TM cross-linking. For NTPDase3, proline residues at positions 53 and 481 help mediate this linkage, needed for optimal activity, which couples binding of nucleotide binding at the enzymatic active site to dynamic movements and/or rearrangement of the TM helices.

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