Identification of a tyrosine residue responsible for \(N\)-acetylimidazole-induced increase of activity of ecto-nucleoside triphosphate diphosphohydrolase 3

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Abstract

Chemical modification in combination with site-directed mutagenesis was used to identify a tyrosine residue responsible for the increase in ecto-nucleoside triphosphate diphosphohydrolase 3 (NTPDase3) nucleotidase activity after acetylation with a tyrosine-selective reagent, \(N\)-acetylimidazole. The NTPDase3 ATPase activity is increased more than the ADPase activity by this reagent. Several fairly well conserved tyrosine residues (252, 255, and 262) that are located in or very near apyrase conserved region 4a (ACR4a) were mutated. These mutants were all active, but mutation of tyrosine 252 to either alanine or phenylalanine eliminated the activity increase observed after \(N\)-acetylimidazole treatment of the wild-type enzyme. This suggests that the acetylation of tyrosine 252 is responsible for the increased activity. Stabilization of quaternary structure has resulted in increased enzyme activities for the NTPDases. However, mutation of these three tyrosine residues did not result in global changes of tertiary or quaternary structure, as measured by Cibacron blue binding, chemical cross linking, and native gel electrophoretic analysis. Nevertheless, disruption of the oligomeric structure with the detergent Triton X-100 abolished the increase in activity induced by this reagent. In addition, mutations that abolished the \(N\)-acetylimidazole effect also attenuated the increases of enzyme activity observed after lectin and chemical cross-linking treatments, which were previously attributed to stabilization of the quaternary structure. Thus, we speculate that the acetylation of tyrosine 252 might induce a subtle conformational change in NTPDase3, resulting in the observed increase in activity.

Abbreviations: ACR – apyrase conserved region; BSA – bovine serum albumin; Con A – Concanavalin A; DMEM – (Dulbecco’s modified Eagle medium); DMSO – dimethyl sulfoxide; DSS – disuccinimido suberate; DTT – (dithiothreitol); MOPS – (3-[\(N\]-morpholino] propanesulfonic acid; \(N\)-AI – \(N\)-acetylimidazole; NTPDases – (nucleoside triphosphate diphosphohydrolases); Pi – (inorganic phosphate); PVDF – polyvinylidene fluoride; TBS – (tris buffered saline)

Introduction

The eNTPDases are a family of enzymes that hydrolyze extracellular nucleoside 5’ di- and triphosphates in the presence of divalent cations [1]. There are integral membrane protein NTPDases, embedded in both the cell surface membrane and internal membranes, as well as NTPDases that can be secreted extracellularly as soluble nucleotidases [2–7]. Four of the eight human NTPDases described to date (NTPDase1–3, 8) are integral membrane, cell-surface glycoproteins, with large extracellular domains and two transmembrane domains located near the N- and C-termini [1, 8].

Several areas of sequence conservation in the NTPDases have been noted. Besides the four apyrase conserved regions (ACRs) originally described [9], and ACR5 described later [10, 11], there are also two other conserved protein motifs in the NTPDases that have been named ACR1a and ACR4a [12]. No amino acid residues in the ACR1a region have thus far been assigned any functional significance. However, ACR4a was found to include a conserved glycine residue (glycine 263 in NTPDase3 [12]) whose mutation to alanine caused complete inactivation of the enzyme, presumably by large-scale disruption of native tertiary and quaternary structures of the nucleotidase [12]. A similar, but slightly less pronounced effect was found by mutation of another conserved glycine residue to alanine in NTPDase3, i.e., glycine 462, located in ACR5 [12]. The enzymatic activities, and therefore presumably the physiological functions, of the cell-surface NTPDases are dependent on their oligomeric state (quaternary structure).
Conditions that stabilize NTPDase homo-oligomers increase activity, while conditions that destabilize NTPDase oligomers decrease nucleotidase activities [13]. For example, solubilization with Triton X-100 detergent disrupts the native oligomeric structure and dramatically decreases nucleotidase activities for both NTPDase1 (CD39 [14]) and NTPDase3 [15]. Lectins such as the tetravalent Con A protein can induce lattices of specific glycoproteins, even in the presence of complex mixtures of glycoproteins exposed to Con A [16]. Thus, several studies have reported that Con A increases NTPDase activity [17–19], presumably by stabilization of the quaternary structure. At least in NTPDase3, this Con A effect seems to be mediated via the conserved N81 glycosylation site, located near ACR1 [20]. It has also been shown that chemical cross-linking can stabilize quaternary structure, and therefore the lysine specific cross-linking agent, DSS, has been used to assess possible changes in quaternary structure as a consequence of site-directed mutagenesis of NTPDase3 [12, 21–24]. Of course, the enzymatic activities of the NTPDases are also dependent on their tertiary structures. Cibacron blue (Reactive Blue 2) is a triazine dye that has affinity for the nucleotide binding site of many proteins, and has been shown to inhibit nucleotide handling enzymes in general [25, 26], as well as specific NTPDases [27, 28]. Cibacron blue binding to NTPDase3 is retained after solubilization and monomerization with Triton X-100 [12]. As a consequence, this dye has been used as a measure of native tertiary structure for NTPDase3, since it does not bind to heat-denatured NTPDase3 or to certain mutants that appear to have disrupted tertiary structures [29].

While screening chemical modification reagents for effects on NTPDase3 nucleotidase activities, we made the unusual and unexpected observation that reaction of wild-type NTPDase3 with the tyrosine selective reagent, N-acetylimidazole, increases enzyme activity. Previously, treatment with aspirin (acetylsalicylic acid) was shown to inhibit rat platelet NTPDase [30], possibly via acetylation. Therefore, we speculate that Tyr 252, as well as the amino acid residues nearby Tyr 252 in ACR4a, may be important for full enzymatic activity of NTPDase3 due to their subtle influence on native tertiary and/or quaternary structure.

Materials and methods

The QuikChange™ site-directed mutagenesis kit was purchased from Stratagene. Oligonucleotides were synthesized by the DNA Core Facility at the University of Cincinnati. Lipofectamine Plus Reagent, Dulbecco’s modified Eagle medium (DMEM), calf serum, and antibiotics/antimycotics were obtained from Gibco/Life Technologies. The mammalian expression vector pcDNA3 was obtained from Invitrogen. The chemical cross-linking reagent disuccinimidyl suberate (DSS) and the SuperSignal chemiluminescence reagents were purchased from Pierce Chemical Company. Cibacron Blue Gel (Affi-Gel Blue), pre-cast SDS-PAGE 4%–15% gradient mini-gels, and goat anti-rabbit horseradish peroxidase conjugated secondary antibody were obtained from Bio-Rad Laboratories. N-Acetylimidazole (N-AI), Ampicillin, nucleotides, and other reagents were from Sigma.

Site-directed mutagenesis of NTPDase3

Mutagenesis of NTPDase3 in pcDNA3 vector was performed using the Quick Change site-directed mutagenesis kit (Stratagene) as described previously [12, 15, 23, 24, 29, 32]. The sense nucleotides used for mutagenesis are as follows:

Y252A, 5’-CTGTATGGCTACGTCGCTACAACGCTCTACAACACAC-3’;
Y255A, 5’-TACGTATACAGCCTAGCAGCACAACACACAGCTTCCAG-3’;
Y262A, 5’-CACAGCTTCCAGTGCCAGGCAGCCGGAATTGAGGCT-3’;
Y252F, 5’-TATGGCTACGTATCCAGCCTACAGCACAACACACAGCTTCCAG-3’;
Y255F, 5’-GTATACACGCTTCCATACACACACACACAGCTTCCAG-3’;
Y262F, 5’-AGCTTCCAGTGCTTTGGCCGGAATTGAGGCT-3’;

Altered codons are underlined, but the complementary antisense oligonucleotides also necessary for mutagenesis are not shown. The presence of the desired mutation and lack of undesired mutations were confirmed by DNA sequencing. The mutated NTPDase3 cDNA was used to transform competent cells as described by the manufacturer (Stratagene).

Transient transfection

COS-1 cells were grown and transfected with wild-type and mutant NTPDase3 cDNA, as previously described.
An empty pcDNA3 vector was transfected into COS cells and used as a background control for nucleotidase assays. Cells were harvested 48 h post-transfection and crude total membrane preparations were obtained as described [12, 15, 23, 24, 29, 32].

**Protein assay**

Protein concentrations were determined using the Bio-Rad Coomassie blue dye binding assay, using bovine serum albumin as the standard, with the modifications of Stoscheck [33].

**Nucleotidase assays**

Nucleotidase activities were determined by measuring the concentration of inorganic phosphate (Pi) released from the ATP or ADP substrates in the presence of Mg$^{2+}$ or Ca$^{2+}$. Assays were performed at 37 °C as previously described [12, 15, 23], modified from Fiske and Subbarow [34]. Nucleotidase activities were corrected for COS-1/pcDNA3 background (provided by the empty pcDNA3 vector transfected into COS-1 cells) and for differences in expression levels relative to those of wild-type, as determined by Western blotting.

**Western blot analysis**

For Western blot, proteins were resolved in a 4%–15% linear gradient SDS-PAGE gradient gel (BioRad 4–15%) and transferred to a polyvinylidene fluoride (PVDF) membrane. Blots were probed with an anti-C-terminal peptide NTPDase3 antibody, as previously described [32].

**Chemical cross-linking**

COS cell membrane preparations (at a total protein concentration of 0.1 mg/ml) were diluted in 20 mM MOPS, 5 mM MgCl$_2$ buffer, pH 7.4, and incubated for 20 min at 22 °C with 200 μM disuccinimidol suberate (DSS) freshly dissolved in DMSO. Non-cross-linked samples were treated with the same volume of DMSO, and the final DMSO concentration of all samples was less than 2% of the total sample volume. The cross-linking reaction was stopped by incubation with an excess (10 mM) of lysine for 5 min at 22 °C. Samples were then either analyzed for intermolecular cross-linking by reducing SDS-PAGE and Western blot analysis, or assayed for Mg$^{2+}$-ATPase activity as described above.

**Analysis of Cibacron blue binding**

Cibacron blue binding assays have been described previously [12, 23, 29].

**Native gel electrophoresis of wild-type and mutant NTPDases**

NTPDase3 membrane preparations were solubilized for 10 min at room temperature in digitonin (1% final concentration), a detergent known to preserve the native quaternary structure and the activity of the NTPDases [13, 14]. Soluble proteins were isolated by centrifugation and run on 6% Laemmli native gel containing 0.1% digitonin as described previously [12].

**Treatment of wild-type and mutant NTPDase3 with Concanavalin A**

Concanavalin A (Con A) was prepared at a concentration of 5 mg/ml in 20 mM MOPS buffer containing 100 mM NaCl, 1 mM MnCl$_2$ and 1 mM CaCl$_2$, pH 7.4. Wild-type and tyrosine mutant NTPDase3 total membrane preparations (2 μg) were incubated with 5 μl of 5.0 mg/ml Con A at 37 °C for 15 min before the addition of substrate. Nucleotidase assays were performed in the presence of 5 mM Ca$^{2+}$ or Mg$^{2+}$, at a final nucleotide concentration of 2.5 mM.

**N-acetylimidazole treatment**

Membrane preparations were diluted to a total protein concentration of 0.1 mg/ml in pH 7.4 MOPS buffer containing 5 mM MgCl$_2$. At 0 time (before addition of N-acetylimidazole) 20-μl aliquots were diluted into 260 μl of an ice-cold stop solution containing an excess of lysine (14.5 mM) to quench the reaction, 100 mM KCl, 5 mM MgCl$_2$, 1 mM EGTA and 20 mM Tris–HCl, pH 7.0. N-acetylimidazole (2 mM) was then added to the remainder of each membrane preparation at 22 °C. At 5, 10, 20, 30, 45 and 60 min, 20 μl aliquots were diluted with 260 μl of stop solution as described above. At 61 min, hydroxylamine was added to a final concentration of 200 mM to the remainder of the N-acetylimidazole-treated membrane preparation to quench the reaction and reverse the acetylation of tyrosine residues. At 62, 65, 75, 90 and 120 min, 21-μl aliquots were diluted with 259 μl stop solution as before. Mg$^{2+}$-ATPase and Mg$^{2+}$-ADPase assays were performed for all time points.

**Time dependence of Mg-ATPase activity**

Kinetics of Mg$^{2+}$-dependent ATPase hydrolysis before and after treatment with N-acetylimidazole or the lysine-specific cross linker, DSS, were measured in a Beckman DU-800 spectrophotometer, using a NADH-linked-enzyme spectrophotometric assay [35], as described previously for NTPDase2 [22] and NTPDase3 [20]. The NADH-linked-enzyme assay was used to allow the continuous monitoring of ATPase activity as a function of time. After pre-incubation to bring the cuvettes and samples to 37 °C, the
reactions were initiated by addition of a small volume of NTPDase3 enzyme.

Solubilization and nucleotidase assays

Cell membranes (at a total protein concentration of 0.1 mg/ml) were solubilized in 1% Triton X-100, 5 mM MgCl2 and 20 mM MOPS buffer pH 7.4 at 22 °C for 10 min with occasional mixing, followed by centrifugation at 150,000 g for 30 min at 22 °C. Mg2+ and Ca2+ ATPase and ADPase activities were measured in presence of 0.1% Triton X-100, after adding nucleotide to a final nucleotide concentration of 0.435 mM, using a malachite green phosphate assay [36] to measure nucleotidase activities, since Triton X-100 causes turbidity in the modified Fiske and Subbarow assay [34].

Results

Rationale for selection and analysis of tyrosine mutants

We mutated several tyrosine residues (Tyr 252, 255, and 262) in the vicinity of a computer predicted, myc-type, helix–loop–helix dimerization domain (238NTSDIMQVS [31]), which is also nearby a region of the enzyme (ACR4a) previously identified as containing a glycine residue important for folding or structure of NTPDase3 (Gly 263, [12], see Table 1). We hypothesized that modification of one or more of these tyrosine residues might be responsible for the increased enzyme activity.

Protein expression levels

Expression levels of the NTPDase3 mutants were calculated by quantification of Western blots as previously described [12] and compared to wild-type NTPDase3 using an Alpha Innotech FluorChem 8800 Imager. The values presented are the average of three separate transfections (Table 2), and the specific activities for ATP and ADP of the NTPDase3 mutants were corrected for variations in expression levels relative to those of the wild-type (see Table 2).

Nucleotidase activities of NTPDase3 mutants

ATPase and ADPase nucleotidase activities of all mutants were determined in the presence of either Mg2+ or Ca2+ (Table 2). All tyrosine mutants were active and displayed nucleotidase activities similar to the wild-type NTPDase3, with expression levels 52%–85% of the wild-type.

N-acetylimidazole increases Mg2+-ATPase/ADPase activity

N-acetylimidazole increases Mg2+-ATPase/ADPase activity of wild-type NTPDase3 in presence of Mg2+. After 60 min of reaction, Mg2+-ATPase and Mg2+-ADPase activities are increased by approximately 1.9-fold (Figures
Values given represent the means ± standard deviations of three separate transfections (which were matched to their own wild-type and empty pcDNA3 vector controls). Activities were measured in presence of 5 mM MgCl₂ or CaCl₂ at a final concentration of 2.5 mM ATP or ADP. Values were normalized for different NTPDase3 protein expression levels by dividing the nucleotidase activity (expressed in the table as mol Pi/mg protein/h) by the expression level relative to the wild-type enzyme.

Table 2. Nucleotidase activities of wild-type (WT) and mutant NTPDase3 enzymes.

| NTPDase3 enzyme | % Wt. level of expression | Normalized Mg²⁺-ATPase activity (% wt.) | Normalized Ca²⁺-ATPase activity (% wt.) | Normalized Mg²⁺-ADPase activity (% wt.) | Normalized Ca²⁺-ADPase activity (% wt.) |
|----------------|---------------------------|---------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| Wild-type      | 100                       | 144 ± 10 (100)                        | 413 ± 35 (100)                         | 57 ± 5 (100)                           | 83 ± 15 (100)                          |
| Y252F          | 60 ± 5                    | 138 ± 10 (95)                         | 360 ± 30 (87)                          | 48 ± 5 (84)                            | 84 ± 8 (101)                           |
| Y255F          | 85 ± 7                    | 170 ± 12 (118)                        | 397 ± 24 (96)                          | 97 ± 12 (170)                          | 96 ± 10 (115)                          |
| Y262F          | 84 ± 5                    | 158 ± 9 (109)                         | 394 ± 23 (95)                          | 59 ± 8 (103)                           | 84 ± 18 (101)                          |
| Y252A          | 52 ± 6                    | 140 ± 12 (97)                         | 345 ± 36 (83)                          | 51 ± 7 (89)                            | 86 ± 7 (103)                           |
| Y255A          | 67 ± 5                    | 172 ± 15 (119)                        | 392 ± 26 (95)                          | 104 ± 15 (182)                         | 106 ± 10 (127)                         |
| Y262A          | 70 ± 4                    | 159 ± 7 (110)                         | 410 ± 25 (99)                          | 60 ± 12 (105)                          | 85 ± 20 (102)                          |

1 and 2, upper panels) and 1.35-fold (Figures 1 and 2, lower panels), respectively. A substantial portion of the increase in activity is rapidly reversible by hydroxylamine added at 61 min (Figures 1 and 2), suggesting that tyrosine modification, rather than lysine modification, is responsible for the effect, since tyrosine modification, unlike lysine modification by N-AI, is reversible by hydroxylamine. As is evident from Figure 2, N-acetylimidazole is unable to increase the nucleotidase activity of the Y252F mutant, in contrast to the wild-type, Y255F, and Y262F mutant NTPDase3. Interestingly, the N-acetylimidazole-induced increase is also greatly attenuated in the Y255A mutant, as well as being eliminated in the Y252A mutant (Figure 1).

To investigate the reaction kinetics of Mg²⁺-ATPase after N-acetylimidazole-mediated chemical modification of wild-type NTPDase3, continuous measurement of the Mg-ATPase activity was made using the linked enzyme assay. The data obtained was fit using a double exponential decay function (Figure 3), suggesting two processes causing decreases in enzymatic activity at 37 °C, which occur on different time scales. N-Acetylimidazole decreases the rate of decay of activity, resulting in the measured increase in activity (Figures 1 and 2).
Is N-acetylimidazole modification associated with higher oligomer formation?

To investigate whether the N-acetylimidazole modification triggers higher oligomer formation, wild-type NTPDase3 was treated with N-acetylimidazole for various times (0, 5, 30, 45 and 60 min) and then run on a native gel. All samples ran with same electrophoretic mobility as untreated NTPDase3, indicating that N-acetylimidazole does not initiate higher order oligomer formation as measured by this technique. In addition, the electrophoretic mobilities of wild-type NTPDase3 and the tyrosine mutants are indistinguishable in this native gel electrophoresis, suggesting that the mutants have the same gross oligomeric structure as wild-type NTPDase3. Also, a lysine-specific cross-linking agent, disuccinimido suberate (DSS), was used to assess gross changes in quaternary structure. After reducing SDS-PAGE and Western blotting, wild-type and all NTPDase3 mutants made in this study were similarly cross-linked into dimers, suggesting that the gross quaternary structures of the mutants are similar to those of the wild-type (all data mentioned above is not shown due to lack of demonstrable differences between the wild-type and mutant enzymes).

**Analysis of tertiary structure of the mutants by Cibacron blue binding**

To explore the possibility of global misfolding induced by point mutations leading to the inability to bind the nucleotide analogue triazine dye, Cibacron blue, Cibacron blue binding assays were performed as previously described [12]. Misfolded mutants are unable to bind Cibacron blue affinity matrix, as is denatured NTPDase3 (exemplified by the boiled, wild-type enzyme [12]). All the mutants described in this study bound to the Cibacron blue matrix like the wild-type enzyme (data not shown), suggesting that there are no gross changes in tertiary conformations of the mutants affecting the ability to bind the nucleotide analogue, Cibacron blue.

**Effect of N-acetylimidazole on Triton X-100 solubilized wild-type NTPDase Mg-ATPase activity**

To investigate whether N-acetylimidazole can increase Mg$^{2+}$-ATPase activity of wild-type NTPDase3 after dissociation into monomers by Triton X-100, wild-type NTPDase3 was solubilized with Triton X-100 either prior to, or after, treatment with N-acetylimidazole. The results given in Table 3 indicate that N-acetylimidazole cannot increase the Mg$^{2+}$-ATPase activity of Triton X-100 solubilized (monomeric) NTPDase3, and that after N-AI is used to increase the activity of the membrane-bound wild-type NTPDase3, that increase in activity is abolished by subsequent Triton X-100 solubilization. Therefore, it appears that the increase in enzyme activity seen upon N-acetylimidazole treatment may be dependent on native quaternary structure, or upon the native tertiary structure.
since the native tertiary structure may be modified by disruption of the quaternary structure by Triton X-100.

**Effect of Concanavalin A on NTPDase3 wild-type and tyrosine mutants**

Concanavalin A is a tetravalent protein that binds to glycans and can stabilize the oligomeric structure of glycoproteins by induction of the formation of protein oligomeric lattices [16]. Concanavalin A was previously shown to increase the nucleotidase activity of several membrane bound, oligomeric NTPDases [18, 22, 37, 38]. The elimination of a single glycosylation site located near ACR1 (N81 in NTPDase3) was shown to greatly diminish the ability of Con A to increase the nucleotidase activity of NTPDase3 [20]. Thus, we used Con A as another probe to determine if the N-AI effect may be related to a quaternary structure effect. The data in Figure 4 are consistent with the N-acetylimidazole data in Figures 1 and 2, i.e., the mutants which do not respond to N-AI also respond less to the stimulating effect of Con A, suggesting some subtle conformational changes in the Y252F, Y252A, and Y255A mutants, which may subtly affect the quaternary structure.

**Effect of DSS chemical cross-linking on the nucleotidase activity of tyrosine mutant and wild-type NTPDase3**

The lysine specific chemical modification reagent, DSS, was used to treat wild-type and mutant NTPDases to observe the effects on Mg-ATPase activities. As can be seen in the top panel of Figure 5, DSS treatment increased the activity of the wild-type as well as the Y255F and Y262F mutants, but decreased the activity of the Y252F mutant, whose activity is also not stimulated by N-acetylimidazole (see Figure 2). In addition, the correlation between stimulation of activity by N-AI and by DSS cross-linking also exists for the tyrosine to alanine mutants, since both Y252A and Y255A, which are not stimulated by N-AI (see Figure 1), are also not stimulated by DSS cross-linking (bottom panel of Figure 5).

**Discussion**

In this study we used site-directed mutagenesis to mutate several conserved tyrosines to investigate which tyrosine residue(s) is(are) responsible for mediating the increase in Mg\(^{2+}\)-ATPase activity caused by acetylation with the chemical modification reagent, N-acetylimidazole (Table 1). These tyrosine residues (see Table 1) are in the vicinity of the glycine residue that was previously proposed to be important for NTPDase3 higher-order structure (due to results obtained upon mutation of glycine 263 in ACR4a [12]), and are also located near the computer-predicted helix-loop-helix dimerization domain [31] of NTPDase3 (238NTSDIMQVS246). These amino acids were first changed to alanine to compare results to our previous studies where NTPDase3 residues were typically mutated to alanine [12, 23, 24, 29, 32]. However, for the purpose of introducing the least structural perturbations and yet allowing us to delineate the residue(s) modified by N-acetylimidazole, these tyrosine residues were also mutated to phenylalanine. As can be seen in Table 2, mutation of none of these three tyrosine residues to either alanine or phenylalanine inactivated the enzyme, indicating that they are not an essential part of the catalytic site.

Upon treatment with N-acetylimidazole, wild-type NTPDase3 displays a time-dependent increase in Mg\(^{2+}\)-ATPase and Mg\(^{2+}\)-ADPase activities (see Figures 1 and 2). A substantial portion of the increase in activity is rapidly reversible by hydroxylamine treatment, suggesting that tyrosine modification, rather than lysine modification, is responsible for the effect. The Y252F mutant does not exhibit this N-acetylimidazole-induced increase in activity, while the nearby Y255F and Y262F mutants behave like the wild-type enzyme (Figure 2). This suggests that modification (acetylation by N-acetylimidazole) of tyrosine at position 252 mediates the effect. However, it is not clear why the increase in activity is not completely reversible with hydroxylamine treatment, if the effect is solely mediated by a single tyrosine acetylation, as the mutagenesis results suggest. Consistent with the phenylalanine mutants, the Y252A mutant is unresponsive to N-AI treatment. However, the Y255A mutant, unlike the Y255F mutant, is also largely unresponsive to N-AI treatment (Figure 1). The reason for this is not known, but we speculate that the Y255A mutation, being less conservative in nature and introducing a substantially smaller side chain at that position than the Y255F mutation, could cause a perturbation in the folding in that area of the protein, resulting in a microenvironment at Y252 that is either less conducive for the reaction of Y252 with N-AI, or alternatively, less conducive.
to the change in local conformation that presumably occurs after acetylation of Y252 by N-AI.

There are no obvious, global changes in tertiary or quaternary structures in any of the mutants described in this study, since all the mutants have expression levels and enzyme activities not very different from the wild-type enzyme (Table 2). In addition, they all behave like wild-type NTPDase3 in Cibacron blue binding assays, as well as on native gels, and have similar relative susceptibility to intermolecular DSS cross-linking (data not shown since all characteristics were not different from the wild-type).

However, we found that Triton X-100 solubilization abolished the N-acetylimidazole-mediated increase in Mg\(^{2+}\)-ATPase activity of wild-type NTPDase3, and that monomerization by Triton X-100 solubilization subsequent to stimulation of activity by N-AI negates the N-AI-induced increase in activity (Table 3). This is important since solubilization with Triton X-100 detergent has been shown to cause a decrease in enzyme activity accompanying disruption of the native oligomeric structure to monomeric enzyme for both NTPDase1 (CD39 [14]) and NTPDase3 [15]. This suggests that the N-acetylimidazole-mediated increase in enzyme activity might be associated with a subtle effect on the quaternary structure, which is not detected by the global nature of the DSS cross-linking and native gel analyses. Consistent with this is the fact that the N-acetylimidazole induced increase of the ATPase activity is more pronounced than the increase of the ADPase activity (see Figures 1 and 2), since, based on Triton X-100 solubilization (monomerization) experiments, it appears that ATPase activity is more dependent on native quaternary structure than ADPase activity for both NTPDase1 [14] and NTPDase3 [15]. Also consistent with the possibility that the N-acetylimidazole-mediated increase in enzyme activity might be associated with a subtle effect on the quaternary structure is the observation that N-AI does not increase the activity (data not shown) of bacterially expressed NTPDase6 [39], which is a soluble, monomeric NTPDase that contains the conserved tyrosines corresponding to residues 252 and 255 in NTPDase3 (see Table 1). In addition, there is precedence for N-acetylimidazole acetylation of proteins affecting quaternary structure and stability of oligomeric proteins, since Pal et al. reported that this reagent acetylated surface exposed tyrosine residues, resulting in a destabilization of the native subunit assembly of the alpha-crystallin protein [40]. Alternatively, monomerization by Triton X-100 may also modify the native tertiary structure of NTPDase3, and it could be that this alteration in the tertiary structure leads to the observed abolition of the N-acetylimidazole-mediated increase in Mg-ATPase activity.

Concanavalin A, a tetravalent ligand known to stabilize the oligomeric structure of glycoproteins by induction of high-order protein oligomer lattice formation [16], increases the nucleotidase activity of several eNTPDases [18, 19, 37, 38, 41-43]. This Con A effect has been previously attributed to stabilization of the native quaternary structure [13, 20]. Thus, we used this lectin as an additional probe of subtle conformational effects of the mutations in this study. The results obtained (see Figure 4) are consistent with, and complementary to, the N-AI results in Figures 1 and 2. Thus, mutations in this region that affect the ability of N-AI acetylation to increase NTPDase3 enzyme activity, possibly via subtle stabilization of the native quaternary structure, also manifest themselves with regard to the ability of Con A to stimulate enzyme activity. As a result, Con A has a lesser effect on the activity of the Y252F mutant than on the Y255F, Y262F, and wild-type NTPDase3 (Figure 4, top panel).

Chemical cross-linking using the lysine-specific agent, DSS, is also consistent with the N-AI and Con A results, since the tyrosine mutants that are unable to respond to N-AI also do not respond like the wild-type NTPDase3 to DSS cross-linking with a similar increase in activity (Figure 5). Instead of the increase in activity of the wild-type enzyme, these mutants display reduced activities after lysine-specific cross-linking (DSS treatment). This reduction in activity could be due to reaction at other lysine residues, the negative effect of which is masked in the wild-type enzyme by the larger positive effect on activity after reaction with a different set of lysine residues.

Very recently, Grinthal and Guidotti [44] found that for the related CD39/NTPDase1 protein, the CD39 dimer appears to be in a relatively rapid equilibrium with monomeric CD39. Thus, those authors proposed that NTPDase1 exists in a relatively unstable dimeric quaternary structure, which allows exchange of monomer protein partners between different dimers. Since the dimer form of oligomeric NTPDases has higher nucleotidase activity than the monomer form, it follows that any mutation or chemical modification that shifts this monomer–dimer equilibrium more towards the dimer would be expected to increase enzyme activity. Thus, one possible explanation for the N-acetylimidazole induced increase in NTPDase3 activity observed in our studies is that acetylation of tyrosine 252 causes a relatively small increase in the stability of the dimer (i.e., slightly shifts the monomer–dimer equilibrium more in favor of the dimer), which is observed as an increase in activity, but is not detected by the relatively crude chemical cross-linking and native gel analyses utilized in this study.

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