# Mutagenesis of Two Conserved Tryptophan Residues of the E-Type ATPases: Inactivation and Conversion of an Ecto-Apyrase to an Ecto-NTPase†

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ABSTRACT: A human brain E-type ATPase (HB6 ecto-apyrase) was subjected to site-directed mutagenesis to assess the functional significance of two highly conserved tryptophan residues (Trp 187 and Trp 459), the only two tryptophans conserved in nearly all E-type ATPases. Mutation of tryptophan 187 to alanine yielded a poorly expressed ecto-apyrase completely devoid of nucleotidase activity. Immunolocalization of the W187A mutant in mammalian COS cells showed a cellular distribution clearly different from that of the wild-type enzyme, with the majority of the immunoreactivity concentrated in the interior of the cell. Unlike the wild-type enzyme, this mutant did not bind the nucleotide analogue Cibacron Blue and was sensitive to proteolytic digestion by chymotrypsin. These results suggest alteration of the tertiary structure, causing the enzyme to be improperly folded and retained within the cell. In contrast, mutation of tryptophan 459 to alanine resulted in an ecto-apyrase with enhanced NTPase activity, but diminished NDPase activity. Immunolocalization of this active mutant ecto-apyrase revealed a cellular pattern similar to that of the wild-type enzyme, distributed along the cell periphery and in cell processes. Coupling this active W459A mutation to a previously described mutation (D219E) resulted in an enzyme which preferentially hydrolyzes nucleoside triphosphates over diphosphates. The D219E/W459A double mutant had an ATPase:ADPase ratio of 11:1 and a UTPase:UDPase ratio of 148:1. In addition, the double mutant is substantially less sensitive to inhibition by azide, a more potent inhibitor of ecto-apyrases than ecto-ATPases. Thus, mutation of only two amino acids of an E-type ATPase essentially converts an ecto-apyrase to an ecto-NTPase.

The E-type ATPases (consisting of the ecto-ATPases and ecto-apyrases[1]) are a class of glycosylated ectoenzymes that have the following characteristics: (1) an extremely active nucleotide hydrolysis site situated on the exterior of the cell, (2) dependency upon divalent cations (usually calcium or magnesium) for activity, (3) insensitivity to the classic inhibitors of the P-, F-, and V-type ATPases, and (4) an ability to hydrolyze a wide range of purine and pyrimidine nucleoside tri- and diphosphates (NTPs and NDPs, respectively) (*1*, *2*). The ecto-ATPases are distinguished from the ecto-apyrases by the fact that the former preferentially hydrolyze NTPs as substrates (NTPs are typically hydrolyzed at rates that are at least 25 times greater than that of NDPs) and are relatively insensitive to inhibition by sodium azide. In contrast, the apyrases hydrolyze both NTPs and NDPs, often at nearly equal rates, and are susceptible to azide inhibition (*2*).

Despite these variations in enzymatic characteristics, the E-type ATPase family members possess a high degree of similarity to one another on the amino acid level. Multiple sequence alignments of several members of the E-type ATPases show several regions of amino acid identity, termed apyrase conserved regions (ACRs) (*3*−*6*). Four such regions (ACR I−IV) were originally identified with several invariant residues found in each, presumably playing important functional roles (*7*). Indeed, a recent site-directed mutagenesis study of a human ecto-apyrase demonstrated the critical role that four such residues in ACR I and ACR IV play in nucleotide hydrolysis (*8*). That study also provided experimental evidence that the E-type ATPases are related to the actin/heat shock protein 70/sugar kinase protein superfamily.

Analysis of multiple sequence alignments of the E-type ATPases reveals that in addition to those invariant amino acids found within the ACR regions, many other residues also appear to be highly conserved, and are likely to be involved in maintaining the proper structure and function of the enzymes (*4*, *6*, *9*). For example, several cysteine and proline residues appear to be conserved among the many family members of the ecto-ATPase and ecto-apyrases. It seems likely that these residues are important for maintaining

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[1] Abbreviations: CAPS, 3-(cyclohexylamino)propanesulfonic acid; cDNA, complementary DNA; E-type ATPase, ecto-ATPase or ecto-apyrase; ECL, enhanced chemiluminescence; ecto-apyrase, ecto-ATPDase or ecto-ATP diphosphohydrolase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HB6, human brain E-type ATPase clone; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride membrane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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the proper tertiary structure of the enzymes. In addition, two tryptophan residues found in the E-type ATPases are highly conserved, and therefore may also be functionally important.

One incompletely explored area of research is the elucidation of those amino acids responsible for the enzymatic differences observed between the ecto-ATPases and the ecto-apyrases. Although the two families of enzymes have a high degree of amino acid identity, some amino acid differences do exist and amino acids distinct to each particular family are evident (*6*). It is likely that these differences account for the variation in enzymatic activities. For example, two E-type ATPase isoforms isolated from *Toxoplasma gondii* differ in only 16 out of 628 amino acids (*10*), yet one (NTPase I) is an ecto-ATPase while the other (NTPase II) is an ecto-apyrase. In addition, a more recent report appeared during the review of this study that describes a specific block of 12 amino acids near the C-terminus of the *T. gondii* NTPases that seem to account for the nucleoside di- and triphosphatase activity in that system (*11*). Since site-directed mutagenesis with the E-type ATPases has recently been accomplished (*8*), this technique may also prove useful in the elucidation of those amino acids that distinguish the two families.

In this report, we describe the site-directed mutagenesis of a human ecto-apyrase and the impact of these mutations on the enzymatic properties and characteristics of the expressed proteins. Despite the high degree of conservation of the two tryptophan residues among the different family members, mutation of each yielded very different results, with one (W187A) disrupting the protein structure and inactivating the enzyme completely and the other (W459A) stimulating NTP hydrolysis activity. In addition, combining the W459A mutation with a previously described mutation (D219E) (*8*) yielded an expressed protein with characteristics indicative of an ecto-NTPase, with an ATP:ADP hydrolysis ratio of nearly 11:1 (unlike the wild-type apyrase with a 3.6:1 ratio), and a decreased sensitivity to azide inhibition. Other nucleoside triphosphates were differentially hydrolyzed to even greater extents. The findings presented here may be useful for the engineering of recombinant E-type ATPases exhibiting varied enzymatic properties, which may be important in the development of therapeutic applications of E-type ATPases and in the modulation of E-type ATPase activity.

## EXPERIMENTAL PROCEDURES

*Materials.* The QuikChange site-directed mutagenesis kit and *Epicurian coli* ultracompetent bacteria were purchased from Stratagene. Synthetic oligonucleotides were produced by the DNA Core Facility at the University of Cincinnati. Plasmid purification kits were purchased from Qiagen, Inc. Lipofectamine Plus Reagent, Dulbecco's Modified Eagle's Medium (DMEM), calf serum, goat anti-rabbit horseradish peroxidase-conjugated secondary antibody, and antibiotics and antimycotics were all obtained from Life Technologies. Goat anti-rabbit CY3-conjugated secondary antibody was from Jackson Immunoresearch. Falcon tissue culture-treated plates were from Becton-Dickinson. The mammalian expression vector pcDNA3 was obtained from Invitrogen. *Not*I and *Kpn*I restriction endonucleases and T4 DNA ligase were purchased from Promega. Sequenase version 2.0 kits were obtained from U.S. Biochemical Corp., while $^{35}$S-labeled dATP was from Dupont NEN. Reactive Blue 2 (Cibacron Blue) was purchased from Sigma, and Reactive Blue 2 affinity chromatography gel (Affi-Gel Blue) was purchased from Bio-Rad. Ampicillin, nucleotides, and other reagents were from Sigma.

*Construction of the HB6 cDNA in pcDNA3.* The isolation of the cDNA for the human brain ecto-apyrase (designated HB6; GenBank accession number AF034840) was described in a previous publication (*6*). The 2.8 kb HB6 cDNA was excised from the pCMV-SPORT plasmid and cloned into the 5.4 kb mammalian expression vector pcDNA3 using *Not*I and *Kpn*I restriction endonucleases. This construct (designated HB6 wild type) was used as the basis for all site-directed mutagenesis experiments.

*Computer Analyses of Sequences.* The deduced amino acid sequence of the HB6 ecto-apyrase (*6*) was compared to the nonredundant database of sequences using the gapped BLASTP program available on the World Wide Web at http://www.ncbi.nlm.nih.gov/BLAST/. Several representative members of the E-type ATPase class were selected, and multiple protein sequence alignments were then performed on regions of the proteins using the CLUSTALW program of the University of Illinois Biology Workbench site at http://biology.ncsa.uiuc.edu/. The default analysis parameters were used for all of the computer analyses that were performed.

*Site-Directed Mutagenesis of HB6.* Mutagenesis of the HB6 wild type was performed by using the QuikChange site-directed mutagenesis kit (Stratagene) as described in our previous publication (*8*). The sense oligonucleotides (26−37 nucleotides in length) used for mutagenesis are as follows: Trp 187 Ala, 5′-GCAAGAAGAAGGGGTATATG-GAGCGATTACAGCCAAC-3′; Asp 219 Glu, 5′-CGGGT-GCCCTGGAGTTAGGTGGTGCC-3′; and Trp 459 Ala, 5′-AGCAGCATAGCCGCGTCTCTTGGCTACATGC-3′, where the altered codons are underlined. The complementary antisense oligonucleotides also necessary for the mutagenesis are not shown. The mutagenized HB6 cDNA was then used to transform ultracompetent cells as described by the manufacturer (Stratagene). Mutagenized HB6 plasmid DNA was purified from 5 mL overnight cultures using the Qiagen miniprep system. The presence of the correct mutation was confirmed in all constructs by dideoxy DNA sequencing using Sequenase version 2.0. The mutagenized insert was then excised from the pcDNA3 vector using *Not*I and *Kpn*I restriction endonucleases, purified on a 0.8% low-melting agarose gel, and religated into the non-PCR-amplified pcDNA3 expression vector using T4 DNA ligase as previously described (*8*).

*COS-1 Cell Expression of Human Brain Ecto-Apyrase (HB6).* COS-1 cells were grown in complete DMEM (Dulbecco's Modified Eagle's Medium supplemented with 10% calf serum and 100 units/mL penicillin G, 100 $\mu$g/mL streptomycin sulfate, and 0.25 $\mu$g/mL amphotericin B as Fungizone) at 37 °C in 10% $CO_2$. Cells were transfected as described previously (*8*) using Lipofectamine Plus Reagent (Life Technologies) and either 4 $\mu$g of wild-type HB6 cDNA, 4 $\mu$g of mutagenized HB6 cDNA, or no cDNA for the control mock-transfected COS-1 cells. Approximately 72 h post-transfection, the COS-1 cells were used for analyses. The COS cell expression system was chosen to permit direct comparison to previous studies with expressed E-type ATPases (*4*, *6*, *8*, *12*−*15*).

Table 1: Multiple Sequence Alignments of Regions of Representative E-Type ATPases[a]

| E-type ATPase Name Species | GenBank Reference | Amino Acid Sequence (ACR III) | Amino Acid Sequence (ACR V) |
|---|---|---|---|
| 1. E-type ATPase (HB6) *Homo sapiens* | AF034840 (*6*) | *177* ISGQEEGVYG**W**ITANYLMGNF | *449* EKEVGNSSIA**W**SLGYMLSLTN |
| 2. Vascular ATP-diphosphohydrolase *Homo sapiens* | P49961 (*33*) | *169* ITGQEEGAYG**W**ITINYLLGKF | *440* IGKIQGSDAG**W**TLGYMLNLTN |
| 3. CD39L1 *Homo sapiens* | U91510 (*34*) | *160* LSGQEEGVFG**W**VTANYLLENF | *403* QKKAADTAVG**W**ALGYMLNLTN |
| 4. Guanosine-diphosphatase *Homo sapiens* | AF016032 (*4*) | *218* ISGKQEGVYA**W**IGINFVLGRF | *509* ALQVYDKEVQ**W**TLGAILYRTR |
| 5. Cell membrane ecto-ATPase *Gallus gallus* | U74467 (*35*) | *156* LSGEEEGVFG**W**ITANYLLENF | *426* QKKAGETSIG**W**ALGYMLNLTN |
| 6. Ecto-ATP-diphosphohydrolase *Gallus gallus* | AF041355 (*3*) | *161* LTGNEEGSFG**W**ITVNYLLETL | *428* SQKAGNADIG**W**TLGFMLNLTN |
| 7. NTPase *Drosophila melanogastor* | AF041048 (*9*) | *197* MDGTDEGIFS**W**FTVNFLLGRL | *433* YKKIDGHEIS**W**ALGCAYNVLT |
| 8. Guanosine diphosphatase *Saccharomyces cerevisae* | P32621 (*36*) | *166* MGGDEEGVFA**W**ITTNYLLGNI | *442* GKKIANKEIG**W**CLGASLPLLK |
| 9. Apyrase precursor *Solanum tuberosum* | P80595 (*7*) | *165* LDGTQEGSYM**W**AAINYLLGNL | *423* QYKNYLVGAA**W**PLGCAIDLVS |
| 10. Nucleoside triphosphatase *Pisum sativum* | P52914 (*37*) | *163* IDGTQEGSYL**W**VTVNYALGNL | *423* EYQDAIVEAA**W**PLGNAVEAIS |
| 11. Nucleoside triphosphatase *Arabidopsis thaliana* | AC004138 | *228* ISGSDEGVYA**W**VVANFALGSL | *532* ANQAGDIPLD**W**ALGAFIQQTA |

[a] The conserved tryptophan residues mutated in this work are boxed. Although not all sequences are shown, the boxed Trp residues are also completely conserved in all vertebrate E-type ATPases sequenced to date. The GenBank accession numbers and references for each E-type ATPase are listed. Apyrase conserved region V (ACR V; *38*, *39*).

*Total Membrane Preparation.* For cell membrane preparations, the method of Smith and Kirley (*8*) was used.

*Nucleotidase Assays.* Nucleotidase activity was determined by measuring the amount of inorganic phosphate released from nucleotide substrates at 37 °C using modifications of the technique of Fiske and Subbarow (*16*). Briefly, 1−3 $\mu$g of cell membranes was added to 300 $\mu$L of assay buffer containing either 20 mM MOPS/NaOH (pH 7.4) and 1 mM EGTA for cation-independent nucleotidase activity or 20 mM MOPS/NaOH (pH 7.4), 1 mM EGTA, and 5 mM $MgCl_2$ for cation-dependent nucleotidase activity. The enzyme assay was initiated by the addition of the respective nucleotide to a final concentration of 2.5 mM and incubated at 37 °C for 30−60 min. The reaction was stopped, and the amount of inorganic phosphate that was released was determined colorimetrically. The cation-stimulated nucleotidase activity was determined by subtracting values obtained with EGTA alone from those with 5 mM $MgCl_2$ and chelator. The minor nucleotidase activities that can be detected in control COS cell membranes (typically 2−5% for NDPs and 10−15% for NTPs) were subtracted from all HB6 wild-type and mutant-transfected COS cell activities (it is not known if this minor activity was due to the presence of an endogenous E-type ATPase). Nucleotide hydrolyzing units are expressed both in units of micromoles of $P_i$ liberated per hour (for easy comparison to previously published results) and in the more standard enzyme units of micromoles of $P_i$ liberated per minute (Table 2).

*Protein Assay.* Protein concentrations were determined using the Bio-Rad CB-250 dye binding technique according to the modifications of Stoscheck (*17*, *18*) using bovine serum albumin as the standard.

*Polyclonal Antibody Production.* The production of the polyclonal antiserum to the HB6 carboxy-terminal amino acid sequence was described in a previous publication (*8*).

Table 2: Enzymatic Characterization of the Wild-Type and Mutant HB6 Ecto-Apyrases[a]

| HB6 ecto-apyrase | ATPase $V_{max}$ (units) | ADPase $V_{max}$ (units) | ATPase:ADPase ratio |
|---|---|---|---|
| wild-type | 97 (1.6) | 26 (0.4) | (3.6 ± 0.4):1.0 |
| Trp 187 Ala | inactive | inactive | − |
| Asp 219 Glu | 179 (2.9) | 26 (0.4) | 6.9:1.0 |
| Trp 459 Ala | 187 (3.1) | 35 (0.6) | 5.4:1.0 |
| Asp 219 Glu/ Trp 459 Ala | 154 (2.6) | 16 (0.3) | 9.5:1.0 |

[a] The values shown were obtained by assaying for nucleotidase activity under saturating substrate conditions (2.5 mM). Units are expressed as micromoles of $P_i$ released per hour per milligram of protein. Standard enzyme units (amount of enzyme that will hydrolyze 1 $\mu$mol of substrate in 1 min) are indicated in parentheses. All values are corrected for the differences in expression levels by quantitation of the 79 kDa HB6 band obtained by scanning densitometry of Western blots (see Figure 1). The wild-type ecto-apyrase data represent the average of five independent experiments. The data presented in this table are from experiments independent from those in Figures 5 and 6.

*Electrophoresis and Western Blot Analysis.* SDS−PAGE was performed according to the method of Laemmli (*19*). Control or HB6-transfected COS-1 cell membrane preparations (usually 5 $\mu$g per lane) were boiled for 5 min in SDS sample buffer containing 30 mM dithiothreitol and 8 M urea and were resolved on a 4 to 15% linear gradient SDS−polyacrylamide gel. After electrophoresis, the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting 3 h at 33 V in cold 10 mM CAPS (pH 11) (*20*). After transfer, the Western blot immunoreactivity was detected by chemiluminescence with Amersham ECL reagents as described previously (*8*).

*Preparation of Cells for Immunocytochemistry.* COS-1 cells were transiently transfected with HB6 wild-type cDNA or mutated cDNA as described above. Approximately 16 h post-transfection, cells were removed from the respective

plates by incubation with trypsin and subsequently diluted into a Lab-Tek II 4 chamber slide (Nalge Nunc International) containing complete DMEM. Approximately 24 h after the slide chamber had been seeded, the cells were processed for immunocytochemistry as described below.

*Immunocytochemistry.* The HB6 ecto-apyrase was localized by indirect immunofluorescence using the following protocol. HB6-expressing COS cells were washed twice in PBS, fixed for 6 min in −20 °C methanol, washed again, and blocked in blocking buffer [1% (w/v) bovine serum albumin and 0.25% (w/v) saponin in PBS] for 1 h at room temperature. Anti-peptide polyclonal antiserum was then added to a final dilution of 1:500 in blocking buffer and incubated for 2 h at room temperature. HB6 control cells received an identical dilution of preimmune antiserum. After being washed with blocking buffer, the cells were exposed for 1 h to a 1:250 dilution of secondary antibody (goat anti-rabbit conjugated to CY3, Jackson Immunoresearch) prepared in blocking buffer. After being washed in blocking buffer followed by PBS alone, the slides were coverslipped in Dabco glycerol mounting media, and examined with a Bio-Rad MRC 600 laser scanning confocal microscope and CoMOS image analysis software.

*Cibacron Blue Treatment of COS Cell Membranes.* Cibacron Blue 3GA (Reactive Blue 2) was purified by thin-layer chromatography, quantitated by absorbance at 610 nm using an extinction coefficient of 13 600 $cm^{-1}$ $M^{-1}$ (*21*), and used for inhibition studies of HB6 apyrase activity. For affinity purification of active HB6 ecto-apyrase, Cibacron Blue gel (Affi-Gel Blue) was used. Briefly, COS cell membranes were solubilized with 1% NP-40 in 20 mM MOPS and 2 mM $MgCl_2$ (pH 7.4) at ambient temperature for 10 min. The solubilization mixture was then centrifuged at 13000*g* for 3 min and then filtered through a 0.45 $\mu$m filter to remove any insoluble proteins. The filtrates were diluted 5-fold into 0.1% NP-40, 20 mM MOPS, and 2 mM $MgCl_2$ (pH 7.4) containing Affi-Gel Blue slurry and incubated at ambient temperature for 15 min. The Affi-Gel Blue containing the bound proteins was then washed with the above buffer three times and then twice with 50 mM Tris-HCl (pH 6.8). Bound proteins were removed by boiling the gel slurry in reducing (30 mM DTT) Laemmli sample buffer. SDS−PAGE and Western blotting were conducted as described above.

*Proteolytic Digestion of Ecto-Apyrase Expressing COS Cell Membranes.* Wild-type and mutant ecto-apyrase cell membranes were resuspended in 50 mM Tris-HCl (pH 8.0), and to each respective reaction mixture was added $^1/_{12}$ volume of chymotrypsin, prepared in 1 mM HCl and 2 mM $CaCl_2$. A chymotrypsin:membrane protein ratio of 1:50 was used for each sample. After incubation at ambient temperature for 5 min, the reactions were stopped by addition of an equal volume of Laemmli SDS sample buffer and the samples were immediately boiled for 5 min. SDS−PAGE and Western blotting were conducted as described above.

## RESULTS

*Amino Acid Sequence Analyses of the E-Type ATPases.* The isolation of the 2809 bp human brain ecto-apyrase (HB6) cDNA and deduced 529-amino acid sequence was described previously (*6*). Analysis of the primary sequence suggests

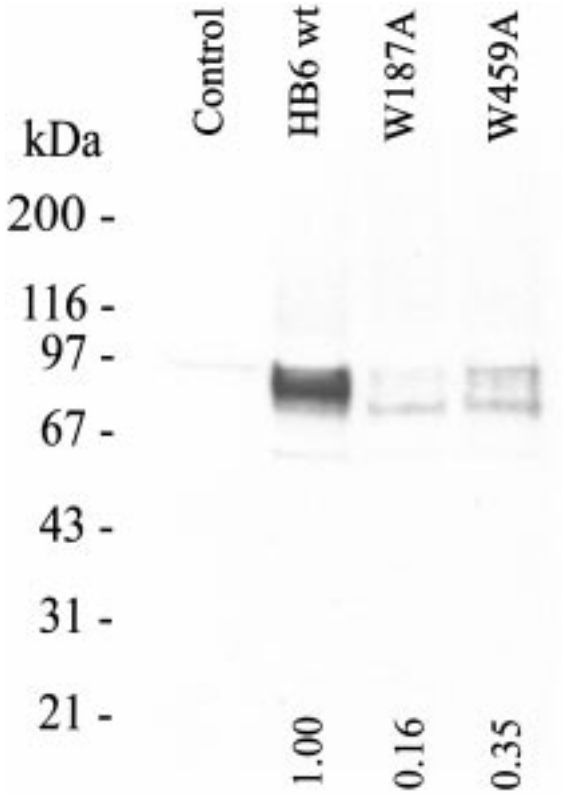


FIGURE 1: Western blot analysis of expressed wild-type and mutant ecto-apyrases using a polyclonal anti-peptide antibody generated against the 15 C-terminal amino acids. Five micrograms of control and HB6 wild-type-, W187A-, and W459A-transfected COS cell membranes were electrophoresed on a 4 to 15% linear gradient gel, transferred to PVDF membrane, and analyzed by Western blotting. The ecto-apyrase is an approximately 79 kDa diffuse glycoprotein band, not present in mock-transfected COS cell membranes. The numbers at the bottom of the blot indicate the normalization values used to correct nucleotidase activity data for the differences in expression levels. Normalization values were obtained by scanning densitometry of the rectangular areas containing each broad immunoreactive protein band and quantitated using AlphaEase version 3.21 from Alpha Innotech Corp. The expression level for the double mutant (D219E/W459A) was also determined (0.47) in the manner described above (not shown). Molecular mass standards are denoted.

that this enzyme is equally related to both the ecto-ATPases and CD39 ecto-apyrases and could perhaps be a progenitor from which these members are derived (*6*). Analyzing regions of diverse E-type ATPase protein sequences after multiple sequence alignments shows that there are several amino acids strictly conserved among each member (Table 1). Careful analyses of the protein sequences also show that there are only two highly conserved tryptophan residues present in these E-type ATPases, corresponding to W187 and W459 in HB6 (Table 1). These two residues were targeted for mutation on the basis of these observations.

*Expression of Wild-Type and Mutant HB6 cDNA in COS-1 Cells.* The tryptophan residues at amino acid positions 187 and 459 were singly replaced with an alanine residue by site-directed mutagenesis and the mutant enzymes expressed in mammalian COS cells. To determine if the site-directed cDNA mutants were expressed in COS cells at levels similar to that of the HB6 wild-type protein, cell membrane preparations of transfected cells were electrophoresed and Western blot analyses conducted on the membrane proteins using an anti-peptide antiserum. As shown in Figure 1, the

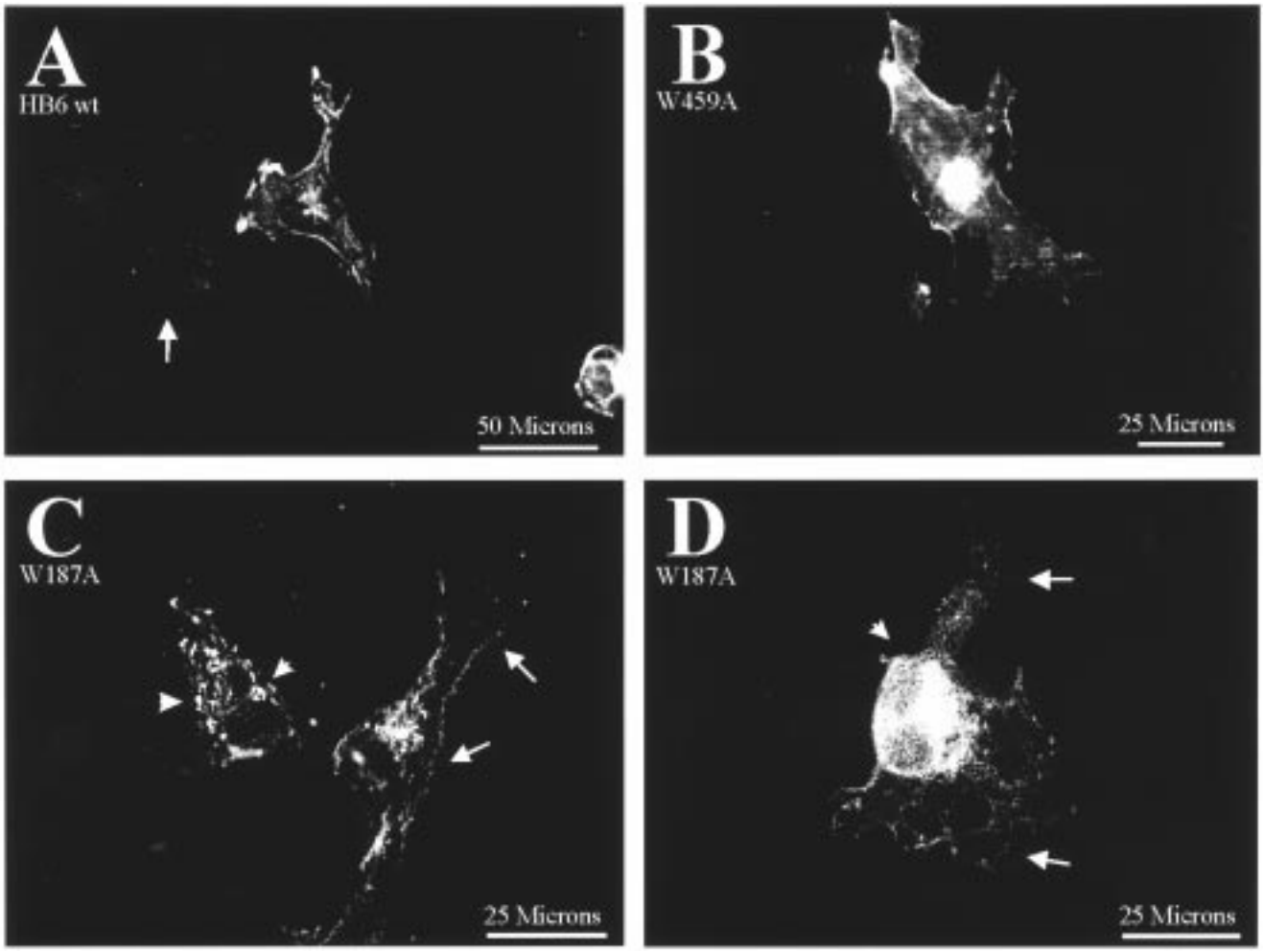


FIGURE 2: Immunolocalization of the wild-type and mutant ecto-apyrase in mammalian COS cells. COS cells were transfected with the HB6 ecto-apyrase or Trp mutant cDNAs and analyzed 48 h later using indirect immunofluorescence as described in Experimental Procedures. In the wild-type-transfected cells (A), the ecto-apyrase is seen to be distributed throughout the cell surface and clearly visible in the cell membrane border and cell processes. A nontransfected cell (nonfluorescent) is denoted by an arrow in panel A. W459A mutant-transfected cells exhibit a similar enzyme distribution, with staining along the cell periphery and extensions (B). In contrast, the W187A mutant exhibited poor cell membrane staining (arrows in panels C and D denote poorly stained cell membranes) as well as a lower intensity of cell membrane staining. Two representative W187A mutant cells are shown, both containing a large amount of "trapped" intracellular immunoreactivity (arrowheads). No immunoreactivity was observed when testing mock-transfected cells or transfected cells using the preimmune serum (not shown).

approximately 79 kDa diffuse ecto-apyrase glycoprotein band was consistently detected in both mutants at levels lower than that of the wild-type protein. As assessed by scanning densitometry, the W187A mutant was expressed at a level nearly 6-fold lower than that of the wild-type enzyme, while the expression level of the W459A mutant was reduced to a lesser extent (2.9-fold). These results are unlike our previous report, which showed little difference in the expression levels of several conserved Asp and Gly mutants compared to nonmutated HB6 (*8*). No immunoreactivity was detected in mock-transfected COS cell membranes (Figure 1).

*Enzymatic Characterization of the Wild-Type HB6 and Mutant Ecto-Apyrase Proteins.* To examine the effects of these single amino acid changes on the activity of the HB6 ecto-apyrase, wild-type and mutant-transfected COS cells were harvested as described and the cell membrane preparations examined for nucleotide hydrolysis activity. Mutation of tryptophan 187 to alanine resulted in an ecto-apyrase completely devoid of nucleoside diphosphate and triphosphate hydrolyzing ability. In contrast, mutation of tryptophan 459 to alanine resulted in an enzyme with an increased ability to hydrolyze both ATP and ADP, albeit to different extents (Table 2). Since ATP hydrolysis was stimulated to a greater degree than ADP hydrolysis, the ATPase:ADPase ratio changed from the wild-type average of 3.6:1.0 to 5.4:1.0 (Table 2). No significant changes in enzymatic activity were detected after preincubation of transfected cell membranes with mild detergent (0.02% saponin), demonstrating that there was no latent E-type ATPase activity (data not shown).

*Cellular Localization of the HB6 Ecto-Apyrase and Tryptophan Mutants.* The greatly reduced expression level of the inactive W187A mutant was not observed for previous mutations (*8*) and was unexpected. To examine the cellular localization of the expressed wild-type and mutant enzymes in transfected cells, wild-type and W187A and W459A mutant HB6 cDNAs were transiently transfected into mammalian COS cells and analyzed by immunocytochemistry. As shown in the confocal immunofluorescence images shown in Figure 2 (panel A), the wild-type glycosylated ecto-apyrase was detected by diffuse staining over the cell and along the borders of the cells. Immunoreactivity can be seen along the ruffles and thin processes of the cells, consistent with

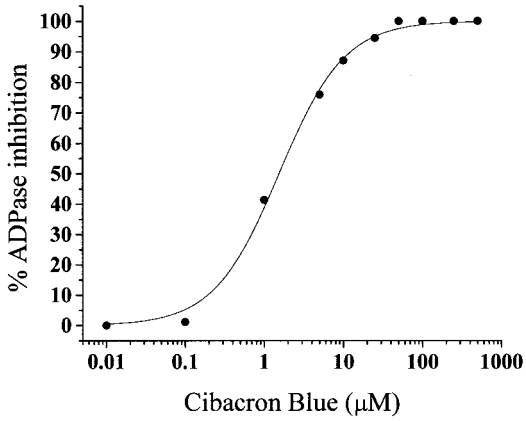


FIGURE 3: Inhibition of wild-type HB6 ecto-apyrase by purified Cibacron Blue. Three micrograms of wild-type apyrase expressing COS cell membranes was incubated with various concentrations of Cibacron Blue for 10 min at 37 °C prior to initiation of ADP hydrolysis by the addition of a final concentration of 2.5 mM ADP. Values are expressed as the percent inhibition of the ecto-apyrase activity.

localization in the cell membrane. Similarly, the fully active W459A mutant displayed immunoreactive protein also localized to the borders and extensions of the cell membrane (panel B). In contrast, the W187A mutation displayed a higher amount of apparent intracellular staining (panels C and D). Some faint immunoreactivity was observed at the cell membrane, but the majority of the enzyme was detected intracellularly, presumably "trapped" in vesicles. No immunoreactivity was evident in mock-transfected cells or when probing transfected cells with preimmune sera (not shown).

*Cibacron Blue 3GA (Reactive Blue 2) Inhibition and Affinity Purification of HB6 Ecto-Apyrase.* On the basis of the lack of enzymatic activity and routing to the cell membrane, we hypothesized that the W187 residue may be important for the tertiary structure of the enzyme, and thus, the W187A mutant may be misfolded and subsequently trapped intracellularly. This would explain the reduced expression level, the loss of enzymatic activity, and the abundance of intracellular staining. To further test this hypothesis, we examined the ability of the HB6 enzyme to bind to the triazine dye Reactive Blue 2 (Cibacron Blue). Cibacron Blue has been previously shown to bind to and inhibit the enzymatic activity of several E-type ATPases (*22*, *23*) and other nucleotide-dependent enzymes (*24*, *25*). To examine the effect of this reactive nucleotide analogue on the wild-type HB6 ecto-apyrase activity, COS cell membranes were incubated with TLC-purified Cibacron Blue and assayed for enzymatic activity. Cibacron Blue inhibited the HB6 ecto-apyrase ADPase activity with an $IC_{50}$ value of 1.5 $\mu$M (Figure 3). An apparent Hill coefficient of 1.06 $\pm$ 0.06 suggests a single class of inhibitor binding sites. The inhibitory binding of the Cibacron Blue compound was also used as a measure of proper tertiary structure of the HB6 ecto-apyrase. As shown in Figure 4 (panel A), active HB6 wild type and the W459A mutant were effectively bound by the affinity matrix Affi-Gel Blue. Enzymatically inactive HB6 wild type (boiled) and the inactive W187A mutant, however, were not bound by the ligand. This demonstrates that the W187A mutant enzyme is not in the proper conformation for binding the Cibacron Blue ligand, and suggests that this mutant is not folded properly.

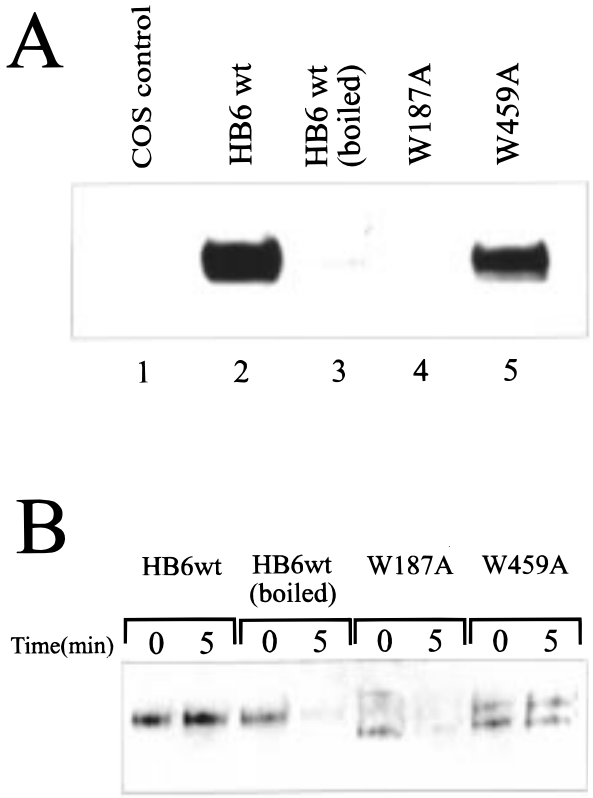


FIGURE 4: Western blot analysis of expressed ecto-apyrases. (A) Reactive Blue 2 affinity purification of wild-type and mutant enzymes. (B) Chymotryptic digestion of wild-type and mutant ecto-apyrases. (A) Control and wild-type HB6- and mutant HB6-transfected COS cell membranes were solubilized with 1% NP40 and affinity purified using Cibacron Blue matrix as described in Experimental Procedures. The bound proteins were analyzed by Western blotting with the C-terminal anti-peptide antibody. Equal amounts (5 $\mu$g) of control and HB6 wild-type membranes were solubilized, but varying amounts of mutant membranes were solubilized depending upon the expression level of the particular mutation (i.e., greater amounts of the COS cell membranes containing mutant ecto-apyrase were solubilized to normalize for lower expression levels, which would result in similar Western band intensities if the mutants had bound to the affinity matrix). (B) HB6 wild-type, W187A, and W459A mutant ecto-apyrase cell membranes were incubated at a chymotrypsin:protein ratio of 1:50 for 0 and 5 min at ambient temperature. The reactions were then stopped by boiling in SDS sample buffer and the mixtures analyzed by SDS−PAGE and immunoblotting. Equal amounts of HB6 wild-type membranes (0.5 $\mu$g) were subjected to digestion, but varying amounts of mutant membranes (based on the normalization values calculated in Figure 1) were subjected to digestion which resulted in similar band intensities at time zero. The appearance of the wild-type and mutant ecto-apyrase protein bands varied depending upon the quantity of protein loaded, electrophoresis conditions, etc. (compare to panel A).

*Protein Folding As Studied by Proteolytic Digestion.* As a further test of the proper folding and stability of the HB6 ecto-apyrase and tryptophan mutants, chymotryptic digestion was employed. This method provides information about the accessibility of phenylalanine, tryptophan, and tyrosine residues to proteolytic attack, and indirectly about the folding of the protein. Various concentrations of HB6 wild type and Trp mutant cell membranes were incubated with chymotrypsin at room temperature and analyzed by Western blotting for sensitivity to the protease. As shown in Figure 4 (panel B), the HB6 wild-type ecto-apyrase exhibits little proteolysis after 5 min. In contrast, denatured HB6 wild type (prepared

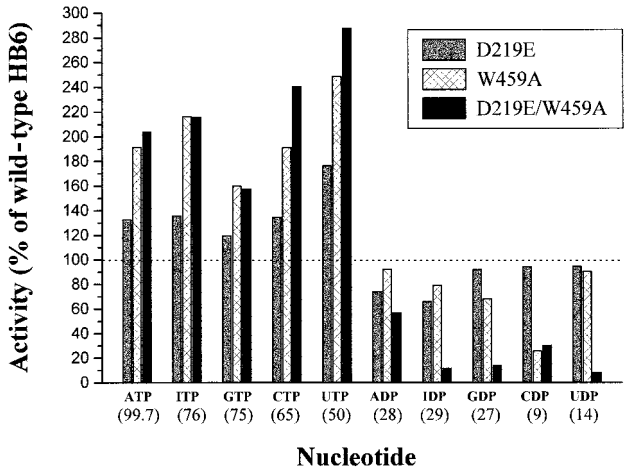


FIGURE 5: Nucleoside di- and triphosphatase activity of the D219E and W459A mutants and the D219E/W459A double mutant. Nucleotidase activities were determined for the HB6 wild-type ecto-apyrase and the three active mutants using each respective nucleotide at a concentration of 2.5 mM as described in Experimental Procedures. ATP was the preferred nucleotide substrate for the wild-type enzyme under these conditions. The specific activity (in units of micromoles per hour per milligram, $n = 3$) of the wild-type HB6 enzyme is indicated in parentheses below each respective nucleotide. Each of the three mutants was a more active NTPase when compared to the wild-type ecto-apyrase. NDPase activity was reduced to varying degrees among these mutants as well. This phenomena caused a marked shift in the NTPase:NDPase ratios (see Figure 6).

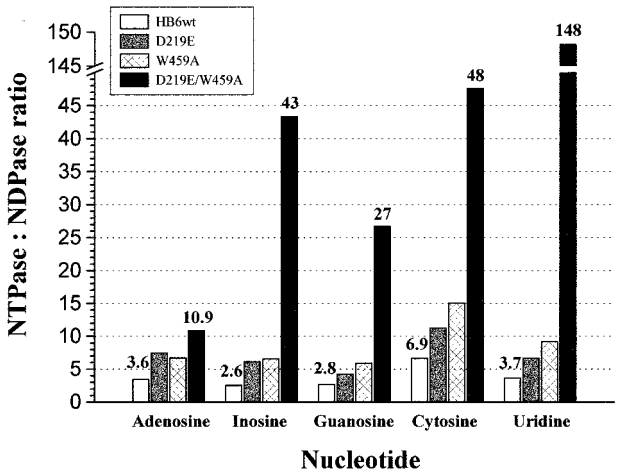


FIGURE 6: Nucleotidase ratios (NTPase:NDPase) of wild-type HB6 ecto-apyrase and mutants using several purine and pyrimidine nucleotides. The wild-type HB6, as well as the D219E, W459A, and D219E/W459A mutant cDNAs, was transfected into mammalian COS cells and assayed as described in Experimental Procedures using each respective substrate at a concentration of 2.5 mM. The values obtained were corrected for background COS cell activity and expression levels determined by Western blotting. The HB6 wild type (white bars) possessed similar NTPase:NDPase ratios (2.6:1.0 to 6.9:1.0), regardless of the nucleotide being examined. Both the D219E (gray bars) and W459A (hatched bars) mutations increased the NTPase:NDPase ratios for all nucleotides, by increasing the rate of NTP hydrolysis while subsequently decreasing the rate of NDP hydrolysis (see Figure 5). Most striking was the NTPase:NDPase hydrolysis ratio for the double mutant D219E/W459A (black bars). The respective nucleotidase ratios for both the wild-type HB6 and D219E/W459A double mutant are indicated above the columns.

by boiling for 5 min prior to incubation with the protease) exhibited a marked sensitivity to chymotrypsin, with the majority of the ecto-apyrase digested after 5 min. Similarly, the inactive W187A mutant was nearly completely digested after proteolysis for 5 min, suggesting an unfolded tertiary structure very unlike the native ecto-apyrase. The enzymatically active W459A mutant, however, exhibited little digestion after chymotrypsin treatment, similar to the wild-type ecto-apyrase.

*D219E/W459A Double Mutant.* The unique nucleotide hydrolyzing characteristics of the W459A mutant (i.e., preferential hydrolysis of ATP over ADP compared to that of the wild type) and a previously characterized mutant (D219E) with similar characteristics (*8*) led us to construct a double mutant of the HB6 apyrase. This mutant D219E/W459A possessed characteristics similar to those of both the D219E and W459A single mutants with the exception of the nucleotide triphosphate to diphosphate hydrolysis ratios. As shown in Table 2, the double mutant also hydrolyzed ATP to a greater extent than the wild-type enzyme but was less efficient at the hydrolysis of ADP. Hence, this caused the ATPase:ADPase ratio to shift to nearly 11:1.0 (Table 2).

*Nucleotide Hydrolysis Ratios for the Wild-Type HB6 Ecto-Apyrase and Mutants.* To examine what effect these mutations had on the ability of the enzyme to hydrolyze other nucleoside tri- and diphosphates, wild-type and mutant-transfected COS cell membranes were assayed in the presence of various nucleotide substrates. Characteristic of the E-type ATPases, the HB6 wild-type ecto-apyrase was able to hydrolyze a broad range of nucleoside tri- and diphosphates (Figure 5). ATP was the preferred substrate for the wild-type enzyme under these conditions. Interestingly, both the D219E and W459A single mutations and the D219E/W459A double mutation had a significant effect on the differential ability to hydrolyze nucleoside triphosphates versus diphosphates. These single mutations caused similar increases in nucleoside triphosphate hydrolysis activity and decreases in the rates of nucleoside diphosphate hydrolysis. This is most evident when NTP to NDP hydrolysis ratios of the mutants were examined. As seen in Figure 6, the wild-type HB6 enzyme had an ATPase:ADPase activity of 3.6:1.0. In contrast, the D219E and W459A single mutations increased the NTP:NDP hydrolysis ratios for each nucleotide that was examined (for ATP, to 6.9 and 5.4, respectively). Most strikingly, the double mutant D219E/W459A caused a significant change in the ratio of NTP:NDP hydrolysis. Cytosine and inosine nucleotide ratios were increased to 48:1.0 and 43:1.0, respectively. This is compared to the HB6 wild-type ratios of 6.9:1.0 and 2.6:1.0 respectively, for these two nucleotides. Although the adenosine and guanosine purine nucleotide ratios were somewhat less dramatic (11:1.0 and 27:1.0, respectively), they were still very much increased. The most impressive change in nucleotide ratios was evident with uridine nucleotide (148:1.0 for D219E/W459A compared to 3.7:1.0 for wild-type apyrase).

*Azide Inhibition of the HB6 Ecto-Apyrase and the D219E/W459A Double Mutant.* The sensitivity of the ecto-apyrase to sodium azide was examined by preincubating the wild type and the D219E/W459A double mutant in various inhibitor concentrations for 10 min at 37 °C. As shown in Figure 7, the ATP and ADP hydrolyzing activity of the wild-type ecto-apyrase was relatively sensitive to azide incubation.

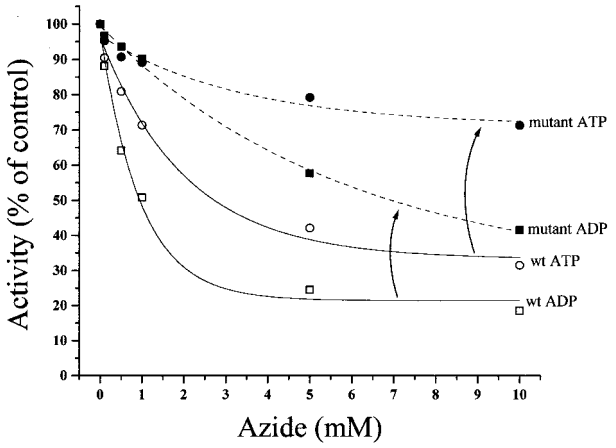


FIGURE 7: Effect of sodium azide on ecto-apyrase ATPase and ADPase activity from wild-type- and D219E/W459A double mutant-transfected cell membranes. Cell membranes from wild-type HB6- or D219E/W459A double mutant-transfected COS-1 cells were harvested as described and assayed in the presence of various concentrations of $NaN_3$ using 2.5 mM ATP or ADP as the substrate. The inhibitor was preincubated with the membranes for 10 min prior to initiation of the hydrolysis reaction by addition of nucleotide. ATPase activity is represented by circles (white for the wild type and black for the mutant), and ADPase activity is represented by squares (white for the wild type and black for the mutant). Note that the wild-type ecto-apyrase ATPase and ADPase activities are more sensitive to inhibition by sodium azide at all the concentrations that were tested (solid lines). Nearly 30 and 50% of the ATPase and ADPase activity of the wild-type ecto-apyrase was inhibited by treatment with 1 mM azide, in contrast to the D219E/W459A mutant which lost only approximately 10% of its ATPase and ADPase activity. The curves are fits to a single-exponential decay model. Arrows make clear the effect of the double mutation on ATPase and ADPase activities and point from the wild-type (solid lines) to the D219E/W459A mutant (dotted lines) nucleotidase curves.

Approximately 30% of the wild-type ATPase and 50% of the wild-type ADPase activity were inhibited by incubation with 1 mM azide. In contrast, the extent of inhibition of the double mutant by azide was substantially decreased (approximately 10% inhibition at 1 mM azide). The W459A single mutant displayed a similar decrease in azide sensitivity (data not shown).

## DISCUSSION

In this study, we report the functional importance of two highly conserved amino acids of the E-type ATPases. Trp 187 and Trp 459 (numbered according to the HB6 ecto-apyrase primary sequence) were singly mutated to alanine and analyzed for their effects on the expressed enzymes. Surprisingly, each mutation yielded significantly different results, either completely inactivating the ecto-apyrase (W187A) or stimulating the NTPase activity (W459A).

Both of these Trp point mutations led to a reduced level of expression in mammalian cells compared to that of the wild-type ecto-apyrase. The W187A and W459A mutations were reduced 6- and 2.9-fold, respectively. In vivo immunofluorescent analyses of wild-type and mutant-transfected COS cells displayed very different cellular localizations. The HB6 wild type and W459A mutant each appeared to be distributed throughout the cellular membrane border and processes. In contrast, for the W187A mutant, immunofluorescent staining was visibly concentrated in the cell interior. A much lower level of staining was observed in the cellular periphery. This suggests that the W187A mutant is not processed and trafficked properly, and is retained by the cell. Also, the expressed W187A mutant enzyme did not bind to a Cibacron Blue affinity matrix, similar to the denatured (boiled) wild-type HB6 apyrase. In contrast, the wild type and W459A mutant were equally bound by this affinity matrix, further suggesting that the W187A mutant is improperly folded. Furthermore, the W187A mutant exhibited an unusual sensitivity to chymotryptic digestion, similar to boiled, denatured HB6 wild-type ecto-apyrase, likely due to an inability of the mutant enzyme to fold into and maintain a protease-resistant native conformation.

It is not unprecedented that a single Trp mutation would result in the inactivation of an enzyme's activity, either directly or indirectly. In tissue-type plasminogen activator, mutation of a single tryptophan residue disrupted the ligand binding properties and stability of the native protein (*26*), while a single Trp mutation in creatine kinase caused a loss of enzymatic activity by dissociation of the (active) dimer form into (inactive) monomers (*27*). There is also precedence in the literature for amino acid point mutations resulting in the misfolding of proteins and subsequent retention and degradation by the cellular machinery. For example, mutation of single amino acids of a chloride channel known as CFTR leads to a misfolding of the protein and retention in the endoplasmic reticulum (*28*). Similarly, a single tryptophan mutation (W556S) of the plasma membrane LDL receptor (found in some patients suffering from familial hypercholesterolemia) has been shown to result in complete retention of the protein in the endoplasmic reticulum of transfected COS cells (*29*). Several studies with the yeast plasma membrane proton ATPase have shown that point mutations could result in misfolding of the enzyme, susceptibility to proteolysis, a reduced expression level and enzymatic activity, and retention within the cell (as assessed by indirect immunofluorescence) (*30*, *31*). Thus, the effects mediated by the W187A mutation reported here are very similar to those effects caused by point mutations in the yeast plasma membrane ATPase.

It is not known why the W459A mutant was expressed at a lower level, since the enzyme is enzymatically active and appeared to be correctly folded and distributed to the cellular membrane similar to the wild-type HB6. Of note is the fact that the D219E mutant and D219E/W459A double mutant were also expressed at reduced levels (1.3- and 2.1-fold, respectively). This is in contrast to our previous report in which we examined the mutagenesis of two conserved Asp and Gly residues of the E-type ATPases (*8*). The expression levels of these enzymatically inactive mutants were nearly equivalent to that of the wild type. We speculate that the increased rate of NTP hydrolysis of the three mutations described in this paper may have repressed their own expression level due to interference with COS cell metabolism caused by hydrolysis of intracellular NTPs during processing of the mutant ecto-ATPases. Possibly related to this, we have noted that bacteria containing plasmids encoding some E-type ATPases grow (divide) slowly, apparently due to very low levels of expression of the E-type ATPases, even though the plasmids containing the E-type ATPase cDNAs are not bacterial expression vectors (unpublished observations).

One very interesting finding reported here is the near conversion of an ecto-apyrase to an ecto-NTPase mediated by only two amino acid point mutations. The wild-type HB6 ecto-apyrase hydrolyzes NTPs and NDPs at ratios between 2:1 and 6:1, depending upon the particular nucleotide that is being examined. With the construction of the double mutant D219E/W459A described in this report, the NTP:NDP hydrolysis ratio ranges from a low of 10.9:1 for adenosine nucleotides to a high of 148:1 for the uridine nucleotides. These dramatic effects were not observed for each point mutation analyzed singly; it is only apparent when the two mutations are combined (see Figure 6). In addition, the D219E/W459A double mutant exhibited a reduced sensitivity to sodium azide inhibition (Figure 7). Sodium azide is an inhibitor of the E-type ATPases that has been shown to preferentially inhibit the ecto-apyrases relative to the ecto-ATPases (*2*, *11*, *32*). Our findings show that the double mutant generated here has characteristics indicative of an ecto-ATPase, since this mutant exhibits a greatly reduced sensitivity to this inhibitor. Thus, it is possible, via two point mutations, to modify an ecto-apyrase to an enzyme with properties more like those of an ecto-ATPase. Although the aspartic acid and tryptophan residues mutated in this study are conserved in both the ecto-ATPases and ecto-apyrases (and thus are not the endogenous amino acid residues distinguishing the enzymatic activities of the two families), this report demonstrates the feasibility of converting an ecto-apyrase to an ecto-ATPase by mutating only a few amino acids.

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