

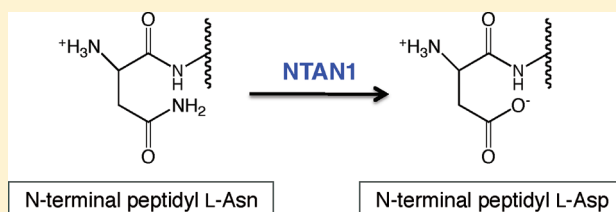
Expression and Biochemical Characterization of the Human Enzyme N-Terminal Asparagine Amidohydrolase

Jason R. Cantor,[†] Everett M. Stone,[†] and George Georgiou^{*,†,‡}

[†]Department of Chemical Engineering and [‡]Institute for Cellular and Molecular Biology, Section of Molecular Genetics and Microbiology, and Department of Biomedical Engineering, University of Texas, Austin, Texas 78712, United States

S Supporting Information

ABSTRACT: The enzymatic deamidation of N-terminal L-Asn by N-terminal asparagine amidohydrolase (NTAN1) is a feature of the ubiquitin-dependent N-end rule pathway of protein degradation, which relates the in vivo half-life of a protein to the identity of its N-terminal residue. Herein, we report the bacterial expression, purification, and biochemical characterization of human NTAN1 (hNTAN1). We show here that hNTAN1 is highly selective for the hydrolysis of N-terminal peptidyl L-Asn but fails to deamidate free L-Asn or L-Gln, N-terminal peptidyl L-Gln, or acetylated N-terminal peptidyl L-Asn. Similar to other N-terminal deamidases, hNTAN1 is shown to possess a critical Cys residue that is absolutely required for catalysis, corroborated in part by abolishment of activity through the Cys75Ala point mutation. We also present evidence that the exposure of a conserved L-Pro at the N-terminus of hNTAN1 following removal of the initiating L-Met is important for the function of the enzyme. The results presented here should assist in the elucidation of molecular mechanisms underlying the neurological defects of NTAN1-deficient mice observed in other studies, and in the discovery of potential physiological substrates targeted by the enzyme in the modulation of protein turnover via the N-end rule pathway.



Targeted proteolytic degradation is essential for the regulation of a variety of cellular processes by achieving the correct balance between protein folding and the degradation of misfolded or damaged proteins.¹ In eukaryotes, the ubiquitin (Ub)—proteasome system (UPS) is the primary pathway for the time-specific elimination of targeted proteins and the N-end rule pathway is a subset of this system that relates the in vivo half-life of a protein to the identity of its N-terminal residue.^{2,3} Degradation signals (degrons) that can be targeted by the N-end rule pathway belong to one of two discrete subsets: N-terminal degrons (N-degrons) and internal degrons.^{2,4} The pathway has been observed in all organisms examined, from prokaryotes to eukaryotes, though the former exists in a Ub-independent manner.⁵ In eukaryotes, an N-degron consists of three determinants: a destabilizing N-terminal residue (Figure 1), one (or more) internal Lys residues (the site of poly-Ub chain formation), and a conformationally flexible region in the vicinity of this internal Lys.^{6,7} An N-degron can be generated from a precursor, called a pre-N-degron, through specific protease-mediated post-translational modifications.⁸

The N-end rule has a hierarchical structure (Figure 1). In eukaryotes, N-terminal L-Asn and L-Gln function as tertiary destabilizing residues through their deamidation by N-terminal amidohydrolases (Nt-amidases) into the secondary destabilizing N-terminal residues L-Asp and L-Glu, respectively.^{9–11} The secondary destabilizing activity of N-terminal L-Asp and L-Glu requires their conjugation to L-Arg by the ATE1-encoded Arg-tRNA protein transferase (R-transferase).^{12–14} In mammals and

other eukaryotes that produce nitric oxide (NO), N-terminal L-Cys can also function as a tertiary destabilizing residue through its oxidation to Cys-sulfinate or Cys-sulfonate in an O₂- or NO-dependent manner, whereby the oxidized L-Cys can be arginylated by ATE1.^{8,13} Together with other primary destabilizing residues, the N-terminal L-Arg allows for protein substrate recognition by specific E3 Ub ligases and subsequent degradation.¹⁵

A number of genetic studies with knockout mice have implicated the involvement of the N-end rule pathway in cardiac development and signaling, angiogenesis,^{12,16} meiosis,¹⁷ DNA repair,¹⁸ neurogenesis,¹⁶ pancreatic functions,¹⁹ learning and memory,^{10,20} female development,¹⁷ muscle atrophy,²¹ and olfaction.²² Other functions of the pathway include (i) selective degradation of misfolded proteins,²³ (ii) sensing of nitric oxide, oxygen, and heme,^{12–14,24} (iii) regulation of short peptide import,^{25,26} (iv) fidelity of chromosome segregation,²⁷ (v) regulation of apoptosis through NTAN-1-mediated degradation of the caspase-mediated C-terminal fragment of *Drosophila* inhibitor of apoptosis 1 (DIAP1),²⁸ and (vi) regulation of leaf senescence in plants.²⁹

In contrast to *Saccharomyces cerevisiae*, which possesses a single enzyme (Nta1) that can deamidate either L-Asn or L-Gln at the N-terminus of a protein substrate,⁹ vertebrates possess one distinct enzyme for the specific deamidation of N-terminal L-Asn

Received: November 16, 2010

Revised: March 3, 2011

Published: March 04, 2011

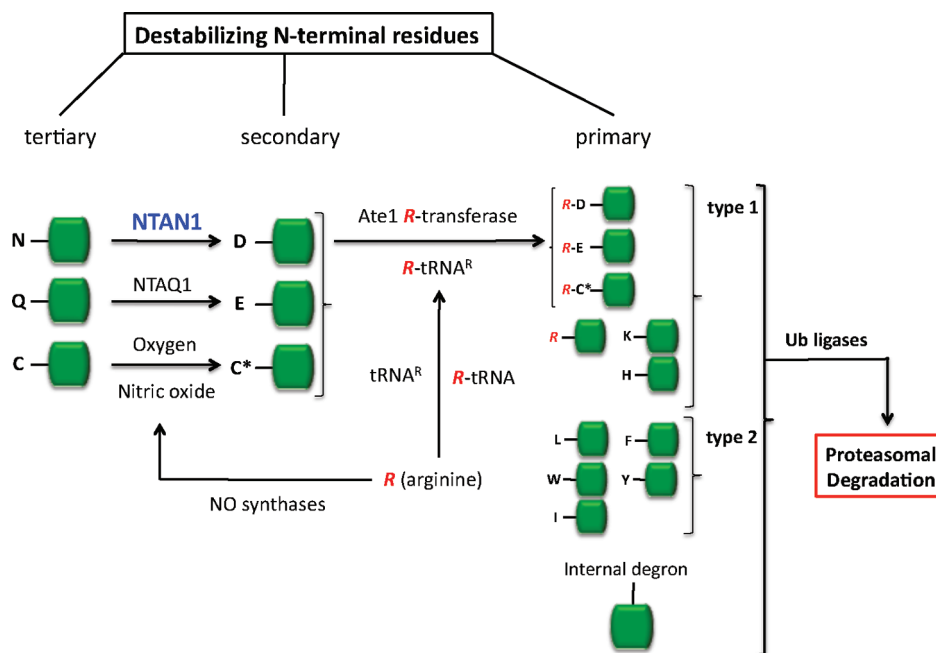


Figure 1. Mammalian N-end rule pathway. N-Terminal residues are denoted by single-letter abbreviations. Green boxes denote the remainder of the protein substrate. C* denotes an oxidized N-terminal Cys (either Cys-sulfinate or Cys-sulfonate). The Cys oxidation requires nitric oxide (NO) and oxygen or its derivatives. “Tertiary”, “secondary”, and “primary” denote mechanistically distinct subsets of destabilizing N-terminal residues (see the introductory section). The enlarged and blue-colored NTAN1 denotes the N-terminal asparagine amidohydrolase characterized in this study.

(NTAN1) and one for the specific deamidation of N-terminal L-Gln (NTAQ1).¹¹ NTAN1-deficient mice were found to display neurological defects such as impairment of spontaneous activity and spatial memory and defects in socially conditioned exploratory behavior.¹⁰

In an earlier study, an enzyme with N-terminal L-Asn hydrolase activity from porcine liver was purified and partially characterized.³⁰ A gene encoding a putative NTAN1 enzyme, *Ntan1*, was subsequently isolated from a mouse cDNA library and shown to complement a *S. cerevisiae* Δ *nta1* mutant strain.³¹ Herein, we describe the expression and biochemical characterization of the human NTAN1 (hNTAN1). Among our results, we have identified an essential L-Cys residue that is critical for the hydrolysis of N-terminal L-Asn by hNTAN1 and thus may act as a nucleophile in catalysis, in analogy with both the *S. cerevisiae* Nta1⁹ and the dedicated murine N-terminal L-Gln deamidase NTAQ1.¹¹

EXPERIMENTAL PROCEDURES

Materials. Synthetic oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Restriction endonucleases, *Vent* DNA polymerase, T4 DNA ligase, and dNTPs were from New England Biolabs (Ipswich, MA). Nickel-nitrilotriacetic acid (Ni^{2+} -NTA) resin was from QIAGEN (Valencia, CA). Strep-Tactin Superflow high-capacity suspension and D-desthiobiotin were obtained from IBA GmbH (St. Louis, MO). Anti-FLAG M2 magnetic beads, FLAG peptide, Val⁵-angiotensin II (AII), and Asn¹Val⁵-angiotensin II (N¹-AII) were purchased from Sigma-Aldrich (St. Louis, MO). Gln¹Val⁵-angiotensin II (Q¹-AII), Ac-Asn¹Val⁵-angiotensin II (Ac-N¹-AII), and a 9mer peptide (N¹-DIAP) corresponding to the N-terminus of the caspase-mediated C-terminal fragment of DIAP1²⁸ were purchased from Abgent Inc. (San Diego, CA).

Difco 2xYT growth medium was from Becton Dickinson (Franklin Lakes, NJ). All other reagents were obtained from Sigma-Aldrich unless otherwise noted.

Construction of hNTAN Expression Plasmids. A gene encoding the human protein N-terminal asparagine amidohydrolase (hNTAN1) (933 bp) with a fused N-terminal His₆ affinity tag was synthetically assembled using a set of codon-optimized overlapping oligonucleotides designed with DNA-Works as described previously.³² *Nco*I and *Eco*RI restriction sites were incorporated into the outermost sense and antisense oligonucleotides, respectively. Sense primers (Table S1 of the Supporting Information) were designed (i) to introduce the hNTAN1 initiation codon within an *Nco*I site, thereby eliminating the N-terminal His₆ affinity tag (NTAN-N-NoHis), and (ii) to modify the ribosome binding site (RBS) and incorporate an upstream *Xba*I site to improve the translation initiation rate of hNTAN1 in *Escherichia coli* (NTAN-RBS2). Antisense primers were designed to append a C-terminal His₆ affinity tag (NTAN-C-H), a C-terminal strepII affinity tag (NTAN-C-SII), and a C-terminal FLAG-strepII tandem affinity tag (NTAN-C-FSII), all followed by a *Not*I site. Lastly, a sense primer (NTAN-MP-RBS2) was designed to eliminate the GGA codon immediately following the initiation ATG codon (within an *Nco*I site) that was incorporated to maintain a correct open reading frame in the cassettes described above. Individual polymerase chain reaction (PCR) products for each cassette were digested accordingly and ligated into pET28-a (Novagen). The RBS and the N- and C-terminal amino acid sequences of each hNTAN1 construct created are listed in Table 1. *E. coli* BL21(DE3) was used for protein expression and purification. Genes encoding hNTAN1 mutants C75A, C75S, C75T, and C160A were constructed by overlap extension PCR using the pNTAN2FLAGSI plasmid as a template and the primer pairs listed in Table S1 of the Supporting Information.

Table 1. hNTAN1 Constructs and Their Relevant Expression Characteristics in *E. coli* BL21(DE3)^a

plasmid	RBS	amino acid sequence	hNTAN1 purity (%)
pHisNTAN ^b	AAGGAGA	MGGSSHHHHHHSSGPLLV...SGPS	30
pNTANHis	AAGGAGA	MGPLLV...SGPSGGSSHHHHHH	30
pR1NTANSII	AAGGAGA	MGPLLV...SPGSSAWSHPQFEK	30
pR2NTANSII	AACAATAATAAGGAGATAAGAA	MGPLLV...SPGSSAWSHPQFEK	70
pNTANFLAGSI	AACAATAATAAGGAGATAAGAA	MGPLLV...SPGSGGSDYKDDDDKSAWSHPQFEK	>98
pNTAN2FLAGSI	AACAATAATAAGGAGATAAGAA	MPLLV...SPGSGGSDYKDDDDKSAWSHPQFEK	>98

^a The sequences of the sense and antisense primers used for the modified termini and RBS are found in Table S1 of the Supporting Information. The resultant amino acid sequences are shown; residues following the ellipses are indicative of the C-terminus of the sequence. The purity of hNTAN1 was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, where band intensities were estimated by densitometry imaging (Quantity 1, Bio-Rad). ^b Trace activity detected for His₆-hNTAN1 through addition of the final purified product (10 μ g of total protein) to 1 mM N¹-AII for 90 min at 37 °C in 50 mM Tris-HCl and 150 mM NaCl (pH 7.5). Reactions were quenched with 12% TCA (w/v) and then evaluated by HPLC for the presence of a peak corresponding to the expected product, AII. For all other plasmids, the presence of hNTAN1 activity could be detected through the addition of final purified product (10–100 ng of total protein) to 100 μ M N¹-AII for 1 min at 37 °C in the same buffer and evaluated similarly.

Expression and Purification of Recombinant hNTAN1.

E. coli BL21(DE3) cells transformed with pNTAN2FLAGSI were cultured overnight at 37 °C in 2xYT medium supplemented with 30 μ g/mL kanamycin and used to inoculate 500 mL of fresh medium (1:100 dilution). When the absorbance at 600 nm (A_{600}) reached 0.5–0.7, the cells were transferred to 25 °C and allowed to equilibrate for 20 min, at which point the culture was supplemented with IPTG to a final concentration of 1 mM to induce protein expression. After incubation for 16 h at 25 °C, the cells were harvested by centrifugation at 10000g for 10 min. hNTAN1 was subsequently purified by tandem strepII affinity chromatography and immobilized anti-FLAG as follows. Cell pellets were resuspended in 15 mL of buffer W [100 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA (pH 8) on ice] and then lysed by three passes through a French pressure cell. Debris was removed by centrifugation at 40000g for 45 min, and the resulting supernatant (soluble fraction) was decanted and applied to a 5 mL polypropylene column (Pierce) packed with 300 μ L of strep-tactin superflow high-capacity resin.³³ The column flow-through was collected and reappplied to the column for three total passes. The resin was then washed with 30 bed volumes of buffer W and 10 bed volumes of buffer W2 (buffer W containing 100 μ M D-desthiobiotin) before the resin was incubated with 10 bed volumes of buffer E (buffer W containing 2.5 mM D-desthiobiotin) and the eluant was collected. All purification steps were performed at 4 °C. The elution fraction was applied to an Amicon Ultra 10K MWCO filter, concentrated to a final volume of 1 mL in buffer W, and then incubated with 200 μ L of anti-FLAG M2 magnetic beads for 1 h at room temperature with gentle rotation. The magnetic beads were then collected using a magnetic separator (DynaL MPC-S, Invitrogen) to remove the supernatant and washed with 30 bed volumes of buffer W before the purified protein was collected by competitive elution with 5 bed volumes of buffer W containing 100 μ g/mL FLAG peptide by gentle rotation for 10 min at room temperature. The final eluant was applied to an Amicon Ultra 10K MWCO filter, concentrated to a final volume of 100 μ L in storage buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.5 mM DTT (pH 7.5)], and stored at 4 °C. Procedures for the purification of recombinant hNTAN1 fused to either a His₆ or a strepII affinity tag can be found in the Supporting Information. Protein concentrations were determined using a calculated extinction coefficient of 32430 M⁻¹ cm⁻¹.³⁴

Activity Assays. For kinetic analyses, hNTAN1 (40–50 nM purified enzyme) was incubated with substrate (concentrations from 0 to 5 \times K_M) at 37 °C in assay buffer [50 mM Tris-HCl and 150 mM NaCl (pH 7.5)] in a total volume of 100 μ L, and at desired times, reactions were quenched with 5 μ L of 12% (w/v) trichloroacetic acid (TCA). Product formation was assessed by HPLC as follows. For the substrate Asn¹Val⁵-angiotensin II (N¹-AII), an aliquot of the quenched reaction mixture was brought to a final volume of 100 μ L with assay buffer and then injected into a Phenomenex C18 reverse-phase column followed by elution with 5% ACN and 95% H₂O for 2 min, increasing to 21% ACN and 79% H₂O over 3 min, increasing to 24.7% ACN and 75.3% H₂O over 11 min, and finally increasing to 95% ACN and 5% H₂O over 3 min before returning to 5% ACN and 95% H₂O over 3 min. Both solvents contained 0.1% trifluoroacetic acid (TFA). The product concentration was determined using the integration area at 280 nm and a standard curve generated under the same conditions with Val⁵-angiotensin II (AII). For substrates L-Asn and L-Gln, following quenching, reaction products were derivatized with *o*-phthalaldehyde (OPA) and analyzed by HPLC as described previously.³² A description of the HPLC program employed in evaluating activity for the substrate N¹-DIAP can be found in the Supporting Information. All reactions were conducted in at least triplicate, and the observed rates were fit to the Michaelis–Menten equation using Kaleidagraph (Synergy).

The effect of pH on hNTAN1 activity was determined in 50 mM Bis-Tris (pH 6.0–6.5), 50 mM Tris-HCl (pH 7.0–8.5), or 50 mM CAPSO (pH 10) and 150 mM NaCl. Enzyme was preincubated in buffer, and reactions were initiated by the addition of preincubated enzyme (1 nM) to 25 μ M N¹-AII at 37 °C in the appropriate pH buffer. After 1.5 min, reactions were quenched and analyzed by HPLC as described previously.

The effects of various divalent salts, EDTA, and D-desthiobiotin on hNTAN1 activity were determined by preincubating enzyme (20 nM) in assay buffer at 4 °C for 3 h with either EDTA (1 mM), D-desthiobiotin (3 mM), or a divalent salt (1 mM). Reactions were initiated by the addition of the preincubated enzyme (1 nM) to 100 μ M N¹-AII in assay buffer at 37 °C. After 4 min, the reactions were quenched and analyzed by HPLC as described above.

Circular Dichroism (CD). Far-UV CD spectra were recorded at 200–260 nm using a J-815 CD spectrometer (Jasco, Easton, MD) equipped with a temperature-controlled cell holder set to

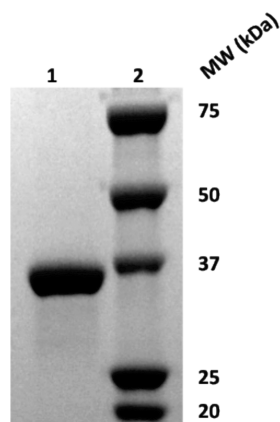


Figure 2. SDS–PAGE analysis of hNTAN1 following tandem affinity purification. In lane 1, hNTAN1 was expressed with a C-terminal tandem FLAG-strepII affinity tag from pNTANFLAGSI and purified as described in detail in Experimental Procedures. Equivalent product homogeneity was observed following analogous purification of hNTAN1 expressed from pNTAN2FLAGSI (Figure S4 of the Supporting Information). Lane 2 contained molecular mass markers.

room temperature, using 15 μ M enzyme in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.5 mM DTT (pH 7.5).

RESULTS

Construction of a Synthetic hNTAN1 Gene. The human NTAN1 gene (GenBank accession number NM_173474) contains a number of rare *E. coli* codons, including 13 such Arg codons (AGA, AGG, and CGA). The presence of rare codons has been shown to be detrimental to the expression of several recombinant proteins.³⁵ A synthetic hNTAN1 gene fused to a 5' sequence encoding a His₆ affinity tag was constructed and expressed in *E. coli* BL21(DE3). Western blot analysis with an anti-His tag antibody as described previously³⁶ revealed the presence of a band with the expected molecular mass (\sim 35 kDa) in both the soluble and insoluble fractions (Figure S1 of the Supporting Information). However, the purified His₆-hNTAN1 enzyme was found to be minimally active toward the substrate N¹-AII (Table 1). In contrast, fusion of a His₆ affinity tag at the C-terminus of hNTAN1 led to the expression of enzyme (Figure S2 of the Supporting Information) with a much higher specific activity toward N¹-AII, indicating that an unoccluded N-terminus may be important for catalysis. In subsequent studies, it was found that hNTAN1 activity was inhibited by Ni²⁺ or Co²⁺, and therefore, for purification purposes, the C-terminal His₆ tag was replaced with a strepII peptide affinity tag (pR1NT-ANSII) and, accordingly, EDTA was included in all purification buffers.

To improve the expression yield, we used the RBS calculator³⁷ to design an optimized ribosomal binding site for more efficient translation initiation. Upon expression of hNTAN1-strepII using the engineered RBS sequence from plasmid pR2NTANSII, the expression yield of hNTAN1 increased 2–3-fold, while the protein purity following strepII affinity chromatography concomitantly increased from 30 to \sim 70% (Table 1) as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure S3 of the Supporting Information). To further improve the purity of hNTAN1 following affinity chromatography, we constructed a plasmid encoding a tandem C-terminal

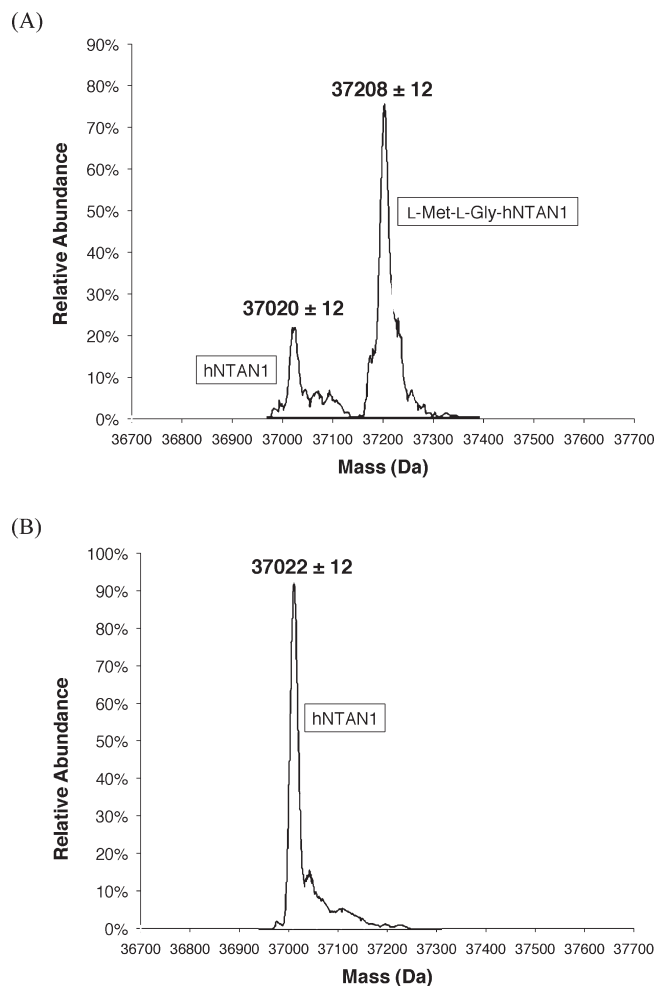


Figure 3. Deconvoluted ESI-MS spectra of hNTAN1. (A) hNTAN1 expressed from pNTANFLAGSI and isolated using tandem affinity purification (see Experimental Procedures). (B) hNTAN1 expressed from pNTAN2FLAGSI and isolated with an identical tandem affinity purification protocol. Samples were analyzed in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.5 mM DTT (pH 7.5). Calculated masses of observed species were as follows: 37026 Da for hNTAN1 and 37214 Da for L-Met-L-Gly-hNTAN1.

FLAG-strepII affinity tag (pNTANFLAGSI). With this construct, the eluant obtained from the strepII affinity chromatography step was loaded onto magnetic beads with an immobilized anti-FLAG monoclonal antibody and eluted with buffer containing a large excess of synthetic FLAG. Using this tandem affinity chromatography procedure, we obtained \sim 0.2 mg of recombinant hNTAN1 per liter that was determined to be $>98\%$ homogeneous by SDS–PAGE (Figure 2).

ESI-MS analysis of purified hNTAN1 revealed the presence of two major peaks at m/z 37208 \pm 12 and 37020 \pm 12 at an \sim 3:1 intensity ratio (Figure 3A). The molecular mass of the latter peak was in agreement with the predicted molecular mass of the enzyme (37026 Da). The peak at m/z 37208 \pm 12 was consistent with an hNTAN1 species containing the initiating L-Met and an L-Gly at position 2 that was introduced during cloning (37214 Da). Although the *E. coli* methionine aminopeptidase (MAP) is highly efficient in removing the initiating L-Met residue when L-Gly occupies P1' of a protein substrate, its catalytic efficiency is severely compromised when the P2' residue is L-Pro,³⁸ as was the

Table 2. Summary of the Kinetic Parameters for Hydrolysis of N-Terminal L-Asn Angiotensin II (N¹-AII) by hNTAN1^a

enzyme	K_M (μ M)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1} s^{-1}$)
hNTAN1 (with Gly2)	31 ± 4	2.3 ± 0.07	$(7.4 \pm 1) \times 10^4$
hNTAN1 (without Gly2)	35 ± 3	6.6 ± 0.1	$(1.9 \pm 0.2) \times 10^5$

^aKinetic parameters were derived from a nonlinear fit of the Michaelis–Menten equation to initial rate measurements. Reactions were conducted at 37 °C in 50 mM Tris-HCl and 150 mM NaCl (pH 7.5). The term in parentheses in the enzyme column depicts whether purified hNTAN1 was expressed from pNTANFLAGSII (with Gly2) or from pNTAN2FLAGSII (without Gly2).

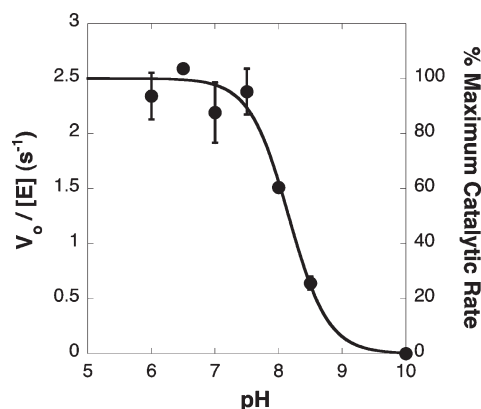


Figure 4. Effect of pH on hNTAN1 activity. hNTAN1 (20 nM) was preincubated at the appropriate pH for 1.5 min at 25 °C. Reactions were initiated by the addition of the preincubated enzyme (1 nM) to 25 μ M N¹-AII at 37 °C in the identical preincubation buffer. After 1.5 min, reactions were quenched and then analyzed by HPLC as described in Experimental Procedures. Activity was evaluated as the initial velocity of the reaction normalized by hNTAN1 concentration. All reactions were conducted in triplicate. The following buffers were used: 50 mM Bis-Tris (pH 6.0–6.5), 50 mM Tris (pH 7.0–8.5), and 50 mM CAPSO (pH 10). All buffers contained 150 mM NaCl.

case in the hNTAN1 enzyme expressed from plasmid pNTAN-FLAGSII. To prevent the accumulation of unprocessed L-Met-L-Gly-hNTAN1, we generated a modified construct (pNTAN-2FLAGSII) in which the L-Gly at position 2 was eliminated. MS analysis of the purified protein obtained from cells transformed with pNTAN2FLAGSII revealed a single peak at the expected molecular mass for hNTAN1 in which the initiating L-Met had been removed by MAP (m/z 37022 \pm 12) (Figure 3B). It is worth noting that *E. coli* MAP is highly efficient in removing the initiating L-Met residue when L-Pro occupies P1' and L-Leu occupies P2'³⁸ given that native hNTAN1 contains L-Pro at position 2 and L-Leu at position 3.

Biochemical Characterization of hNTAN1. The kinetics for hydrolysis of N-terminal L-Asn angiotensin (N¹-AII) by hNTAN1 (Table 2) purified from either pNTANFLAGSII or pNTAN2FLAGSII (Table 1) were determined by HPLC (Figure S5 of the Supporting Information). In both cases, the data could be fit to a Michaelis–Menten model (Figure S6 of the Supporting Information). hNTAN1 exhibited nearly identical K_M values (K_M = 31 μ M for pNTANFLAGSII, and K_M = 35 μ M for pNTAN2FLAGSII) in a manner independent of the expression construct; however, hNTAN1 expressed from pNTAN2-FLAGSII in which the L-Gly at position 2 was eliminated

Table 3. Effects of Divalent Metals, EDTA, and D-Desthiobiotin on hNTAN1 Activity^a

effector	% activity remaining
MgCl ₂	100
CaCl ₂	100
CoCl ₂	58 \pm 2.8
MnSO ₄	40 \pm 14
NiSO ₄	39 \pm 4.2
CuCl ₂	0 ^b
ZnSO ₄	0
EDTA	100
D-desthiobiotin	100

^ahNTAN1 (20 nM) was preincubated in 50 mM Tris-HCl and 150 mM NaCl (pH 7.5) at 4 °C for 3 h with either EDTA (1 mM), D-desthiobiotin (3 mM), or a divalent salt (1 mM). Reactions were initiated by addition of the preincubated enzyme (1 nM) to 100 μ M N¹-AII in 50 mM Tris-HCl and 150 mM NaCl (pH 7.5) at 37 °C. After 4 min, reactions were quenched and then analyzed by HPLC as described in Experimental Procedures. ^bThe area of the product peak was less than or equal to that observed in an HPLC trace of N¹-AII only under the equivalent reaction conditions.

exhibited a nearly 3-fold greater k_{cat} (k_{cat} = 2.3 s^{-1} for pNTAN-FLAGSII, and k_{cat} = 6.6 s^{-1} for pNTAN2GLAGSII). This result indicates that the presence of the conserved L-Pro residue (Figure S7 of the Supporting Information) at the N-terminus of hNTAN1 is important for catalysis and is corroborated by the ESI-MS analysis described above.

hNTAN1 was unable to hydrolyze Q¹-AII, Ac-N¹-AII, L-Asn, or L-Gln, indicating that the recombinant human enzyme exhibited a substrate specificity analogous to that of the isoform previously purified from porcine liver.³⁰ Moreover, hNTAN1 was unable to hydrolyze N¹-DIAP (data not shown), suggesting the possibility that NTAN1-mediated deamidation of the DIAP1 substrate in vivo²⁸ may be additionally dependent on interactions between NTAN1 and other (unidentified) protein factors.

The effect of pH on hNTAN1 activity with N¹-AII is shown in Figure 4, which revealed that the enzyme exhibited an activity optimum between pH 6.0 and 7.5, followed by a sharp decline in activity at alkaline pH. Our approximation of the pH dependence of hNTAN1 activity suggests that the protonation of a single group within the enzyme is crucial for catalysis, resulting in a pH dependence curve with a single descending limb pK_a of 8.2 ± 0.06 .

As summarized in Table 3, low micromolar concentrations of Zn²⁺ and Cu²⁺ completely inhibit hNTAN1 activity, while 50 μ M Ni²⁺, Co²⁺, or Mn²⁺ only partially inhibited the hydrolysis of N¹-AII. Further, Mg²⁺, Ca²⁺, EDTA, and D-desthiobiotin did not impart any noticeable effects on the activity of hNTAN1.

Phylogenetic Conservation and Mutagenesis of Conserved Cys Residues in hNTAN1. Given prior validation that the functionally similar enzymes *S. cerevisiae* Nta1⁹ and murine NTAQ1¹¹ each possess an essential catalytic cysteine residue, we explored the possibility that hNTAN1 contained an analogous activity-dependent cysteine residue. A Position Specific Iterative BLAST (PSI-BLAST)³⁹ search was conducted with the complete amino acid sequence of hNTAN1 to determine the phylogenetic conservation across each of the enzyme's five cysteines, as those residues exhibiting the highest degree of conservation would be

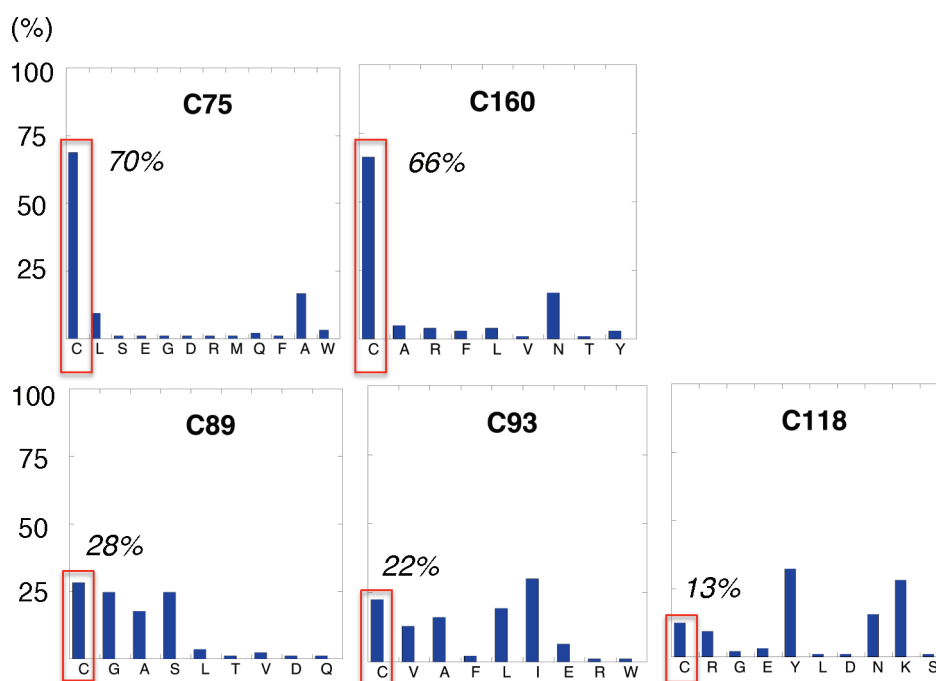


Figure 5. Phylogenetic conservation of hNTAN1 Cys residues. A PSI-BLAST search was conducted with the complete amino acid sequence of hNTAN1 (UniProtKB entry Q96AB6) at EMBL-EBI (<http://www.ebi.ac.uk/Tools/sss/psiblast/>). The “UniProt Knowledgebase” was selected as the Protein Database, and the PSI-BLAST threshold was set to 10. An alignment of hNTAN1 with the PSI-BLAST results ($n = 106$) was generated with ClustalW2⁴⁰ and used to evaluate the degree of conservation across each Cys residue in hNTAN1. Each plot depicts the residue identities and corresponding frequencies observed at the hNTAN1 Cys position denoted at the top of each plot. The italicized percentage value within each plot indicates the frequency of Cys (highlighted by the red outline) across all sequences at the given hNTAN1 position.

Table 4. Relative Activity of hNTAN1 Mutants^a

enzyme	% relative activity (vs wild type)
wild type	100
C160A	100
C75A	0 ^b
C75S	0
C75T	0

^aReactions of hNTAN1 (40–50 nM) with 100 μ M N¹-AII were conducted at 37 °C in 50 mM Tris-HCl and 150 mM NaCl (pH 7.5). After 1 min, reactions were quenched and then analyzed by HPLC as described in Experimental Procedures. ^bThe area of the product peak was less than or equal to that observed in an HPLC trace of N¹-AII only under the equivalent reaction conditions.

implicated as having a greater likelihood of functional and/or structural essentiality. Sequence alignment of hNTAN1 with the PSI-BLAST results ($n = 106$) revealed two distinct cysteine conservation groupings: (i) C75 and C160 (70 and 66% Cys frequencies, respectively) and (ii) C89, C93, and C118 (28, 22, and 13% Cys frequencies, respectively) (Figure 5). C75 and C160 in hNTAN1 were substituted with L-Ala, and the relative hydrolysis of N¹-AII by the respective protein mutants was assessed (Table 4). While replacement of C160 with L-Ala had no effect on the catalytic activity, the C75A variant enzyme was completely inactive. This finding indicates that C75 is essential for enzymatic activity and likely to constitute the catalytic nucleophile. The substitution of C75 with other nucleophilic amino acids, L-Ser or L-Thr, failed to restore activity.

Table 5. Calculated Secondary Structure of Wild-Type (WT) and C75A hNTAN1 from CD Spectra.

	% helix	% sheet	% turn	% random
WT	30	20	20	30
C75A	30	20	20	30

Analysis of the CD spectrum of recombinant hNTAN1 indicated 30% helical, 20% sheet, 20% turn, and 30% random structure (Table 5). The C75A mutation resulted in no obvious change in CD spectra or secondary structure composition (Figure 6), further supporting the suggestion that the inactivity of this variant was strictly due to the removal of the potentially nucleophilic thiol group and not to changes in folding.

DISCUSSION

A number of biochemical and genetic studies over the past 20 years have begun to elucidate the significance of the N-end rule pathway in numerous physiological processes and have revealed many of the critical components involved in this subset of the UPS-dependent proteolytic quality control system. Proteolysis by the N-end rule pathway is dependent upon distinct types of degradation signals (degrons). In vertebrates, N-terminal L-Asn and L-Gln can function as tertiary destabilizing residues of the N-end rule pathway through enzymatic deamidation by either NTAN1 (Asn-specific) or NTAQ1 (Gln-specific) to yield the secondary destabilizing N-terminal L-Asp and L-Glu, respectively.

Almost 20 years ago, a mammalian monomeric enzyme purified from the cytosolic fraction of porcine liver cells³⁰ was

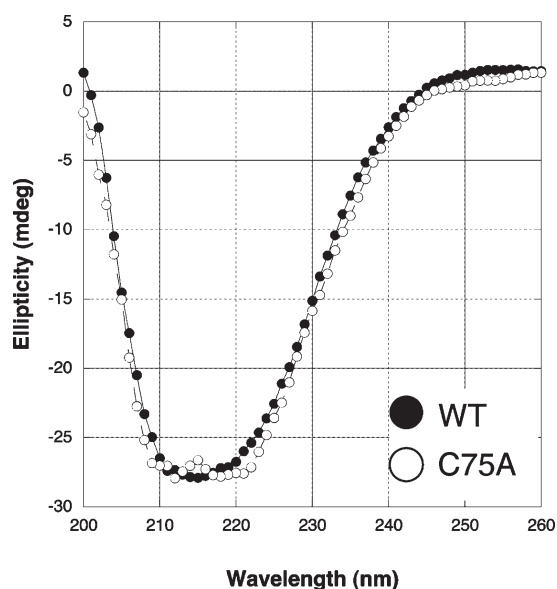


Figure 6. Far-UV CD analyses of wild-type (WT) and C75A hNTAN1. CD spectra of each sample (15 μ M) were recorded at 25 $^{\circ}$ C in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.5 mM DTT (pH 7.5). These spectra reflect three accumulations per sample: WT hNTAN1 (●) and C75A hNTAN1 (○).

shown to exhibit L-Asn-specific Nt amidase activity. More recently, a murine cDNA that complemented a *S. cerevisiae* Δ nta1 mutant was identified, though the protein was not characterized enzymologically.³¹ Interestingly, the murine gene that was able to complement the *S. cerevisiae* Δ nta1 mutant displays only 10% amino acid sequence identity to the recently described murine L-Gln-specific Nt amidase, NTAQ1,¹¹ and only 12% amino acid sequence identity to *S. cerevisiae* Nta1,⁹ which deamidates both L-Asn and L-Gln at the N-terminus of a protein substrate.

Because of its high degree of sequence homology to other mammalian NTAN1s (e.g., murine and porcine), a putative NTAN1 gene from *Homo sapiens* (hNTAN1) has been assigned, though direct experimental evidence of its activity and biochemical characterization had been lacking. Here, we report that bacterial expression and kinetic characterization of hNTAN1. Expression of hNTAN1 in *E. coli* at preparative levels was achieved through the use of a codon-optimized gene and an optimal RBS and translation initiation region for efficient protein synthesis. Two lines of evidence suggested that the N-terminus of hNTAN1 is important for its enzymatic activity. First, enzyme expressed with an N-terminal epitope tag exhibited substantially lower activity relative to that of the enzyme expressed with a C-terminal epitope tag, as evaluated by deamidation of N¹-AII (Table 1). Second, the presence of an N-terminal L-Met-L-Gly dipeptide, which partially interfered with processing by the *E. coli* methionine aminopeptidase (MAP), resulted in an enzyme preparation containing an approximately 3:1 ratio of L-Met-L-Gly-hNTAN1 to MAP-processed hNTAN1 with an exposed L-Pro at the N-terminus. This mixed preparation had a specific activity nearly 3-fold lower than that of a preparation of the authentic human enzyme. Thus, we concluded that the exposure of the conserved L-Pro residue at the N-terminus is important for the function of the enzyme.

Kinetic analysis revealed that hNTAN1 is highly selective for the hydrolysis of N-terminal peptidyl L-Asn but fails to deamidate free L-Asn or L-Gln, N-terminal peptidyl L-Gln (in Q¹-AII), or acetylated N-terminal peptidyl L-Asn (in Ac-N¹-AII). The human enzyme displayed an approximately 10-fold lower K_M and a 100-fold lower k_{cat} for the N¹-AII peptide relative to the results reported by Stewart et al.³⁰ for the porcine enzyme under similar assay conditions. However, we note that the total yield of the purified porcine enzyme reported in that study was less than 2 μ g, raising the possibility that inaccuracies in the quantification of very small amounts of protein used in that study may have led to an overestimation of the k_{cat} value. Nonetheless, it is also possible that the difference in catalytic activity may be due to the slight differences in the primary sequence between the human and porcine NTAN1 isoforms (92% amino acid sequence identity) or caused by the fact that the recombinant human enzyme may be lacking a factor present in liver tissue.

Recombinant hNTAN1 displayed a pH optimum in the range of 6.0–7.5, with a sharp decline in activity with a shift to a more alkaline pH, indicating that protonation of a single group is important for catalysis. PSI-BLAST analysis of hNTAN1 homologues revealed two highly conserved L-Cys residues. Site-specific mutagenesis subsequently demonstrated that the substitution of C75 with L-Ala completely abolishes activity but does not affect the secondary structure of the enzyme as determined by circular dichroism spectra. Further, neither L-Ser nor L-Thr could replace C75 and concomitantly maintain enzyme function, revealing that this residue is absolutely required for catalysis. Therefore, similar to both yeast Nta1 and the recently reported NTAQ1, human NTAN1 appears to possess a critical nucleophilic L-Cys.

Although NTAN1 has been implicated in the modulation of protein turnover via the N-end rule pathway, very little is known with respect to (i) the exact molecular mechanisms or interactive processes underlying the neurological defects observed in *Ntan1*^{−/−} mice,¹⁰ (ii) the catalytic mechanism and nature of the active site structure of NTAN1, which shares little sequence similarity to other amidases, and (iii) the identity of actual physiological substrates targeted by the enzyme.

While it is conceivable to scan the proteome for amino-terminal sequences of the type L-Met-L-Asn (or L-Met-L-Gln), the prediction of physiological substrates targeted by NTAN1 (or NTAQ1) is inherently complicated because of the potentially vast contribution of endoproteolysis in generating N-terminal degrons of these types. This point was illustrated by the discovery of NTAN1-mediated degradation of DIAP1 in the regulation of apoptosis.²⁸ DIAP1 is cleaved by a caspase to generate a C-terminal fragment with N-terminal L-Asn, which is then targeted through the NTAN1–ATE1 pathway in ultimately generating a primary destabilizing N-terminal L-Arg for subsequent substrate protein degradation. To date, DIAP1 remains the only known substrate of N-terminal L-Asn deamidation. However, we describe here that hNTAN1 was unable to deamidate the N-terminal L-Asn of a synthetic 9mer corresponding to the N-terminus of the caspase-mediated C-terminal fragment (in N¹-DIAP), suggesting the possibility that NTAN1-mediated deamidation of the DIAP1 substrate in vivo may be dependent on additional interactions between the enzyme and other (unidentified) protein factors.

The enzymological characterization of N-end rule pathway components such as hNTAN1 should assist in the elucidation of their physiological roles, allow for the in vitro reconstruction of the pathway itself, and, subsequently, aid in the discovery of

potential physiological substrates targeted by its components. Further, recombinant expression of the human homologues of these components could present an opportunity to specifically investigate mechanisms and features of human pathological states associated with protein misfolding and aggregation, some of which may be associated with the N-end rule pathway.

■ ASSOCIATED CONTENT

S Supporting Information. Procedures for the purification of recombinant hNTAN1 fused with either a His₆ or a strepII affinity tag, a table of the primers used for the work described in this study, and seven figures depicting the detection of soluble His₆-hNTAN1 by Western blot, the purity of hNTAN1-His₆ determined by SDS–PAGE, the purity of hNTAN1-strepII expressed from a plasmid with engineered RBS determined by SDS–PAGE, the purity of hNTAN1-FLAG-strepII expressed from pNTAN2FLAGSI determined by SDS–PAGE, an HPLC trace overlay of N¹-AII-, AII-, and hNTAN1-mediated deamidation of N¹-AII, the Michaelis–Menten curves generated for determination of hNTAN1 kinetic parameters, and an alignment of multiple vertebrate NTAN1 sequences revealing a conserved Pro2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (512) 471-6975. Fax: (512) 471-7963. E-mail: gg@che.utexas.edu.

Funding Sources

This work was supported by National Institutes of Health Grant CA139059 and the Cancer Prevention and Research Initiative of Texas. J.R.C. also acknowledges the U.S. Department of Homeland Security (DHS) for a Graduate Fellowship under the DHS Scholarship and Fellowship Program.

■ ACKNOWLEDGMENT

We thank Stony Lo for generating ESI-MS mass spectra and Eun Jeong Cho for assistance with obtaining CD spectra.

■ ABBREVIATIONS

Ac-N¹-AII, N-acetyl-Asn¹Val⁵-angiotensin II (Ac-NRVYVHPF); ACN, acetonitrile; ATE1, arginyltransferase 1; AII, Val⁵-angiotensin II (DRVYVHPF); CD, circular dichroism; DIAP1, *Drosophila* inhibitor of apoptosis 1; ESI-MS, electrospray ionization mass spectrometry; IPTG, isopropyl β-D-1-thiogalactopyranoside; MAP, methionine aminopeptidase; N¹-AII, Asn¹Val⁵-angiotensin II (NRVYVHPF); N¹-DIAP, 9mer corresponding to the N-terminus of the caspase-mediated C-terminal fragment of DIAP1 (NNTNATQLF); Nta1, N-terminal amidase 1; Nt-amidase, N-terminal amidohydrolase; NTAN1, N-terminal asparagine amidohydrolase; NTAQ1, N-terminal glutamine amidohydrolase; PSI-BLAST, position-specific iterative BLAST; Q¹-AII, Gln¹Val⁵-angiotensin II (QRVYVHPF); Ub, ubiquitin; UPS, ubiquitin–proteasome system.

■ REFERENCES

- (1) McClellan, A. J., Tam, S., Kaganovich, D., and Frydman, J. (2005) Protein quality control: Chaperones culling corrupt conformations. *Nat. Cell Biol.* 7, 736–741.
- (2) Varshavsky, A. (1996) The N-end rule: Functions, mysteries, uses. *Proc. Natl. Acad. Sci. U.S.A.* 93, 12142–12149.
- (3) Mogk, A., Schmidt, R., and Bukau, B. (2007) The N-end rule pathway for regulated proteolysis: Prokaryotic and eukaryotic strategies. *Trends Cell Biol.* 17, 165–172.
- (4) Ravid, T., and Hochstrasser, M. (2008) Diversity of degradation signals in the ubiquitin-proteasome system. *Nat. Rev. Mol. Cell Biol.* 9, 679–690.
- (5) Varshavsky, A. (1997) The N-end rule pathway of protein degradation. *Genes Cells* 2, 13–28.
- (6) Bachmair, A., and Varshavsky, A. (1989) The degradation signal in a short-lived protein. *Cell* 56, 1019–1032.
- (7) Prakash, S., Inobe, T., Hatch, A. J., and Matouschek, A. (2009) Substrate selection by the proteasome during degradation of protein complexes. *Nat. Chem. Biol.* 5, 29–36.
- (8) Tasaki, T., and Kwon, Y. T. (2007) The mammalian N-end rule pathway: New insights into its components and physiological roles. *Trends Biochem. Sci.* 32, 520–528.
- (9) Baker, R. T., and Varshavsky, A. (1995) Yeast N-terminal amidase. A new enzyme and component of the N-end rule pathway. *J. Biol. Chem.* 270, 12065–12074.
- (10) Kwon, Y. T., Balogh, S. A., Davydov, I. V., Kashina, A. S., Yoon, J. K., Xie, Y., Gaur, A., Hyde, L., Denenberg, V. H., and Varshavsky, A. (2000) Altered activity, social behavior, and spatial memory in mice lacking the NTAN1p amidase and the asparagine branch of the N-end rule pathway. *Mol. Cell. Biol.* 20, 4135–4148.
- (11) Wang, H., Piatkov, K. I., Brower, C. S., and Varshavsky, A. (2009) Glutamine-specific N-terminal amidase, a component of the N-end rule pathway. *Mol. Cell* 34, 686–695.
- (12) Kwon, Y. T., Kashina, A. S., Davydov, I. V., Hu, R. G., An, J. Y., Seo, J. W., Du, F., and Varshavsky, A. (2002) An essential role of N-terminal arginylation in cardiovascular development. *Science* 297, 96–99.
- (13) Hu, R. G., Sheng, J., Qi, X., Xu, Z., Takahashi, T. T., and Varshavsky, A. (2005) The N-end rule pathway as a nitric oxide sensor controlling the levels of multiple regulators. *Nature* 437, 981–986.
- (14) Hu, R. G., Wang, H., Xia, Z., and Varshavsky, A. (2008) The N-end rule pathway is a sensor of heme. *Proc. Natl. Acad. Sci. U.S.A.* 105, 76–81.
- (15) Kwon, Y. T., Reiss, Y., Fried, V. A., Hershko, A., Yoon, J. K., Gonda, D. K., Sangan, P., Copeland, N. G., Jenkins, N. A., and Varshavsky, A. (1998) The mouse and human genes encoding the recognition component of the N-end rule pathway. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7898–7903.
- (16) An, J. Y., Seo, J. W., Tasaki, T., Lee, M. J., Varshavsky, A., and Kwon, Y. T. (2006) Impaired neurogenesis and cardiovascular development in mice lacking the E3 ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6212–6217.
- (17) Kwon, Y. T., Xia, Z., An, J. Y., Tasaki, T., Davydov, I. V., Seo, J. W., Sheng, J., Xie, Y., and Varshavsky, A. (2003) Female lethality and apoptosis of spermatocytes in mice lacking the UBR2 ubiquitin ligase of the N-end rule pathway. *Mol. Cell. Biol.* 23, 8255–8271.
- (18) Ouyang, Y., Kwon, Y. T., An, J. Y., Eller, D., Tsai, S. C., Diaz-Perez, S., Troke, J. J., Teitell, M. A., and Marahrens, Y. (2006) Loss of Ubr2, an E3 ubiquitin ligase, leads to chromosome fragility and impaired homologous recombination repair. *Mutat. Res.* 596, 64–75.
- (19) Zenker, M., Mayerle, J., Lerch, M. M., Tagariello, A., Zerres, K., Durie, P. R., Beier, M., Hulskamp, G., Guzman, C., Rehder, H., Beemer, F. A., Hamel, B., Vanlieferinghen, P., Gershoni-Baruch, R., Vieira, M. W., Domic, M., Auslender, R., Gil-da-Silva-Lopes, V. L., Steinlicht, S., Rauh, M., Shalev, S. A., Thiel, C., Ekici, A. B., Winterpacht, A., Kwon, Y. T., Varshavsky, A., and Reis, A. (2005) Deficiency of UBR1, a ubiquitin ligase of the N-end rule pathway, causes pancreatic dysfunction,

malformations and mental retardation (Johanson-Blizzard syndrome). *Nat. Genet.* 37, 1345–1350.

(20) Balogh, S. A., Kwon, Y. T., and Denenberg, V. H. (2000) Varying intertrial interval reveals temporally defined memory deficits and enhancements in NTAN1-deficient mice. *Learn. Mem.* 7, 279–286.

(21) Lecker, S. H., Solomon, V., Price, S. R., Kwon, Y. T., Mitch, W. E., and Goldberg, A. L. (1999) Ubiquitin conjugation by the N-end rule pathway and mRNAs for its components increase in muscles of diabetic rats. *J. Clin. Invest.* 104, 1411–1420.

(22) Tasaki, T., Sohr, R., Xia, Z., Hellweg, R., Hortnagl, H., Varshavsky, A., and Kwon, Y. T. (2007) Biochemical and genetic studies of UBR3, a ubiquitin ligase with a function in olfactory and other sensory systems. *J. Biol. Chem.* 282, 18510–18520.

(23) Eisele, F., and Wolf, D. H. (2008) Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1. *FEBS Lett.* 582, 4143–4146.

(24) Lee, M. J., Tasaki, T., Moroi, K., An, J. Y., Kimura, S., Davydov, I. V., and Kwon, Y. T. (2005) RGS4 and RGSS are in vivo substrates of the N-end rule pathway. *Proc. Natl. Acad. Sci. U.S.A.* 102, 15030–15035.

(25) Du, F., Navarro-Garcia, F., Xia, Z., Tasaki, T., and Varshavsky, A. (2002) Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14110–14115.

(26) Turner, G. C., Du, F., and Varshavsky, A. (2000) Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. *Nature* 405, 579–583.

(27) Rao, H., Uhlmann, F., Nasmyth, K., and Varshavsky, A. (2001) Degradation of a cohesin subunit by the N-end rule pathway is essential for chromosome stability. *Nature* 410, 955–959.

(28) Ditzel, M., Wilson, R., Tenev, T., Zachariou, A., Paul, A., Deas, E., and Meier, P. (2003) Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. *Nat. Cell Biol.* 5, 467–473.

(29) Yoshida, S., Ito, M., Callis, J., Nishida, I., and Watanabe, A. (2002) A delayed leaf senescence mutant is defective in arginyl-tRNA: protein arginyltransferase, a component of the N-end rule pathway in *Arabidopsis*. *Plant J.* 32, 129–137.

(30) Stewart, A. E., Arfin, S. M., and Bradshaw, R. A. (1994) Protein NH₂-terminal asparagine deamidase. Isolation and characterization of a new enzyme. *J. Biol. Chem.* 269, 23509–23517.

(31) Grigoryev, S., Stewart, A. E., Kwon, Y. T., Arfin, S. M., Bradshaw, R. A., Jenkins, N. A., Copeland, N. G., and Varshavsky, A. (1996) A mouse amidase specific for N-terminal asparagine. The gene, the enzyme, and their function in the N-end rule pathway. *J. Biol. Chem.* 271, 28521–28532.

(32) Cantor, J. R., Stone, E. M., Chantranupong, L., and Georgiou, G. (2009) The human asparaginase-like protein 1 hASRGL1 is an Ntn hydrolase with β -aspartyl peptidase activity. *Biochemistry* 48, 11026–11031.

(33) Schmidt, T. G., and Skerra, A. (2007) The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nat. Protoc.* 2, 1528–1535.

(34) Gill, S. C., and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* 182, 319–326.

(35) Kane, J. F. (1995) Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* 6, 494–500.

(36) Griswold, K. E., Mahmood, N. A., Iverson, B. L., and Georgiou, G. (2003) Effects of codon usage versus putative 5'-mRNA structure on the expression of *Fusarium solani* cutinase in the *Escherichia coli* cytoplasm. *Protein Expression Purif.* 27, 134–142.

(37) Salis, H. M., Mirsky, E. A., and Voigt, C. A. (2009) Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol.* 27, 946–950.

(38) Frottin, F., Martinez, A., Peynot, P., Mitra, S., Holz, R. C., Giglione, C., and Meinnel, T. (2006) The proteomics of N-terminal methionine cleavage. *Mol. Cell. Proteomics* 5, 2336–2349.

(39) Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-

BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.

(40) Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948.