Homology Modeling and Site-directed Mutagenesis of Pyroglutamyl Peptidase II

INSIGHTS INTO OMEGA- VERSUS AMINOPEPTIDASE SPECIFICITY IN THE M1 FAMILY

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Pyroglutamyl peptidase II (PPII), a highly specific membrane-bound omegapeptidase, removes N-terminal pyroglutamyl from thyrotropin-releasing hormone (<Glu-His-Pro-NH₂), inactivating the peptide in the extracellular space. PPII and enzymes with distinct specificities such as neutral aminopeptidase (APN), belong to the M1 metallopeptidase family. M1 aminopeptidases recognize the N-terminal amino group of substrates or inhibitors through hydrogen-bonding to two conserved residues (Gln-213 and exopeptidase motif Glu-355 in human APN), whereas interactions involved in recognition of pyroglutamyl residue by PPII are unknown. In rat PPII, the conserved exopeptidase residue is Glu-408, whereas the other one is Ser-269. Given that variations in M1 peptidase specificity are likely due to changes in the catalytic region, we constructed three-dimensional models for the catalytic domains of PPII and APN. The models showed a salt bridge interaction between PPII-Glu-408 and PPII-Lys-463, whereas the equivalent APN-Glu-355 did not participate in a salt bridge. Docking of thyrotropin-releasing hormone in PPII model suggested that the pyroglutamyl residue interacted with PPII-Ser-269. According to our models, PPII-S269Q and -K463N mutations should leave Glu-408 in a physicochemical context similar to that found in M1 aminopeptidases; alternatively, PPII-S269E replacement might be sufficient to transform PPII into an aminopeptidase. These hypotheses were supported by site-directed mutagenesis; the mutants lost osteopeptidase II activity. These hypotheses were supported by site-directed mutagenesis; the mutants lost osteopeptidase II activity. 

Amino- or omegapeptidases hydrolyze the peptide bond linking the N-terminal amino acids of peptides, selecting either a free terminal amino group or a <Glu₃> residue at the P1 substrate position. They have been grouped in families according to their catalytic mechanisms and amino acid sequences. The M1 family clusters zinc metallopeptidases widely distributed in nature, with homologues found in bacteria, fungi, plants, invertebrates, amphibians, birds, and mammals (1, 2). They are broadly expressed in animal tissues, where they play critical roles in maturation, activation, and degradation of peptides and, therefore, in a variety of physiological processes. Their abnormal expression has been linked to cancer and other pathological conditions such as cardiovascular disorders (3–5). The M1 family contains aminopeptidases (AP) with varied specificities, defined in part by their preference for the type of N-terminal residue; among them are the neutral AP (APN; EC 3.4.11.2), the acidic AP (APA; EC 3.4.11.7) and the basic AP (EC 3.4.11.6), or the leukocyte-derived arginine AP (L-RAP; EC 3.4.1). Each of these aminopeptidases has multiple tissue specific roles in vivo. For example, in brain APN controls vasopressin release through the hydrolysis of angiotensin III (6); peptides belonging to the oxytocinase subfamily play important roles in maintenance of normal pregnancy, memory retention, blood pressure regulation, and antigen presentation (7). All members of the M1 family are aminopeptidases, except for pyroglutamyl peptidase II (PPII, EC 3.4.19.6), a membrane-bound omegapeptidase that catalyzes the inactivation of the neuropeptide thyrotropin-releasing hormone (TRH, <Glu-His-Pro-NH₂) in the extracellular space (8–12). TRH is a neurohormone that regulates adenohypophyseal secretions and a neurotransmitter with effects on multiple central nervous system functions, including cognition and locomotion (13–17). Clinical studies have reported beneficial effects of its administration in several diseases, but these improvements are of short duration. The rapid inactivation of TRH by PPII in the central nervous system extracellular space (17, 18) represents a significant drawback in its potential use as a therapeutic agent. Rat and human PPII cDNAs encode sequences with a high degree of conservation (19, 20). Purification of the brain enzyme indicates that it is a glycoprotein composed of two identical subunits with a molecular mass of 230 kDa when solubilized with trypsin (21, 22).

The primary structure of M1 membrane-anchored metallopeptidases includes a small intracellular N-terminal fragment, one transmembrane segment, and a large extracellular C-terminal region that holds the exopeptidase (GU/A/G/MEN) and catalytic (HEXXHX₁₉E) motifs. Soluble members share sequence homology with the ectodomains of M1 membrane peptidases; this similarity increases remarkably in the region surrounding the conserved motifs (20). For PPII as for

leukocyte-derived arginine aminopeptidase; F3, tricorn interacting factor 3; EGFP, enhanced green fluorescent protein; βNA, β-naphthylamide; SCR, structurally conserved regions; PA, pepstatin A.
other glucinzins, it was proposed that the His residues and the C-terminal Glu within the catalytic motif (**HEXHX**₁⁸**E**⁶⁶) coordinate the Zn²⁺ atom, whereas PPII-Glu-442 activates a water molecule, and PPII-Tyr-528 stabilizes the transition state (23). Sequence alignments among M1 peptidases show that rat PPII shares 34% amino acid identity with human APN, 32% with mouse APA, 31% with human L-RAP, or 26% with human leukotriene A₄ hydrolase (LTA₄H; EC 3.3.2.6) (Fig. 1). In contrast to APN, the most similar counterpart in the family, PPII, is a narrow-specificity enzyme hydrolyzing pyroglutamyl from TRH or very closely related tripeptides or tetrapeptides but not from longer peptides such as luteinizing hormone-releasing hormone, bombesin, or neurotensin (24–27). PPII substrates have the general structure <Glu-X-Y, where X is a moderately bulky and uncharged residue, and Y is Pro, Ala, Thr, Pro-Gly, Pro-NH₂ (TRH), Pro-β-naphthylamine (βNA), or Pro-7-amino-4-methyl coumarin (20, 25, 28–30).

Despite the importance of M1 peptidases in the metabolism of peptides, the structural determinants ensuring their strict exopeptidase action and specificity are poorly explored. LTA₄H, a soluble bifunctional enzyme (epoxide hydrolase and aminopeptidase) was the first M1 metallopeptidase with a crystallographic structure solved (31). Recently, the structure of the tricorn-interacting factor F3 (F3) was also reported (32). The LTA₄H and F3 structures consist of three and four domains. Domains I, II, and IV of F3 are equivalent to the three domains of the LTA₄H structure, whereas the barrel-like β-sheet structure of domain III is a unique feature of F3. Superposition of the single domains of LTA₄H and F3 demonstrates a variable degree of similarity; the N-terminal domain forming a saddle-like structure that covers the active site, and the thermolysin-like catalytic domain, including the zinc-binding residues, are very similar in these proteins. In contrast, the C-terminal domains are partially conserved and differ considerably in their relative positions (32). The remarkable structural similarity between the LTA₄H and F3 catalytic domains, the high sequence conservation around the M1 catalytic domains, and site-directed mutagenesis studies (20, 33–36) suggest that they use a common catalytic mechanism and that different N-terminal residue preferences (neutral (APN), acidic (APA), arginyl (L-RAP), or PPII) are supported by discrete changes near the active site. In the M1 aminopeptidases, specific recognition of the free N-terminal group of substrates and inhibitors involves hydrogen bonding with two conserved residues. The structure of LTA₄H in complex with the competitive inhibitor bestatin shows that Glu-271, located within the exopeptidase motif, and Gln-136 are positioned in the active site; both make hydrogen bonds to the free amine of the inhibitor, which chemically resembles a peptide substrate, suggesting their participation in the binding of the N-terminal group of substrates. Experimental analysis as well as examination of the x-ray structure of LTA₄H-E271Q inactive mutant, indicates that Glu-271 carboxylate is not only involved in the N-terminal recognition but also has a critical role in the aminopeptidase activity (33). It is proposed that the counterparts of LTA₄H-Glu-271, APA-Glu-352, and APN-Glu-355 interact with the free amino group of substrates and inhibitors via a hydrogen bond, with their negative charge stabilizing the transition state (34, 35). Additionally, APA-Glu-215, counterpart of LTA₄H-Gln-136, is involved in the exopeptidase specificity by interacting with the N-terminal amine of the substrate, contributing together with the exopeptidase motif Glu-352 to the correct positioning of substrates and inhibitors in the active site (36). In conclusion, recognition of the α-amino group of substrates, or inhibitors, by M1 aminopeptidases implicates hydrogen bond and charge interaction with a glutamate residue within the exopeptidase motif (anionic binding site) as well as another hydrogen bond with the Glu or Gln residue equivalent to APA-Glu-215 or LTA₄H-Gln-136.

Unlike aminopeptidase substrates, TRH does not have an α-amino group at its N terminus, implying that in PPII substrate recognition differs from the rest of the M1 family members. In PPII, exopeptidase motif Glu (PPII-Glu-408) is conserved, and its replacement by Gin leads to a completely inactive enzyme, whereas the E408D mutant has a very low catalytic activity (due to a decreased V_{max} value, whereas K_{m} is not affected) (23). This suggests that position and negative charge of PPII-Glu-408 carboxylate are critical for catalysis but not for substrate binding. In the absence of structural information to understand the ome-gapeptidase specificity of PPII and to compare it with that of M1 aminopeptidases, we performed multiple alignments of M1 family members and constructed by homology modeling three-dimensional models for part of rat PPII, human APN, and L-RAP ectodomains; TRH was docked in the PPII model. We predicted that substituting one or two PPII-specific residues for M1 family residues was sufficient to migrate from PPII to alanyl-aminopeptidase specificity. Site-directed mutagenesis experiments supported these theoretical predictions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA). DNA purifications were done using Qiagen (Valencia, CA) kits: QIAquick PCR purification kit, QIAquick gel extraction kit, and plasmid Mini and Midi kits. TaqDNA polymerase, pcDNA3.1/HisA expression vector, and liposome transfection reagent Lipofectamine 2000 were from Invitrogen. pEGFP-N3 expression vector and anti-enhanced green fluorescent protein (EGFP) antibody (BD Living Colors) were from Clontech (Palo Alto, CA). Monoclonal anti-poly-His/alkaline phosphatase conjugate (clone HIS-1), phenylmethylsulfonyl fluoride, iodoacetamide, pepstatin A (PA), and deoxyribonuclease I (DNase I) were obtained from Sigma. pBluescript II KS and SK were from Stratagene (La Jolla, CA). Synthetic substrates Ala-βNA, (Glu-βNA, <Glu-βNA, and TRH-βNA were from Bachem (Bundendorf, Switzerland). Actinonin was from Alexis Biochemicals (San Diego, CA). Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate were from Roche. Millipore-P membranes were from Millipore Corp. (Bedford, MA). For DNA sequencing, we used a Dye Terminator Cycle Sequencing Ready Reaction kit from Applied Biosystems (Foster City, CA). Multiple alignments were performed with ClustalW.

**Modeling of Catalytic Domains of PPII, PPII Mutants, APN, and L-RAP (Lys-392)—**By homology modeling, three-dimensional models were constructed for the catalytic domains of rat PPII and human APN (residues 244–623 and 190–567) using as template the x-ray crystallographic structure of human LTA₄H (31). PPII and APN models contained only the region most conserved between template and PPII or APN because we were interested in studying the structure-function relationship for the active sites.

The method was similar to that used for APA modeling (37). InsightII (Accelrys Software Inc., San Diego, CA) was used to construct the models. We aligned the sequences of APN or PPII with that of LTA₄H taking into account the multiple alignment between several proteins of the M1 family and the secondary structure of the template as well as experimental information from previous site-directed mutagenesis studies. Aligned regions, in which we assumed that the secondary structure was conserved, were defined as structurally conserved regions (SCRs), and unaligned regions were defined as loops. Initial models were obtained by transfer of coordinates from the template to PPII or APN sequences in the SCRs and completed by adding the missing loops using the loop
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The completed systems (protein, Zn\(^{2+}\), solvent, we deleted water molecules surrounding the ions (radius 2 Å). The minimized model was placed in a water box (dimensions: 80, 95, 80 Å), and 5 or 15 water molecules were substituted with sodium ions to achieve neutrality. Once in NAMD, the refinement procedure continued with several energy minimization steps followed by molecular dynamics. We began by fixing the backbone of the protein, whereas side chains, water molecules, and ions were variables; in a subsequent step the whole system was relaxed. Periodic boundary conditions were used with the same cutoff to truncate non-bonded interactions. The dielectric constant was 1. We checked the stability of the models during molecular dynamics (500 ps); backbone atoms did not have residual mean square deviations greater than 1.5 Å. PPII mutants were modeled by substituting the desired side chain in PPII model followed by energy minimization and 300 ps of molecular dynamics. Because of the sequence similarity between L-RAP and LTA4H sequences, the crystal structure of human LTA4H was also chosen as a template for construction of a three-dimensional model of human L-RAP (Lys-392) (residues 118 – 494); three-dimensional modeling was performed by using CPH model 2.0 homology modeling server (38).

Docking of TRH in PPII Model—To understand the structural basis of TRH recognition by PPII, we performed docking studies. Using InsightII, TRH was manually docked in the PPII catalytic domain model according to bestin position in the template structure (31). All water molecules surrounding the peptide in a radius of 2 Å were deleted, and the PPII-TRH system was subjected to minimization steps followed by molecular dynamics (500 ps).

Expression Plasmids and Site-directed Mutagenesis—Expression vectors for rat PPII (pN3/rPPII), poly-His-tagged rPPII (pcDNA3.1/HisA-rPPII), and EGFP fused in-frame to the C terminus of rPPII (pEGFPN3/cPPII) were constructed as previously described (39). In pcDNA3.1/HisA-rPPII, the rPPII coding sequence was in-frame C-terminal to the poly-His tag. pEGFP-N3 expression vector for EGFP was used as the control vector in transfections.

Site-directed mutagenesis was done according to the one-step overlap extension PCR method (40). The PCR amplifications were performed with 35 cycles of denaturation (92 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 1 min). Fragment XmaI/SacI of rat PPII DNA was cloned into both pBlueScript II/ks and SK. These vectors served as PCR templates in the presence of one universal primer (T7, 5’-GTAATACGACTCACTATAGGGC-3’ and 5’-AAAGCTTCTGCAACACATCTTCCA-3’). K463Q, 5’-GTGTTGATCGAAGGGCTTACACATCCATCTTCCA-3’; K463R, 5’-GTTGCTCAGCTGCACTCTTCCA-3’. PCR products were digested (Xmal/Sacl), separated by gel electrophoresis, and purified using the gel extraction kit. Each mutant sequence was subcloned back into the wild-type, poly-His, or EGFP-tagged PPII expression vector. Double mutants were constructed sequentially using the same protocol. The presence of the mutation and the absence of nonspecific mutations were confirmed by DNA sequencing.

Cell Culture, Transfection, Membrane Preparation, and Fluorescence Microscopy—COS-7 or C6 glioma cells were cultured and transfected as previously described (39). Cells were collected 48 h post-transfection, and total membranes were prepared essentially as described (41). Briefly, cells were homogenized in 50 mM potassium phosphate buffer, pH 7.5 (buffer A), 0.3 mM phenylmethylsulfonyl fluoride, 1 μM iodoacetamide, 1 μM PA, 2.5 mM MgCl\(_2\), 0.1 mg/ml DNase I by freezing and thawing on ice (3 ×). Total membranes were collected by centrifugation (90,000 ×g, 45 min), the pellet was washed once with buffer A, 1 M NaCl, and centrifugation was repeated. Finally, the pellet was homogenized in buffer A and stored at –80 °C until use. Protein concentrations were determined by the Bradford assay (42). For microscopic analysis, cells were observed under an eclipse TE300 microscope (Nikon, Melville NY) equipped with the cool snap software at 40× magnification. EGFP fluorescence was detected using the EPI-FL filter block (Nikon).

S DS-PAGE and Western Blot Analysis—Membrane preparations were submitted to reducing SDS-polyacrylamide gel electrophoresis, proteins were transferred, and poly-His or EGFP-tagged PPII (wild type or mutants) was detected as described (39). The protein expression levels were estimated by densitometric scanning with a Fluor-S MultiImager (Bio-Rad).

Peptidase Activity Determination—PPII activity was determined using 400 μM TRH-βNA as substrate in a coupled assay with excess dipeptylaminopeptidase IV (EC 3.4.14.5) essentially as described (41); assay buffer (50 mM Na\(_3\)PO\(_4\), pH 7.5) included 0.2 mM N-ethyl maleimide, an inhibitor of pyroglutamyl peptidase I (EC 3.4.19.3), and 0.2 mM bacitracin, an inhibitor of prolyl endopeptidase (EC 3.4.21.26); both are soluble enzymes able to degrade TRH in vitro. Alanyl- or glutamyl-aminopeptidases activities were assayed with 400 μM Ala-βNA or 400 μM Glu-βNA in 100 mM Tris-HCl, pH 7.5. To determine pyroglutamyl peptidase activity, assay buffer (50 mM Na\(_3\)PO\(_4\), pH 7.5) included 1 mM N-ethylmaleimide; the enzymatic reaction was initiated by the addition of 400 μM Glu-βNA. All enzymatic assays were performed at 37 °C under initial velocity conditions in duplicate or triplicate, and their mean was taken as one determination for each independent transfection. Released βNA was determined in a fluorometer (excitation, 335 nm; emission, 410 nm). Activities of wild type PPII and mutants were normalized by total protein or PPII expression levels for non-tagged or poly-His-tagged proteins.

RESULTS AND DISCUSSION

Multiple alignments for the M1 family were performed to study the conservation of the residues involved in the recognition of the α-amino group of substrates and inhibitors by M1 aminopeptidases. Exopeptidase motif Glu (PPII-Glu-408) was conserved among most biochemically characterized M1 metallopeptidases, including PPII orthologues (Fig. 1); these results are consistent with its critical role. Human ami-
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A) M1 metallopeptidases

| M1 Metallopeptidases | PPII Rat | Q10836 | Q9SP7--YWLA--YAMENWGLSIF--VHVEICIQWFGQLTVPWVHDEWDLKGF--1AYXKX | Q9SP7--YWLA--YAMENWGLSIF--VHVEICIQWFGQLTVPWVHDEWDLKGF--1AYXKX |
|----------------------|---------|--------|--------------------------------------------------|--------------------------------------------------|
| APN human            | P15144  |        | QMQRQ--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   | QMQRQ--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   |
| APA mouse            | P16406  |        | DTHY--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   | DTHY--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   |
| L-RAP rat            | Q9U222  |        | QQPRL--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   | QQPRL--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   |
| P-LAP human          | NP_05566|        | DTEPT--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   | DTEPT--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   |
| APE2 yeast           | NP_01725|        | QMEPT--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   | QMEPT--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   |
| AP1 yeast            | NP_01913|        | QMEAT--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   | QMEAT--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   |
| PSA human            | P55766  |        | QFETQ--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   | QFETQ--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   |
| APB human            | Q9N4A   |        | QCCAV--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   | QCCAV--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   |
| LT44 human           | P05926  |        | QCCAV--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   | QCCAV--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   |

rat PPII 100 % S269 E408 E442 E664 Y528
human APN 34 % Q213 E355 E389 E411 Y477
mouse APA 32 % E215 E352 E386 E408 Y471
human L-RAP 31 % E200 E337 E371 E393 Y455
human LT44 26 % Q136 k271 k236 k316 Y383

B) PPII sequences

| PPII Sequences | Rat | Q10836 | Q9SP7--YWLA--YAMENWGLSIF--VHVEICIQWFGQLTVPWVHDEWDLKGF--1AYXKX |
|----------------|-----|--------|--------------------------------------------------|--------------------------------------------------|
| Mouse          | Q8N093|        | Q9SP7--YWLA--YAMENWGLSIF--VHVEICIQWFGQLTVPWVHDEWDLKGF--1AYXKX |
| Bird           | Q9U266|        | Q9SP7--YWLA--YAMENWGLSIF--VHVEICIQWFGQLTVPWVHDEWDLKGF--1AYXKX |
| Fish           | XP_45244| Q9SP7--YWLA--YAMENWGLSIF--VHVEICIQWFGQLTVPWVHDEWDLKGF--1AYXKX |
| Sea Urchin     | XP_79287| F1P1I--YLACQAEAFEYAEWQFHNLTQVTEEDNQMLNMGF--1E5Y5G   |

FIGURE 1. Multiple alignments of M1 family amino acid sequences around the active site. RefSeq accession numbers are included. The zinc binding ligands are indicated in boldface. Ser-269, Glu-408, and Lys-463 rat PPII residues are denoted with arrows. A, characteristic motifs of M1 family peptidases are underlined; A-LAP, adipocyte-derived leucine aminopeptidase; P-LAP, placental leucine aminopeptidase; APE2, yeast aminopeptidase 2; AP1, yeast aminopeptidase 1; PSA, puromycin-sensitive aminopeptidase; APB, basic AP.

nopeptidase O is an exception, with an Ala that substitutes the glutamic acid residue (43). The second residue involved in the α-N-terminus recognition (APA-Glu-215, APN-Gln-213, or LTA4H-Gln-136) was over helix 3. Two loops (loop 1 and 2) approached PPII-Glu-408 (or APN-E411) was detected (not shown). The active sites were buried but accessible to the solvent, organized by three NH groups and two loops that together displayed the residues involved in catalysis.

Modeling of Rat PPII and Human APN Catalytic Domains Identifies a PPII-specific Salt Bridge in the Active Site—To characterize the structural requirements for omegapeptidase activity or/and specificity, three-dimensional models were constructed for the catalytic domains of PPII and APN based on the crystal structure of LTA4H.

PPII and APN models consisted of a part of the N-terminal region (mainly β-sheets), a globular central domain (or catalytic domain), and part of a C-terminal helical region (Fig. 2). In both models, the N-terminal region was tightly compacted due to hydrogen bonding. The salt bridge interaction involved in the N-terminal domain cohesion (previously described for APA (37)) was detected (not shown). The active sites were buried but accessible to the solvent, organized by three α-helices and two loops that together displayed the residues involved in catalysis.

PPII-H441, PPII-Glu-442, and PPII-H445 (or their counterparts in the APN model: APN-H388, APN-E389, APN-H392) were over helix 1; helix 2 held PPII-Glu-464 (or APN-E411); PPII-Tyr-528 (or APN-Y477) was over helix 3. Two loops (loop 1 and 2) approached PPII-Ser-269 (or APN-Glu-215) and exopeptidase motif residue PPII-Glu-408 (or APN-Glu-355) to the active site. The histidine and glutamate residues of the catalytic motif coordinated the Zn²⁺ ion in both models (Fig. 2). A water molecule was kept stable around the zinc ion, engaged by two proton acceptors, PPII-Ser-269 is involved as the hydrogen donor in the recognition of the active site. This hypothesis may explain the unaltered Kₘ value for Glu-389 to place a water molecule around the zinc ion (not shown).

Substrate Docking Suggests a Mode of Pyroglutamyl Residue of TRH-PPII Interaction—When TRH was docked into PPII catalytic pocket, the salt bridge was not disrupted, Glu-408 carboxylate being 3.1 Å from Lys-463 amine (Fig. 3). TRH kept the same orientation as bestatin in the LTA4H structure. The <Glu residue was stacked between the aliphatic chain of PPII-Glu-408 and PPII-Tyr-404 located at loop 2. The interaction between the <Glu ring and a Tyr residue was similar to that found in the LTA4H-bestatin complex in which the phenyl ring of bestatin is stacked against LTA4H-Tyr-267 (exopeptidase motif). In addition, the NH pyroglutamyl group was hydrogen-bonded with the backbone carbonyl group of the Ala-406 (loop 2) as occurs for the bestatin backbone NH group and the backbone carbonyl group of the LTA4H-Glu-269 exopeptidase motif. Moreover, the zeta carbon of the <Glu ring was a proton acceptor in a hydrogen bond with the PPII-Ser-269 hydroxyl group (Fig. 3). Analysis of the residues with direct interactions with the bestatin phenyl ring in the LTA4H structure or with the pyroglutamyl residue in the PPII model showed PPII-Ser-269 as the only omegapeptidase-specific residue (Table 1, shaded boxes). No differences were found between residues that constitute the binding pockets for the bestatin ring and the TRH <Glu (Table 1, open cells). Therefore, as for M1 aminopeptidases, PPII-TRH modeling predicted involvement of loop 1 PPII-Ser-269 residue in N terminus recognition. In contrast, the loop 2 PPII-Glu-408 "anionic site" was neutralized by Lys-463 and not contributed with Glu-389 to place a water molecule around the zinc ion (not shown).
nition of the pyroglutamyl residue of TRH, whereas Glu-408 negative charge is neutralized by Lys-463 to assure omegapeptidase specificity.

Recent results suggest a ligand binding mode for TRH in PPII active site that differs from the orientation that bestatin adopts in the active site of LTA4H structure (44). The binding mode of bestatin is the binding orientation proposed for substrates of M1 aminopeptidases based on theoretical and experimental data (31–33, 36). Because molecular mechanisms of ligand binding are well conserved during evolution of members of the same protein family (45), our proposal is in better accordance with the M1 family evolutionary binding mode, as further supported by the successful substrate specificity change obtained with our designed mutants (see below).

Multiple Alignments and L-RAP Modeling Suggest That in M1 Aminopeptidases Loop 2 Gln Substitution by Glu Is Required to Neutralize the Presence of Lys in Helix 2—To confirm our hypothesis we performed a detailed search of the protein sequence data base, including putative M1 metallopeptidases from genomes. The alignment confirmed that exopeptidase motif Glu is conserved in most members of the family (with the exception of aminopeptidase O). However, it showed that although most M1 peptidase sequences contained an Asn residue in the position corresponding to PPII-Lys-463 (helix 2), a subset of bacterial peptidases had Lys (Figs. 1 and 4A). Experimental data on Escherichia coli PepN confirm its aminopeptidase specificity (46). Likewise, a putative APN sequence (slamdance gene) from fruit fly has Lys instead of the conserved Asn residue (47). Finally, two human L-RAP sequences differ in residue 392, which corresponds to PPII-Lys-463, that difference being Asn for one sequence (L-RAP-(Asn392)) or Lys for the other (L-RAP-(Lys-392)). There is no information regarding L-RAP-(Lys-392) specificity, but characterization of L-RAP-(Asn-392) demonstrates a preference for substrates with N-terminal Arg (48).

The existence of M1 aminopeptidase sequences with a Lys counterpart in helix 2 appears to contradict the hypothesis that PPII-Lys-463 and omega specificity correlate. To clarify the role of the Lys-392 residue in L-RAP-(Lys-392), we constructed a simplified three-dimensional

FIGURE 2. Models of rat PPII and human APN catalytic regions and sites. Upper panels, three-dimensional models for the catalytic regions of PPII and APN, generated using as template the human LTA4H x-ray crystal structure in complex with bestatin. The zinc ions are shown as dark spheres. Lower panels, zoom on the catalytic site. Helices 1 and 2 (H1, H2) display the catalytic motif residues, and helix 3 (H3) places the tyrosine residue implicated in the stabilization of the transition state, whereas loops 1 and 2 (L1, L2) approximate the residues implicated in recognition of the N terminus of substrates and inhibitors in aminopeptidases. The Glu-408–Lys-463 salt bridge in the active site of PPII is boxed, and zinc ions are shown as spheres.
model for its catalytic domain. The L-RAP-(Lys-392) model gave evidence of a 3.5-Å salt bridge interaction between loop 1 L-RAP-Glu-200 (equivalent to PPII-Ser-269 or APN-Gln-213) and L-RAP-Lys-392 and a 4-Å salt bridge interaction between loop 2 L-RAP-Glu-337 (equivalent to PPII-Glu-408 or APN-Glu-355) and L-RAP-Lys-392. These distances suggest that the strongest salt interaction occurred between L-RAP-Lys-392 and L-RAP-Glu-200, leaving the exopeptidase motif residue L-RAP-Glu-337 charged, as in other aminopeptidases (Fig. 4B). Interestingly, an extensive multiple alignment, including more than 150 PepN-like bacterial sequences, showed that a Lys in helix 2 was always accompanied by a Glu residue in loop 1, except for PPII sequences, where Ser is found in loop 1 (Figs. 1 and 4A and supplemental Fig. 1). These data support the putative interaction between helix 2 Lys-392 and loop 1 Glu-200 in L-RAP-(Lys-392). A similar interaction may occur for bacterial PepN sequences with a Lys residue in helix 2. Thus, loop 1-Glu combined with helix 2-Lys may maintain an anionic binding site in loop 2 and a hydrogen bonding property in loop 1, allowing aminopeptidase specificity. Taken together, our theoretical results show correlations that may be relevant for the origin of specificity in the M1 metallopeptidase family. Although PPII (omega) sequences have Ser and Lys residues localized in positions 269 (loop 1) and 463 (helix 2), almost all M1 aminopeptidases have Glu/Gln and Asn in these positions. Moreover, contrary to the conserved Glu within the exopeptidase motif (loop 2), a negative charge in loop 1 may not be necessary for aminopeptidase specificity unless a positive charge is localized in helix 2.

**Mutations of Ser-269 or Lys-463 Do Not Change Rat PPII Expression**—To validate our predictions, site-directed mutagenesis was performed to test the role of PPII-Ser-269 and -Lys-463 in PPII activity and whether their substitution with their APN or L-RAP counterparts promotes aminopeptidase-like specificity. To study the functional role of these residues in the activity and specificity of PPII, Lys-463 was replaced with Asn (the common residue in the family), Arg, or Glu; Ser-269 was substituted for Ala or residues usually found in the family (Gln or Glu). The S269Q/K463N and S269Q/K463R double mutants were also generated. The effect of these mutations on expression, maturation, and cellular distribution of PPII was studied.

Western blot analysis in reducing conditions showed that in transiently transfected COS-7 cells, wild type and mutated PPIIs were detected as a single band of 145 kDa, corresponding to the glycosylated PPII monomer (19). We observed very similar expression levels for His-tagged wild type PPII and each of the eight mutated His-tagged PPII (Fig. 5). Similar results were obtained in C6 glioma cells (not shown).

We also compared the subcellular distribution of wild type PPII with that of PPII-K463N, S269A, S269Q, and S269E mutants by transfecting C6 glioma cells with pEGFPN3/rPPII or expression vectors for mutant PPII. When analyzed by fluorescent microscopy, proteins displayed the same subcellular distribution, being localized mainly at the plasma membrane (Fig. 6 and not shown). Additionally, the aminopeptidase activity displayed by the mutants PPII-S269E, PPII-S269Q/K463N, or PPII-S269Q/K463R (see below) discounted a misfolding caused by these mutations. Together these data show that any change of PPII activity by mutating the Ser-269 or Lys-463 residues cannot be explained by alterations of expression or trafficking.

**Mutations of Ser-269 or Lys-463 Residues Differentially Affect PPII Activity**—To determine the activity of wild type and mutant proteins, C6 glioma or COS-7 cells were transiently transfected with each of the expression vectors pN3/rPPII, pcDNA3.1/HisA-rPPII (wild type or mutant), or pEGFP-N3 as a control vector. The relative effects of mutations on specific activities were independent of the cell type and whether transfection with pN3/rPPII or pcDNA3.1/HisA-rPPII vectors generated a robust PPII activity (Fig. 5A). Substitutions of Lys-463 with...
neutral amino acids (Asn or Gln) generated mutants that did not hydrolyze TRH-NA, suggesting that disruption of the predicted salt bridge was responsible for the loss of activity. The K463R substitution also inactivated PPII (Fig. 5A). Substitution of Lys-463 by Arg in the PPII model placed the Arg-463-NH1 side chain group at 3.1 Å from Glu-408 carboxylate. The Arg-463-NH (1) and -NH (2) amines were hydrogen-bonded with the backbone carbonyl groups of Phe-448, Gly-449, and Val-452, whereas the Arg-463-NH(ε) group was located at 3.7 Å of the helix 2 Glu-464 carboxylate (not shown). This configuration suggests that, compared with its position in Lys-463, the positive charge in Arg-463 side chain was delocalized and that the predicted salt bridge with Glu-408 was disrupted in the PPII-K463R mutant. An example in which substituting Lys by Arg does not restore the original interaction is a naturally occurring mutation (K183R) in the ectodomain of the human thyrotropin receptor; the K183R change disrupts a salt bridge involved in the specificity of the receptor (49). Our results together with data published for Glu-408 mutants (23) suggest that loss of the Glu-408-Lys-463 interaction may contribute significantly to the loss of PPII activity when either of these residues is mutated. It is probable that during evolution recognition of a substrate without an N-terminal charge required neutralization of the loop 2 anionic site. In addition, our docking predicted an interaction between Glu-408 side chain and Glu ring (stacking), suggesting that the Glu-408 –Lys-463 salt bridge may participate in the conformation of the Glu binding pocket. However, other roles for the salt bridge cannot be discarded.

With TRH-NA as substrate, PPII-S269Q and PPII-S269E were inactive, in contrast to the PPII-S269A mutant that showed a 40% decrease in specific activity (Fig. 5A). The mutant kinetic parameter values were 72 and 42% of the wild type values for Km and Vmax (wild type PPII, Km = 9.42 ± 0.31 μM and Vmax = 554 ± 133 pmol/min/mg; PPII-S269A, Km = 6.79 ± 1.1 μM and Vmax = 234 ± 77 pmol/min/mg (mean ± S.D., n = 4). These results indicate that the putative hydrogen bond interaction between PPII-Ser-269 and the pyroglutamyl of TRH does not strongly contribute to binding. Moreover, they suggest that Ser-269 is
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A  
Omega
dipeptidase activity

| specific activity (with PPII = 100%) | control | wt PPII | K463N | K463R | K463Q | S269Q-K463N | S269Q-K463R |
|-----------------------------------|---------|---------|-------|-------|-------|-------------|-------------|
| wt PPII                           | 100     | 100     | 100   | 100   | 100   | 100         | 100         |
| Ser-269Q                          | 99      | 99      | 98    | 98    | 98    | 98          | 98          |
| K463N                             | 109     | 109     | 108   | 108   | 108   | 108         | 108         |
| K463R                             | 107     | 107     | 106   | 106   | 106   | 106         | 106         |
| K463Q                             | 105     | 105     | 104   | 104   | 104   | 104         | 104         |
| S269Q-K463N                       | 100     | 100     | 100   | 100   | 100   | 100         | 100         |
| S269Q-K463R                       | 100     | 100     | 100   | 100   | 100   | 100         | 100         |

B  
Alanyl aminopeptidase activity

| specific activity (with PPII = 100%) | actinonin 0 mM | 0.5 mM | 1 mM |
|-------------------------------------|---------------|--------|------|
| wt PPII                             | 100           | 100    | 100  |
| Ser-269Q                           | 99            | 99     | 99   |
| K463N                              | 109           | 109    | 109  |
| K463R                              | 107           | 107    | 107  |
| K463Q                              | 105           | 105    | 105  |
| S269Q-K463N                        | 100           | 100    | 100  |
| S269Q-K463R                        | 100           | 100    | 100  |

FIGURE 5. Expression and omega- or alanyl-aminopeptidase activities of wild type PPII and mutants. Cells (8 × 10⁶ cells/60-mm dish) were transiently transfected with expression vectors for each of the following proteins: EGFP (control), PPII, PPII-S269A, S269Q, S269E, K463N, K463R, S269Q-K463N, and S269Q-K463R. 15 μl of Lipofectamine 2000 were used. Membranes were prepared after 48 h in culture. Data are the mean ± S.E. specific activities (in % of wild type (wt PPII) activity) from three independent transfections. A, PPII activity was determined in membrane fractions from C6 cells expressing the indicated untagged proteins with TRH-JNA as substrate. Data were identical if assay time was increased up to 8 h. Wild type PPII specific activity was 268 ± 4.6 pmol of TRH-JNA hydrolyzed/min/mg of protein. A Western blot was performed after 48 h in culture, membrane fractions from COS-7 cells expressing the indicated His-tagged proteins were subjected to SDS-PAGE in reducing conditions, and His-tagged proteins were detected with a monoclonal anti-poly-His alkaline phosphatase conjugate (data shown correspond to a representative transfection). The table shows the quantification of expression levels of wild type PPII and mutants. Data are the mean ± S.E. expression levels (in % of wild type PPII expression) from three independent transfections. B, alanyl-aminopeptidase activity was determined in membrane fractions from C6 cells expressing the indicated untagged proteins using Ala-JNA as substrate without or with actinonin; control specific activity (without actinonin) was 75.8 ± 12 pmol of Ala-JNA hydrolyzed/min/mg of protein.

involved in the hydrolytic mechanism rather than in substrate recognition. The reduction in the cleavage efficiency of PPII-S269A may be due to an altered ability to position the substrate in a favorable geometry for catalysis. In contrast, the loss of omegapeptidase activity displayed by the S269Q or S269E mutants may be explained in terms of binding: introduction of either Glu or Gln in the Ser-269 position could generate a steric impediment for the Glu residue docking. This is consistent with the detection of aminopeptidase activity in PPII-S269E (see below). Therefore, 269 side-chain shortening from Glu/Gln in aminopeptidases to Ser in PPII correlates with a switch from an N-terminal group to a <Glu in the substrate N terminus (Fig. 7). Our results are compatible with those observed for the counterpart of PPII-Ser-269 in APA, with APA-Glu-215 playing an important role in substrate or inhibitor bind-

ing but also in the catalytic process (36). In agreement with the data for single mutations, PPII-S269Q/K463N and PPII-S269Q/K463R double mutants did not hydrolyze TRH-βNA (Fig. 5A).

Because Arg could not replace Lys-463 and the S269A mutation affected PPII hydrolytic mechanism, this active site appears to have a precise spatial geometry that may explain its high specificity. We propose a differential role for the exopeptidase motif glutamate residue for omega or amino specificities in the M1 family. Although PPII-Glu-408 charge is neutralized by Lys-463 and its aliphatic side chain may contribute to the <Glu ring binding pocket, the loop 2 Glu residue of M1 aminopeptidases is an anionic site that acts together with a loop 1 Glu/Gln residue (counterpart of PPII-Ser-269) in the binding of the NH₂⁺-terminal group of substrates (31–34, 36). Accordingly, we predicted that disruption of the Glu-408–Lys-463 salt bridge (leaving Glu-408 free to interact with an N-terminal group) and substitution of PPII-Ser-269 (to put in place the second carboxylate required for amino recognition) should be sufficient to transform PPII from omega- to aminopeptidase. Alternatively, as in PepN-like aminopeptidases, disruption of the Glu-408–Lys-463 salt bridge by substitution of PPII-Ser-269 with Glu should be sufficient to change PPII specificity.

Single PPII-Ser-269 or Double PPII-Ser-269 and -Lys-463 Mutations Are Sufficient to Transform PPII into an Alanyl-aminopeptidase—Wild type PPII and mutants were evaluated for their ability to hydrolyze Ala-βNA, <Glu-βNA, and Glu-βNA. When Ala-βNA was used as substrate, specific activities in membranes from cells transfected with either pEGFP-N3, pN3/rPPII, or pcDNA3.1/HisA-rPPII were significant and indistinguishable, in agreement with the omegapeptidase specificity of PPII. The specific activities of S269Q, K463N, and K463R mutants did not differ from control values. The PPII-S269E single mutant as well as the PPII-S269Q/K463N double mutant displayed higher specific activity than the controls, confirming our theoretical predictions (Fig. 5B).

Because the basal activity was probably due to the action of endogenous neutral peptidases, to minimize it and test the nature of the new activity we used actinonin, a relatively specific APN inhibitor (50). The addition of 0.5 or 1 mM actinonin concentrations, which did not affect wild type PPI activity, reduced control activity by 65 or 80%, respectively (not shown). In these conditions the specific activities of S269Q, K463N, and K463R mutants did not differ from control values, but the specific activity of PPII-S269E single mutant as well as the PPII-S269Q/K463N double mutant was amplified with respect to controls (Fig. 5B). We also tested the PPII-S269Q/K463R mutant for alanyl-aminopeptidase activity. It displayed an activity similar to that of the PPII-S269Q/K463N mutant, reflecting that Glu-408 neutralization is sensitive to local geometry.

Because the ratio of the Ala-βNA-hydrolyzing activity of “PPII-aminopeptidases” over control activities was increased by the APN inhibitor, the aminopeptidase activity of these mutants was not due to an induction of endogenous APN activity but to an intrinsic activity of the mutant proteins. The different specific activities for PPII-aminopeptidases can be attributed to differences in substrate affinities and/or hydrolysis rates; however, their kinetic characterization was prevented by the remaining endogenous activities that contributed to substrate hydrolysis.

Our experimental results are consistent with the proposal that the positive charge in helix 2 of PepN-like aminopeptidases is neutralized by the Glu residue at loop 1, resulting in a charged Glu residue at loop 2 and a hydrogen bond property in a second carboxylate group at loop 2, a configuration similar to that found for most mammalian aminopeptidases. Probably K463N and K463Q single mutants did not hydrolyze Ala-βNA because the Ser residue in loop 1 (Ser-269) did not promote the interaction between the exopeptidase glutamate Glu-408 and the

2 R. Cruz, R. unpublished information.
terminal amine of the substrate. However, we cannot discard low aminopeptidase activity levels in PPII-Lys-463 mutants. In agreement, replacement of APA-Glu-215 with alanine or aspartate led to a strong decrease in the efficiency of hydrolysis by the mutant enzyme (36). Conversely, substitution of Ser-269 by Gln in the PPII model did not disrupt the Glu-408–Lys-463 salt bridge (not shown), possibly explaining the inability of this mutant to hydrolyze Ala-βNA.

In the presence of 1 μM actinonin, the addition of 100 μM TRH inhibited PPII-S269E and PPII-S269Q/K463N alanyl-aminopeptidase activities by 12% approximately, whereas it reduced omegapeptidase activity of wild type PPII activity by about 35% (supplemental Table 1). The lower but significant competition levels measured for both PPII-aminopeptidases may reflect that the Glu-408–Lys-463 salt bridge is not essential for binding, but that a short side chain at position 269 facilitates TRH–<Glu binding and the subsequent catalysis.

When Glu-βNA was used as substrate, hydrolytic activities of membranes from cells transfected either with pEGFP-N3, pN3/rPPII, or pcDNA3.1/HisA-rPPII were low but significant and indistinguishable from each other, in agreement with the omegapeptidase specificity of PPII. Specific activities were similar for mutants and control preparations (supplemental Fig. 2). The fact that PPII-aminopeptidases did not hydrolyze Glu-βNA provided evidence that S1 subsites are different in APA and PPII mutants, giving useful information for their identification in the M1 metallopeptidase family.

<Glu-βNA hydrolysis was undetectable in membranes from cells transfected with either pEGFP-N3, pN3/rPPII, or pcDNA3.1/HisA-rPPII. These data are consistent with the soluble nature of PPI, the addition of a PPI inhibitor in the assay buffer, and the very low catalytic turnover that PPII displays with <Glu-βNA as substrate (22). None of PPII mutants had detectable activity (not shown).

In agreement with previous data (31–34, 36), our results demonstrate that loop 1 and loop 2 (PPII-269 and PPII-408 positions) actively participate in the generation of the M1 family metallopeptidase specificity, contributing both to the correct positioning of the substrate in the active site and to the catalytic process. Whereas the exopeptidase negative charge must be neutralized to enable omegapeptidase activity, the presence of an anionic binding site promotes aminopeptidase specificity. Moreover, results with the K463R mutant imply that neutralization of the anionic binding site is sensitive to local geometry. In addition, omegapeptidase specificity is only exhibited when Ser (or shorter residues) is at position 269 (loop 1). In contrast, aminopeptidases have Glu or Gin at loop 1, but other residues are still functional (36) (Fig. 7). The fruit fly slamdance gene protein, previously classified according to
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sequence similarity as an aminopeptidase N (47), has Ser (Ser-309) and Lys (Lys-508) residues in loop 1 and helix 2 (Fig. 4A); based on our results, we predict this enzyme to be an omegapeptidase. It is worth noting that, even if there is no evidence that TRH is expressed in the fruit fly, putative functions of TRH in mammals include the homeostatic control of epileptic seizures (51). Because the slamide enzyme and the observed phenotype.

Conclusions—A major goal of protein engineering is to generate enzymes with targeted specificities. Rational redesign using homology modeling combined with site-directed mutagenesis and directed evolution have both been used to transform the substrate specificity of enzymes (52–55). Our results demonstrate that the PPII and APN models are useful tools for investigating the activity and specificity mechanisms in the M1 family and, in comparison with that of other M1 family members, to understand the difference in specificities. Our results confirm the essential role of the Glu residue within the exopeptidase motif for aminopeptidase specificity. Additionally, the correlation between bacterial and animal M1 aminopeptidases suggests conserved enzymatic mechanisms. Finally, we identify two residues in PPII active site implicated in the generation of omegapeptidase specificity. One of these is a serine residue specific for PPII (Ser-269), and the second one, Lys-463, creates a putative salt bridge with Glu-408, a salt bridge that is specific for PPII. These three residues in PPII active site implicated in the generation of omegapeptidase specificity. One of these is a serine residue specific for PPII (Ser-269), and the second one, Lys-463, creates a putative salt bridge with Glu-408, a salt bridge that is specific for PPII. Finally, we identify two residues in PPII active site implicated in the generation of omegapeptidase specificity. One of these is a serine residue specific for PPII (Ser-269), and the second one, Lys-463, creates a putative salt bridge with Glu-408, a salt bridge that is specific for PPII. These three positions (269, 408, and 463 for rat PPII) critically interact to establish amino or omega specificity in the M1 family.

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