Histidine 450 Plays a Critical Role in Catalysis and, with Ca^{2+}, Contributes to the Substrate Specificity of Aminopeptidase A

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ABSTRACT: Aminopeptidase A (EC 3.4.11.7, APA) is a 130 kDa membrane-bound protease that contains the HEXXH consensus sequence found in the zinc metalloprotease family, the zincins. In addition to the catalytic zinc atom, APA contains a Ca^{2+} ion that increases its enzymatic activity. Aligning the sequences of the mouse APA, APN, and other monozinc aminopeptidases led to the identification of a conserved histidine (His 450 in mouse APA). Replacing this residue with a phenylalanine (Phe 450) by site-directed mutagenesis resulted in markedly lower levels of APA activity and in a change in the sensitivity of APA to Ca^{2+} (the EC_{50} for Ca^{2+} was 25 μM in the wild type and only 279 μM in the mutant). Kinetic studies, with a supramaximal Ca^{2+} concentration (4 mM), showed that the K_m of the mutant enzyme for the substrate α-L-glutamyl-β-naphthylamide was 25 times higher than that of the wild type, whereas the k_{cat} value was much lower (factor of 22). Thus, overall, the wild-type enzyme had a cleavage efficiency that was 571 times higher than that of the mutant. The inhibitory potencies of two different classes of inhibitors, a glutamate thiol and a glutamate phosphonate compound, were significantly lower (factors of 19 and 22, respectively) for the mutated enzyme than for the wild-type enzyme. In contrast, inhibition by lysine thiol was unaffected. These data strongly suggest that His 450 is critical for catalytic activity and is involved in substrate binding via interaction with the P1 carboxylate side chain of the substrate. Furthermore, His 450, together with Ca^{2+}, may contribute to the substrate specificity of APA for N-terminal acidic amino acid residues.

Aminopeptidase A (EC 3.4.11.7, APA)[1] is a 130 kDa homodimeric type II membrane-bound zinc metallopeptidase that specifically cleaves in vitro the N-terminal glutamyl or aspartyl residue from peptide substrates such as angiotensin II and cholecystokinin-8 (*1*, *2*). APA is strongly expressed in peripheral tissues, the brush border of the intestinal and renal epithelial cells, and in the vascular endothelium (*3*) but is also present in brain nuclei with angiotensin II type 1 receptors and angiotensinergic nerve terminals (*4*−*6*). Studies with specific and selective APA inhibitors (*7*) have shown that APA is involved in vivo in the conversion of brain angiotensin II (Ang II) to angiotensin III (Ang III). This has led to the identification of angiotensin III as one of the main effector peptides of the brain renin−angiotensin system, controlling vasopressin release and vasopressinergic neuron activity (*8*, *9*). More recently, we demonstrated that brain Ang III, generated by APA, exerts a tonic central control over blood pressure (BP), at least in conscious spontaneously hypertensive rats, and thus, inhibition of central APA with specific and selective inhibitors leads to a decrease in BP (*10*). This suggested that APA could constitute a possible target for the treatment of hypertension. The determination of the complete amino acid sequence of APA in mice (*11*), humans (*12*, *13*), and pigs (*14*) has led to the identification of the HEXXH consensus sequence, which is found in the zinc metalloprotease family, the zincins (*15*, *16*). Overall, the amino acid sequence of APA is 22−35% homologous to those of other monozinc aminopeptidases such as aminopeptidase N (EC 3.4.11.2, APN) (*17*, *18*), aminopeptidase B (EC 3.4.11.6, APB) (*19*, *20*), thyrotropin-releasing hormone degrading enzyme (EC 3.4.19.6, TRH degrading enzyme) (*21*), leukotriene A4 hydrolase (EC 3.3.2.6, LTA4 hydrolase) (*22*), and placental leucine aminopeptidase/oxytocinase (EC 3.4.11.3, P-LAP) (*23*), with the greatest degree of sequence conservation in the region flanking the zinc-binding motif. The three-dimensional structure of APA is unknown. However, on the basis of site-directed mutagenesis studies, we proposed a general base mechanism for peptide hydrolysis for APA similar to that proposed for the bacterial endopeptidase, thermolysin (EC 3.4.24.4, TLN), deduced from X-ray crystallography studies (*24*). In our model, the zinc atom is coordinated by three ligands (His 385, His 389, and Glu 408) and a water molecule (*25*, *26*). In the presence of the substrate, the negative charge of Glu 386 polarizes the water molecule, allowing its nucleophilic attack on the carbonyl carbon of the scissile peptide bond. The resulting tetrahedral intermediate may then be stabilized by hydrogen bonds with Tyr 471 (*27*). Glu 352 would then be responsible for the exopeptidase specificity of this enzyme, as in APN (*28*). This residue interacts with the free N-terminal part of the substrate (*29*).

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[1] Abbreviations: ACE, angiotensin converting enzyme; APA, aminopeptidase A; APN, aminopeptidase N; APB, aminopeptidase B; TRH, thyrotropin-releasing hormone; LTA4, leukotriene A4; P-LAP, placental leucine aminopeptidase; TLN, thermolysin; ArgNA, α-L-arginyl-β-naphthylamide; GluNA, α-L-glutamyl-β-naphthylamide; $GluPO_3H_2$, glutamate phosphonic acid; GluSH, glutamate thiol; LysSH, lysine thiol.

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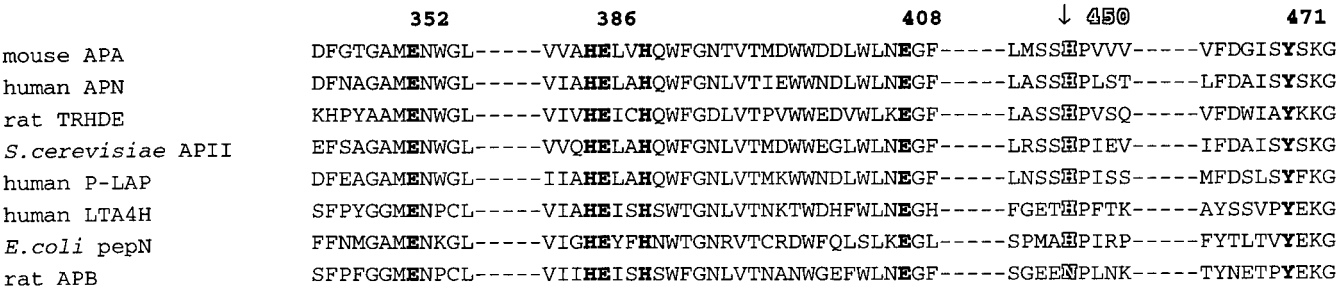


FIGURE 1: Alignment of the mouse APA amino acid sequence with those of other monozinc aminopeptidases. Alignment of the amino acid sequences of mouse APA (*11*), human APN (*55*), rat APB (*19*, *20*), *Escherichia coli* pepN (*56*), human LTA4H (*22*), *Saccharomyces cerevisiae* APII (*57*), rat TRHDE (*21*), and human P-LAP (*23*). The histidine that was mutated and the other residue in the same position are indicated in shadow letters. The three zinc ligands (*25*, *26*), the catalytic glutamate (Glu 386) (*26*), and the tyrosine (Tyr 471) residue involved in the stabilization of the transition state (*27*) are shown in bold type.

Another prominent feature of APA, different from other monozinc aminopeptidases, is its enhanced activity with Ca^{2+} (*30*). Atomic absorption spectrometry with APA has shown that one Ca^{2+} ion is associated with each monomer (*31*). Ca^{2+} increases the extent of hydrolysis of the N-terminal acidic amino acid of substrates, but decreases the extent of hydrolysis of substrates with N-terminal neutral or basic amino acids, suggesting that Ca^{2+} is involved in the binding of the side chain carboxylate of the N-terminal acidic amino acid of substrates.

Studies involving the chemical modification of APN by diethyl pyrocarbonate (DEPC) treatment have demonstrated that there is one essential histidine residue in the active site of this enzyme (*32*). Experiments involving the use of inhibitors to protect against DEPC inactivation have suggested that the modified histidine is probably located in the S1 subsite. Kinetic parameter analysis for the modified APN has indicated that this histidine may be involved in substrate binding. Alignment of the sequences of APA, APN, and other monozinc aminopeptidases (Figure 1) shows that there is a conserved histidine residue (His 450 in mouse APA) located at a constant distance (60 amino acids) from the HEXXH motif except in APB, in which the histidine residue is replaced with an asparagine residue.

Thus, His 450 may be located in the S1 subsite and may be involved in substrate binding via an interaction with the P1 side chain carboxylate of the substrate, the extent of this interaction being increased by Ca^{2+}. We tested this hypothesis by investigating the function of this residue, replacing His 450 with a phenylalanine residue by site-directed mutagenesis. We biochemically and kinetically characterized purified recombinant wild-type and mutated enzymes and determined their sensitivity to Ca^{2+}.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases and DNA-modifying enzymes were obtained from New England Biolabs (Hitchin, England) and were used according to the manufacturer's instructions. DNA Taq polymerase isolated from *Pyrococcus furiosus* (Pfu) was purchased from Stratagene (La Jolla, CA). The liposomal transfection reagent DOSPER was purchased from Roche (Mannheim, Germany). The pcDNA 3.1 His vector and the anti-Xpress antibody were purchased from Invitrogen (Groningen, The Netherlands). Immobilized cobalt affinity columns (Talon) were obtained from Clontech (Heidelberg, Germany). The synthetic substrate, α-L-glutamyl-β-naphthylamide (GluNA), was purchased from Bachem (Bunderdorf, Switzerland).

Cloning and Site-Directed Mutagenesis. The mouse cDNA encoding APA (*25*), from the expression vector SRαBP1, was inserted into the eukaryotic expression vector, pcDNA 3.1 His. The SRαBP1 vector was digested with *Sap*I and *Xho*I, and subsequently, the *Sap*I site was blunt-ended. The *Sap*I blunt-*Xho*I fragment was then inserted into pcDNA 3.1 His, digested with *Bam*HI blunt-*Xho*I. Site-directed mutagenesis was performed by polymerase chain reaction as previously described (*33*). Two overlapping regions of the cDNA were amplified separately using two flanking oligonucleotides: A (5′-CTTTTGTGAAGTCTGTCC-3′, positions 799−816) as a sense primer and B (5′-TGCCTCTTGCAGTGAATCCCA-3′, positions 1533−1553) as a reverse primer; two overlapping oligonucleotides containing the mutation were also used: C (5′-GTCTTCATTTCCAGTGGTC-3′, positions 1340−1359) as a sense primer and D (5′-GACCACTGGAAATGAAGAC-3′, positions 1340−1359) as a reverse primer. The underlined bases encode the new amino acid residue phenylalanine (Phe 450) replacing histidine (His 450) at position 450. Nucleotide numbering refers for the mouse APA sequence (*11*) deposited in GenBank (accession number M29961).

The products of the two first amplifications (A−D and B−C) were used for a further PCR with the two flanking oligonucleotides A and B. For all PCRs, Pfu (1 unit) was used (32 cycles, 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 90 s). A final 754 bp PCR product was obtained, which contained unique restriction sites *Nar*I and *Eco*RV. The fragment was purified from the agarose gel, and the 360 bp *Nar*I−*Eco*RV PCR DNA containing the mutation was used to replace the corresponding nonmutated region (*Nar*I−*Eco*RV) of the full-length APA cDNA. The presence of the mutation and the absence of nonspecific mutations were confirmed by automated sequencing using an Applied Biosystems 377 DNA sequencer and dye deoxy-terminator chemistry.

Cell Culture and Establishment of Pure CHO-K1 Cell Lines Expressing Wild-Type and Phe 450 His-APAs. CHO-K1 cells were maintained in Ham's F12 medium supplemented with 7% fetal calf serum, 0.5 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Boehringer-Mannheim, Mannheim, Germany). To establish pure cell lines expressing the polyhistidine-tagged wild-type (wild-type His-APA) and mutated (Phe 450 His-APA)

enzymes, CHO-K1 cells were transfected with 6 μg of the corresponding plasmids, using a liposomal transfection reagent (DOSPER). Transfected cells were grown in a humidified 5% CO_2/95% air atmosphere and were selected for resistance to 750 μg/mL Geneticin G418 (Gibco-BRL, Cergy Pontoise, France). Individual resistant colonies producing large amounts of APA were cloned by limiting dilution techniques.

Production and Purification of Recombinant APAs. (1) Membrane Preparation from Stable Transfected CHO Cells. Stable transfected cells (600×10^6 cells) were washed with 25 mL of ice-cold phosphate-buffered saline (PBS) and harvested by scraping into 10 mL of cold PBS. They were collected by centrifugation at 1000*g* for 5 min. All subsequent steps were performed at 4 °C. Cells were homogenized in 1 mL of ice-cold 50 mM Tris-HCl buffer (pH 7.6) containing 250 mM sucrose, 6.5 mM $MgCl_2$ with 200 μM PMSF, 2 μM pepstatin, and 20 μM leupeptin, using a Teflon−glass homogenizer (five strokes at 2000 rpm), and then centrifuged at 1000*g* for 5 min. The resulting supernatant was kept on ice, and the corresponding pellet was subjected to a second identical homogenization step and centrifugation. Both supernatants were pooled and centrifuged again at 100000*g* for 60 min. The crude membrane pellet was washed to eliminate any soluble enzymatic activity and solubilized overnight in 5 mL of 20 mM Tris-HCl buffer (pH 8) containing 100 mM NaCl, 0.5% CHAPS, and 10% glycerol (loading buffer). The solubilized preparation was centrifuged at 100000*g* for 60 min.

(2) Purification of Wild-Type and Mutated Recombinant His-APAs. The supernatant resulting from this last centrifugation contained the solubilized recombinant His-APA. It was incubated with 0.5 mL of Talon gel (Clontech) for 60 min with gentle, continuous agitation. The preparation was centrifuged at 700*g* for 5 min, and the liquid phase (flow through) was kept on ice. The resin pellet was washed with 5 mL of loading buffer, then packed into a column, and washed again with 2 mL of loading buffer containing 10 mM imidazole. The wild-type or Phe 450 His-APAs were eluted with 2 mL of the loading buffer containing 200 mM imidazole. Proteins were identified as described by Lowry et al. (*34*) using bovine serum albumin as the standard. Protein fractions were resolved by 7.5% SDS−polyacrylamide gel electophoresis as described by Laemmli (*35*). Proteins were stained with Coomassie Brilliant Blue R-250 or transferred to a nitrocellulose membrane by liquid transfer in 25 mM Tris/glycine buffer (pH 8.3) containing 10% (v/v) methanol. His-tagged recombinant proteins were detected with the anti-Xpress antibody (1:5000 dilution). The immune complex was detected with an anti-mouse antibody coupled to horseradish peroxidase (HRP) (1:12000) and resolved with ECL Western blotting reagents (Amersham).

Enzyme Assay. Wild-type and mutated His-APAs activities were determined in a microtiter plate, by monitoring the rate of hydrolysis of a synthetic substrate, α-L-glutamyl-β-naphthylamide (GluNA), as previously described (*7*). Recombinant purified wild-type (0.06 μg) and Phe 450 His-APAs (2.5 μg) were incubated at 37 °C for 20 min in the presence of various concentrations of GluNA with or without various concentrations of $CaCl_2$ in a final volume of 100 μL of 50 mM Tris-HCl buffer (pH 7.4). The kinetic parameters (K_m and k_{cat}) were determined from Lineweaver−Burk and Eadie Hofstee plots with a final GluNA concentration of 0.08−2 mM. All enzymatic assays were performed under initial velocity conditions. The sensitivities of wild-type and mutated His-APAs to D,L-glutamate thiol (GluSH is 4-amino-5-thiopentanoic acid) (*36*), L-glutamate phosphonic acid ($GluPO_3H_2$ is 4-amino-5-phosphonopentanoic acid) (*37*, *38*), and D,L-lysine thiol (LysSH is 2,6-diaminohexanethiol) (*39*) were determined by establishing dose-dependent inhibition curves using a final GluNA concentration of 0.5 mM and calculating K_i values with Graph Pad Prism 2 software. Statistical comparisons were made with a Student's unpaired *t* test. Differences were considered significant if *P* was less than 0.05. The difference in free energy ($\Delta\Delta G$) for the mutated enzyme was calculated from the equation

$$\Delta\Delta G = RT \ln[(k_{cat}/K_m)_{wild\ type}/(k_{cat}/K_m)_{mutant}]$$

RESULTS

Construction, Expression, and Purification of Recombinant His-APAs. Alignment of the sequence of APA with those of several other monozinc aminopeptidases (Figure 1) in the region surrounding the zinc binding motif showed the presence of a strictly conserved histidine residue (His 450 in mouse APA), located 60 residues downstream from the zinc-binding motif, except in APB, in which the histidine residue was replaced with an asparagine residue. We studied the function of this amino acid in APA, by replacing His 450 with a phenylalanine by site-directed mutagenesis, to abolish the charge and to keep the structure of the active site in its most native form. We first verified that the mutation does not modify expression and glycosylation of the mutant Phe 450. For this purpose, we determined by metabolic labeling of COS-7 cells transiently transfected with either the wild-type or Phe 450 mutated APAs (not tagged with the polyhistidine tail) and immunoprecipitation of cell lysate proteins with a polyclonal anti-rat APA serum of the recombinant APAs as previously described (*26*) that both proteins migrated with an identical apparent molecular mass of 130 kDa (data not shown). Then, we developed a single-step purification assay based on immobilized metal affinity chromatography (IMAC), to enable us to characterize the kinetic and inhibitor binding properties of recombinant purified APAs. A sequence encoding a polyhistidine (six residues) affinity label was added to the 5′-end of the coding region of the wild-type and mutated APAs. Stable CHO-K1 cell lines overexpressing either the wild-type or the Phe 450 APA were established, making possible the rapid purification of recombinant His-APAs from CHAPS-solubilized crude membrane preparations by a single-step procedure using a metal chelate resin column (Talon Co^{2+}). The solubilized proteins were incubated for 30 min with Co^{2+} resin, which is highly selective for proteins containing a stretch of histidine residues (*40*). The resin was then packed into a column and washed with loading buffer (Figure 2A, lane 3), and then with 10 mM imidazole (Figure 2A, lane 4). The His-APA was finally eluted from the column with 200 mM imidazole (Figure 2A, lanes 5 and 6). The eluted protein was >80% pure, as shown by SDS−PAGE (Figure 2A, lanes 5 and 6), with an overall yield of about 14% and a purification factor of 10. Western blotting with anti-Xpress antibody indicated that the purified proteins were indeed

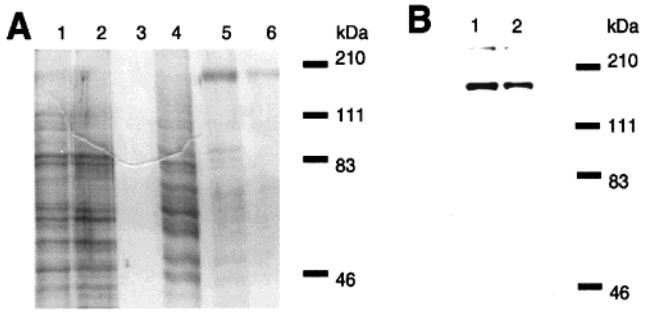


FIGURE 2: Purification of histidine-tagged mouse APA, wild-type (WT) and Phe 450 mutant, by Co^{2+} affinity chromatography. (A) Pure CHO cell lines were established that overproduced recombinant WT His-APA and Phe 450 His-APA. Crude membranes were solubilized and subjected to metal affinity chromatography on a Talon column (Co^{2+}) as described in Experimental Procedures. Material eluted from the Talon column was analyzed by SDS−PAGE in a 7.5% gel, and proteins were stained with Coomassie blue: lane 1, solubilized membranes containing the WT His-APA protein; lane 2, flow-through of the Talon column; lane 3, wash with loading buffer; lane 4, wash with 10 mM imidazole; lane 5, elution of WT His-APA with 200 mM imidazole; and lane 6, elution of Phe 450 His-APA with 200 mM imidazole. (B) Corresponding Western blot using anti-Xpress antibody: lane 1, purified WT His-APA; and lane 2, purified Phe 450 His-APA. The following molecular mass markers were used: myosin (210 kDa), β-galactosidase (111 kDa), bovine serum albumin (83 kDa), and ovalbumin (46 kDa).

Table 1: Kinetic Parameters for Wild-Type and Mutated APAs[a]

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
wild-type			
no Ca^{2+}	697 ± 91	93 ± 4	133
0.025 mM Ca^{2+}	432 ± 14	149 ± 7	345
4 mM Ca^{2+}	171 ± 8	166 ± 6	971
Phe 450			
no Ca^{2+}	NM	NM	NM
0.025 mM Ca^{2+}	5940 ± 658	1.8 ± 0.2	0.3
4 mM Ca^{2+}	4383 ± 293	7.5 ± 0.7	1.7

[a] K_m and k_{cat} values are the means ± the standard error of the mean of three separate experiments with duplicate determinations. NM, not measurable.

recombinant His-APAs (Figure 2B, lanes 1 and 2). As expected, the recombinant purified His-APAs had a slightly higher apparent molecular mass, 168 kDa, than the genuine protein (130 kDa), as previously reported (*26*, *27*, *29*) (Figure 2A, lanes 5 and 6, and Figure 2B, lanes 1 and 2).

Enzymatic Activity of Purified Recombinant His-APAs Measured under Standard Conditions. Under standard conditions, we measured the enzymatic activity of purified recombinant His-APAs using the synthetic substrate GluNA (0.5 mM) in the presence of 4 mM $CaCl_2$, a Ca^{2+} concentration inducing maximal activation of APA. We found that the replacement of His 450 with phenylalanine led to a large decrease in the extent of substrate hydrolysis as compared with that of the wild-type enzyme. We analyzed in more detail the effects of the mutation on enzymatic activity, by determining K_m and k_{cat} values in the presence of 4 mM Ca^{2+} (Table 1). The K_m value of the mutant was 25 times higher than that of the wild type. In contrast, the k_{cat} value of the mutant was reduced (by a factor of 22), resulting in a decrease by a factor of 571 of the k_{cat}/K_m ratio of Phe 450 as compared to that of the wild type. This corresponds to a change in free energy ($\Delta\Delta G$) of 3.8 kcal/mol.

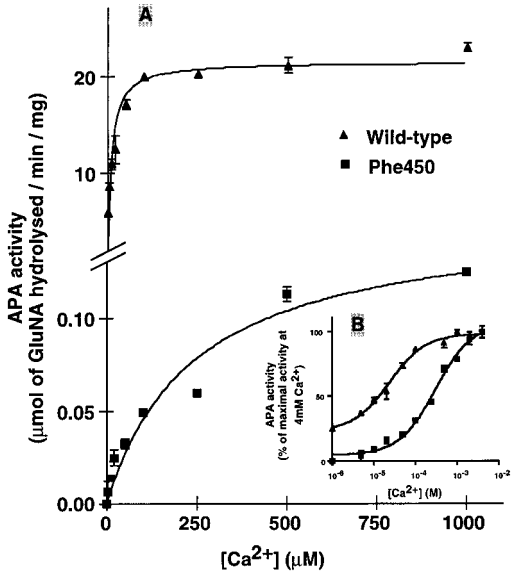


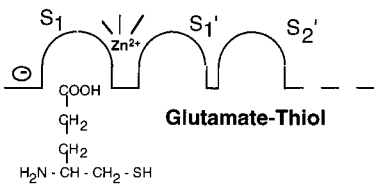
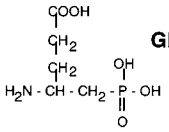
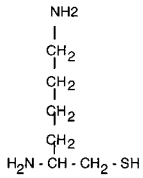
FIGURE 3: Effect of the divalent cation, Ca^{2+}, on WT His-APA and Phe 450 His-APA activities. Purified WT His-APA (0.06 μg) and purified Phe 450 His-APA (2.5 μg) were incubated at 37 °C for 20 min with 5×10^{-4} M GluNA, with various concentrations of $CaCl_2$, in a final volume of 100 μL of 50 mM Tris-HCl buffer (pH 7.4). (A) Effects of increasing Ca^{2+} concentration on recombinant APA activities. The inset (B) shows APA activities expressed as a percentage of maximal activity obtained with 4 mM Ca^{2+} for each purification vs log Ca^{2+} concentration.

Effects of Ca^{2+} Concentration on the Enzymatic Activities of Recombinant His-APAs. As APA activity is dependent on Ca^{2+} concentration (*30*, *31*, *41*), we determined the Ca^{2+} activation profile of both enzymes (Figure 3). Ca^{2+} increased the activity of the mutant by a factor of 10, and that of the wild-type enzyme by a factor of 4. The EC_{50} of Ca^{2+} for the mutant (279 μM) was about 10 times higher than that of the wild type (25 μM). Even in the presence of a supramaximal Ca^{2+} concentration, the enzymatic activity of the mutant did not reach that of the wild type and was very much lower. Kinetic parameters for the wild-type and mutated APAs were measured in the absence of Ca^{2+} or in the presence of 25 μM Ca^{2+} (corresponding to the EC_{50} of Ca^{2+} for the wild-type enzyme) and compared with the values obtained in the presence of 4 mM Ca^{2+} (Table 1). As previously described (*31*), increasing the Ca^{2+} concentration from 0 to 4 mM resulted in a decrease of the K_m value of the wild-type APA by a factor of 4, whereas the k_{cat} value was only affected by a factor of 1.8, resulting in a cleavage efficiency that was 7.3 times higher.

Analysis of Phe 450 enzymatic activity in the absence of Ca^{2+} did not allow the determination of kinetic parameters. Thus, kinetic parameters for the mutant were evaluated for Ca^{2+} concentrations of 0.025 and 4 mM (Table 1). For the mutant, there was no significant change by a factor of 1.4 in K_m values evaluated for these two Ca^{2+} concentrations. In contrast, the k_{cat} value was 4 times higher at 4 mM Ca^{2+} than at 0.025 mM, resulting in a 6-fold higher cleavage efficiency.

These results pointed to the differential behavior of recombinant enzymes with respect to Ca^{2+} activation. This led to a difference between the K_m values of the mutant and wild-type enzymes that was smaller at 0.025 mM Ca^{2+} (factor of 14) than at 4 mM Ca^{2+} (factor of 25). The opposite was

Table 2: K_i Values (μM) for Several Inhibitors with Wild-Type and Mutated APAs[a]

	Wild-type			Phe 450	
	No Ca2+	0.025 mM Ca2+	4 mM Ca2+	0.025 mM Ca2+	4 mM Ca2+
 Glutamate-Thiol	1.67± 0.28	0.36 ± 0.01	0.12 ± 0.01	5.24 ± 0.83**	2.27 ± 0.33***
 Glutamate phosphonic acid	2.87 ± 0.10	0.61 ± 0.06	0.038 ± 0.02	56 ± 13**	0.84 ± 0.04***
 Lysine-Thiol	0.46 ± 0.11	0.47 ± 0.04	7.2 ± 1.6	1.20 ± 0.08**	19.9 ± 4.4 n.s.

[a] K_i values are the means ± the standard error of the mean of six or seven separate experiments with duplicate determinations. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. n.s., no significance.

true for k_{cat} values, the difference between the mutant and wild-type enzymes being larger at 0.025 mM Ca^{2+} (factor of 83) than at 4 mM Ca^{2+} (factor of 22). As a result, the difference in cleavage efficiency for the two enzymes was twice as large at 0.025 mM Ca^{2+} (factor of 1150) as at 4 mM Ca^{2+} (factor of 571), with the mutant enzyme having the lower cleavage efficiency.

Inhibitory Potencies of GluSH, $GluPO_3H_2$, and LysSH in the Presence of Various Ca^{2+} Concentrations. We characterized further the function of the His 450 of APA, by evaluating the inhibitory potencies of various classes of compounds, which differ in their side chains and the nature of the zinc-chelating group, on GluNA (0.5 mM) hydrolysis by the wild-type and mutated enzymes at a supramaximal Ca^{2+} concentration (4 mM). The results are summarized in Table 2. GluSH, a substrate analogue, was 19-fold less potent as an inhibitor of Phe 450 than of the wild-type enzyme. Similarly, the inhibitory potency of $GluPO_3H_2$, a pseudoanalogue of the transition state, was decreased 22-fold for the mutated enzyme. In contrast, LysSH, a weak inhibitor of APA ($K_i = 7.2 \pm 1.6$ μM) possibly due of the presence of a basic side chain that fits into the S1 subsite, inhibited wild-type and mutant enzymes to a similar extent.

We have therefore examined the effect of increasing Ca^{2+} concentrations on the inhibitory potencies of these compounds (Table 2). When the Ca^{2+} concentration was increased from 0.025 to 4 mM, the affinity of GluSH for the wild-type and mutated enzymes was similarly enhanced (2−3-fold). In contrast, the affinity of LysSH for both recombinant enzymes decreased 15-fold. $GluPO_3H_2$ affinity was 16 times higher for the wild type and 66 times higher for the Phe 450 mutant at 4 mM Ca^{2+} than at 0.025 mM Ca^{2+}.

We then compared the extent of inhibition by GluSH and LysSH, two inhibitors differing only in the nature of the P1 side chain amino acid (acid or basic), for both recombinant enzymes at two Ca^{2+} concentrations. We came to two conclusions. (i) For the wild-type enzyme, GluSH and LysSH were inhibitors with similar potencies at 0.025 mM Ca^{2+}, whereas at 4 mM Ca^{2+}, GluSH was 60 times more potent as an inhibitor than LysSH. (ii) For the mutant enzyme, the effect of increasing the Ca^{2+} concentration was much less marked; at 0.025 mM Ca^{2+}, GluSH was 4 times less potent than LysSH, whereas at 4 mM Ca^{2+}, GluSH was only 9 times more potent than LysSH.

DISCUSSION

APA was originally described as a calcium-stimulated enzyme (*30*). Subsequently, molecular cloning and site-directed mutagenesis studies (*11*−*13*, *25*, *26*) showed that APA is a zinc-dependent enzyme, leading to the proposal of a molecular mechanism of action (*26*, *27*), similar to that of TLN (*24*). The study presented here allowed us to complete this model by including the presence of a histidine residue (His 450 in mouse APA) that is critical for catalytic activity and involved in substrate binding via hydrogen bonds with the negatively charged P1 side chain carboxylate of the substrate. Our results suggest that histidine 450, with Ca^{2+}, contributes to the substrate specificity of aminopeptidase A.

As the structure of monozinc aminopeptidases is unknown, site-directed mutagenesis remains an important tool for probing the organization of the active site of these enzymes. Comparison of the sequences of APA, APN, and other monozinc aminopeptidases identified a conserved histidine residue (His 450 in mouse APA). This histidine is not present in aminopeptidase B, a chloride-activated aminopeptidase with a strong specificity for N-terminal basic residues (*42*, *43*). Chemical modification of APN by diethyl pyrocarbonate treatment had previously shown that there is an essential histidine residue at the active site, probably located in the S1 subsite and involved in substrate binding (*32*). Thus, we

investigated the function of His 450 in APA by replacing this residue with a phenylalanine by site-directed mutagenesis. We added a polyhistidine affinity label to the amino terminus of the wild-type and mutated APA amino acid sequences (His-APA), facilitating the simple and rapid purification of recombinant enzymes from crude solubilized membrane preparations by metal chelate affinity chromatography. Purified wild-type His-APA had kinetic parameters and inhibitor binding properties similar to those reported for the native wild-type APA (*26*, *27*, *29*). This shows that the addition of the histidine tag resulted in a functional enzyme that had the same properties, including sensitivity to Ca^{2+}, as the nontagged enzyme, as previously described for other proteins (*44*−*46*). The production of the histidine-tagged wild-type and Phe 450 APAs in established CHO cell lines provided a convenient source for the analysis of the biochemical properties of the purified recombinant enzymes.

Western blot analysis of recombinant enzymes purified from a crude membrane preparation showed that Phe 450 and wild-type APAs are similarly routed to the plasma membrane and glycosylated, suggesting that the replacement of His 450 with a phenylalanine residue does not affect the biosynthesis, the folding, or the stability of the resulting protein. The mutation led to a marked decrease in the extent of hydrolysis of the synthetic substrate, GluNA, under standard conditions [i.e., in the presence of a Ca^{2+} concentration (4 mM) inducing maximal activation of mouse recombinant APA (*31*)]. The K_m value of the mutant Phe 450 was 22 times higher than that of the wild-type enzyme, indicating that His 450 is involved in substrate binding. The k_{cat} value of Phe 450 was lower than that of the wild type by a factor of 25, lowering the k_{cat}/K_m ratio by a factor of 571, indicating that His 450 is also involved in catalytic activity.

These changes correspond to a decrease in binding energy ($\Delta\Delta G$) of 3.8 kcal/mol, consistent with the loss of the strong hydrogen bond between an uncharged side chain of the enzyme and a charged group of the substrate (*47*). The substrate that was used, GluNA, only fits into the S1 subsite. So the hydrogen bond that is concerned may be between the imidazole group of His 450 and the negatively charged P1 side chain carboxylate of the substrate.

We investigated the function of His 450 in substrate binding, by evaluating the mode of binding of various inhibitors at the active sites of the wild-type and mutated APAs. All the inhibitors that were used interact with the S1 subsite but differ in the nature of their zinc-chelating group and in the nature of their side chain. (i) The two β-amino thiol inhibitors, GluSH (*36*) and LysSH (*39*), chelate the zinc ion via their thiol groups and differ in having an N-terminal acidic (GluSH) or basic (LysSH) side chain. (ii) The pseudoanalogue of the transition state, the α-aminophosphonate inhibitor $GluPO_3H_2$ (*36*, *37*), is a potent inhibitor of APA ($K_i = 8$ nM), interacting with the active site via one of its phosphoryl oxygen groups, with the zinc ion and the phenolic oxygen of Tyr 471 (*27*). In the presence of 4 mM Ca^{2+}, the mutation of His 450 to Phe 450 resulted in significantly lower affinities of GluSH and of $GluPO_3H_2$ but did not affect that of LysSH. This demonstrates that the side chain carboxylate of GluSH and of $GluPO_3H_2$ interacts with the imidazole group of His 450, whereas the amino group of the side chain of LysSH cannot establish such an interaction. These data are consistent with the involvement of His 450 in substrate and inhibitor binding at the S1 subsite, via interaction with the N-terminal acidic residue of these compounds.

The involvement of His 450 in the binding of the N-terminal amino acid residue of substrates suggests that it is involved in the specificity of APA for substrates with N-terminal acidic amino acids. Consistent with this hypothesis, the replacement of His 450 with Phe 450 reduced the ability of the mutant enzyme to discriminate between thiol inhibitors with N-terminal acidic or basic amino acid residues.This is illustrated by the smaller difference (factor of 9) between the K_i values of LysSH and GluSH for the mutant enzyme compared to those for the wild-type enzyme (factor of 60) under standard enzyme assay conditions (4 mM Ca^{2+}). Moreover, the comparison of the activities of purified recombinant APAs using GluNA (500 μM) and ArgNA (500 μM) as substrates in the absence of Ca^{2+} shows that the Phe 450 mutant (ratio of GluNA to ArgNA hydrolysis of 2) is more tolerant of a basic substrate than the wild-type APA (ratio of GluNA to ArgNA hydrolysis of 14) (data not shown).

These results are consistent with the His 450 in APA having a role similar to that proposed for histidine 213 (His 213) in the glutamic acid endopeptidase of *Streptomyces griseus* (EC 3.4.21.82, SGEP), the three-dimensional structure of which is known (*48*). SGEP has a distinct preference for glutamate in the P1 position of the substrate, and this preference is ensured by histidine 213 and two serine residues (Ser 192 and Ser 216) located in the S1 subsite (*49*). It is interesting to note the presence in the mouse APA sequence of two serine residues (Ser 448 and Ser 449) located near His 450 which could play a similar role, remaining to be demonstrated.

A prominent feature of APA is its stimulation by Ca^{2+}. Danielsen et al. (*31*) showed that each APA monomer contains one Ca^{2+} ion. Wild-type APA was strongly activated by Ca^{2+}, with a 3−5-fold increase in enzymatic activity if the Ca^{2+} concentration was increased from 0 to 4 mM, as previously reported for the native enzyme (*30*). The stimulatory effect of Ca^{2+} on wild-type APA is due to a decrease in the K_m of the substrate GluNA by a factor of 4, whereas k_{cat} is not significantly affected as shown in other studies (*31*, *50*). Ca^{2+} not only stimulates the hydrolysis of N-terminal acidic amino acids but also inhibits the hydrolysis of N-terminal neutral or basic amino acid residues, imparting greater specificity to the enzyme (*41*). Danielsen et al. have suggested that Ca^{2+}, in the S1 pocket of APA, ensures the specificity of APA for substrates with N-terminal acidic amino acids, by interacting with the side chain carboxylate of the substrate (*31*).

As His 450 is involved in the substrate specificity of APA, we investigated the effect of the mutation on Ca^{2+} activation. The mutated enzyme was sensitive to Ca^{2+} activation, but the concentration required for half-maximal activation (EC_{50}) was much higher than that for the wild-type enzyme (EC_{50} for the wild type of 25 μM vs EC_{50} for Phe 450 of 275 μM). Therefore, the mutated enzyme seems to be able to bind the Ca^{2+} ion, but Ca^{2+} stimulates substrate hydrolysis less efficiently. This suggests that the interaction between Ca^{2+} and the side chain carboxylate of the substrate (*31*) is decreased by the mutation, which abolishes the hydrogen bond between His 450 and the negatively charged side chain carboxylate, possibly causing a change in substrate positioning.

The cleavage efficiency of the mutated enzyme was 6 times higher in the presence of a high concentration of Ca^{2+} (at 4 mM Ca^{2+}, $k_{cat}/K_m = 1.7$ mM^{-1} s^{-1}) than in the presence of a low concentration of Ca^{2+} (at 0.025 mM Ca^{2+}, $k_{cat}/K_m = 0.3$ mM^{-1} s^{-1}), suggesting that Ca^{2+} may partially compensate for the mutation. Nevertheless, in contrast to the situation for the wild-type enzyme, Ca^{2+} activation of the mutant enzyme is due to an increase in substrate hydrolysis velocity (k_{cat}), revealing a different Ca^{2+} activation mechanism.

This again was reflected by the fact that increasing the Ca^{2+} concentration from 0.025 to 4 mM increased the ratio of K_i values for LysSH to GluSH much less for the mutant enzyme than for the wild-type enzyme. These data therefore suggest that His 450 and Ca^{2+} act in synergy to ensure the substrate specificity of APA. Thus, in the absence of His 450, Ca^{2+} is less able to facilitate the discrimination by APA between acidic and basic N-terminal amino acid residues.

This observation can be compared to those reported on the mode of Ca^{2+} activation of the *S. griseus* aminopeptidase (SGAP). SGAP is a rare example of an extracellular metalloprotease, the activity with various substrates (essentially neutral substrates) (*51*, *52*) of which is increased by Ca^{2+} binding. A recent study on this enzyme has shown that the mechanism of Ca^{2+} activation depends on the substrate (*53*). Crystallography has shown that SGAP contains two zinc atoms and that the Ca^{2+}-binding site is located 25 Å from the active site, suggesting that Ca^{2+} may regulate the activity of SGAP via changes in electrostatic potential distribution (*54*). A similar mechanism of activation may exist in APA, in which the ionization of the imidazole of His 450 may be regulated by Ca^{2+}.

Thus, His 450, located in the S1 subsite in APA, seems to be involved in catalysis and substrate binding via hydrogen bonding between the imidazole group of the histidine residue and the negatively charged side chain carboxylate of the N-terminal acidic amino acid of substrates. In this way, His 450 and Ca^{2+} together control the specificity of APA for substrates with N-terminal acidic residues.

Further studies, including molecular modeling and crystallographic analysis of the protein, are required to ascertain the exact role of His 450 in APA.

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