Characterization of Glu^{350} as a Critical Residue Involved in the N-Terminal Amine Binding Site of Aminopeptidase N (EC 3.4.11.2): Insights into Its Mechanism of Action

Nathalie Luciani, Cynthia Marie-Claire, Emmanuel Ruffet, Ann Beaumont, Bernard P. Roques, and Marie-Claude Fournié-Zaluski*

Département de Pharmacochimie Moléculaire et Structurale, INSERM U266−CNRS URA D 1500, UFR des Sciences Pharmaceutiques et Biologiques, 4, avenue de l'Observatoire, 75270 Paris Cedex 06, France

Received July 14, 1997; Revised Manuscript Received November 6, 1997

ABSTRACT: The molecular components ensuring the strict exopeptidase action of aminopeptidase N (APN) and related zinc aminopeptidases of the M_1 family have not yet been clearly established. The specific recognition of the N-terminal amino acid of the substrates by the enzymes has been proposed to involve either the complexation of the free amino group by the catalytic zinc ion or an interaction with an anionic binding site, which could be constituted by an aspartate or glutamate residue. To investigate the existence of such an ionic binding site, site-directed mutagenesis experiments have been performed on acidic residues of pig APN. Given that aminopeptidases of the M_1 family are likely to have a common mechanism of action, only strictly conserved residues were mutated. As compared to the wild-type enzyme, the mutation D^{220}E led only to slight modifications in the kinetic parameters of the enzyme and in the K_i values of various inhibitors, indicating that this residue is not critically involved in the hydrolytic mechanism. In contrast, the mutations E^{350}Q and E^{350}D induced a large decrease in enzyme activity, essentially due to modifications in k_{cat}, whereas the E^{350}A mutation led to an almost completely inactive enzyme. Moreover, among the inhibitors tested, only those acting as transition state analogs showed significant increases in their K_i values. These data are in favor of E^{350} belonging to the "anionic binding site" in APN. A mechanism of action, derived from that of thermolysin, is proposed for these aminopeptidases, which explains the importance of E^{350} in transition state formation, rather than in the Michaelis complex.

Aminopeptidase N (EC 3.4.11.2, APN)[1] is a monomeric or homodimeric type II membrane-bound zinc metallopeptidase expressed in many tissues, with highest levels in intestinal and kidney brush border membranes (*1*). APN has also been found in the brain, the lung, blood vessels, and primary cultures of fibroblasts (*2*) and is identical to human lymphocyte surface cluster differentiation antigen, CD 13 (*3*). Pig APN has been reported to act as a major receptor for transmissible gastroenteritis virus (*4*), and the human form is a receptor for human coronavirus 229E (*5*). The enzyme releases neutral and basic amino acids from the N-termini of peptide substrates and has been shown to be involved in enkephalin metabolism in the brain (*6*, *7*) and to participate in the enzymatic cascade of the renin−angiotensin system in the brain and periphery by cleaving angiotensin III (*8*). APNs from human intestine, rat kidney, rabbit kidney, and pig intestine have 80% sequence identity (*4*, *9*−*12*). APN is a transmembrane protein comprising 963 to 967 amino acids, depending on the species, with a small N-terminal cytoplasmic tail (9 or 10 amino acids), a 23- or 24-amino acid transmembrane domain, and a large extracellular ectodomain that contains the active site.

APN is a member of the M_1 family of zinc metallopeptidases (*13*), which also includes related enzymes such as aminopeptidase A (APA) (*14*), aminopeptidase B (APB) (*15*, *16*), leukotriene A4 hydrolase (LTA_4) (*17*), the puromycin-sensitive aminopeptidase (PSA) (*18*), thyrotropin-releasing hormone degrading enzyme (THR-de) (*19*), the rat vesicle protein Vp 165 (*20*), and *Escherichia coli* pepN (*21*) (Figure 1). Sequence comparisons and site-directed mutagenesis experiments (*22*−*25*) have suggested that these enzymes have the same mode of zinc coordination as a large group of zinc endopeptidases typified by thermolysin (EC 3.4.24.28, TLN), a bacterial protease which has been crystallized with a variety of different inhibitors (*26*). In thermolysin, the zinc is coordinated by three residues, two histidines found in a consensus sequence HEXXH, and a glutamate residue 19 residues C-terminal to the second histidine. The glutamate of the consensus sequence acts as a general base in catalysis. All aminopeptidases of the M_1 family contain the HEXXH consensus sequence (*27*), and the third zinc-binding ligand has been shown by site-directed mutagenesis to be a glutamate 18 residues C-terminal to the second histidine (Figure 1) (*28*). In addition, chemical modifications of APN (*29*) have indicated that the general organization of its active

* Author to whom correspondence should be addressed. Telephone: (33)-01-53.73.96.88./89. Fax: (33)-01-43.26.69.18.

[1] Abbreviations: APN, aminopeptidase N; APA, aminopeptidase A; APB, aminopeptidase B; LTA_4, leukotriene A4 hydrolase; PSA, puromycin-sensitive aminopeptidase; THR-de, thyrotropin-releasing hormone degrading enzyme; Vp 165, rat vesicle protein; TLN, thermolysin; LAP, leucine aminopeptidase; TIMP-1, tissue inhibitor of metalloproteinases.

S0006-2960(97)01705-4 CCC: $15.00
Published on Web 01/13/1998

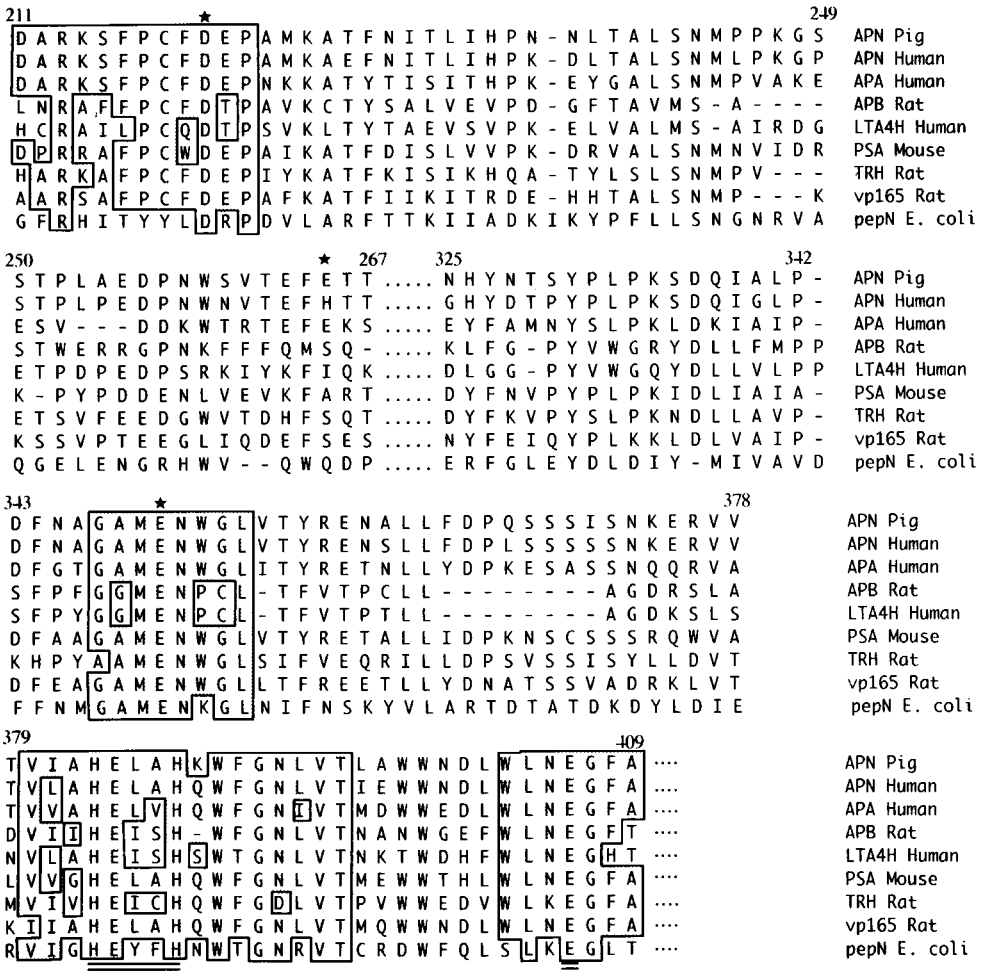


FIGURE 1: Comparison of pig APN sequences with homologous aminopeptidases. Alignment of the sequences of pig APN (*4*), human APN (*9*), human APA (*14*), rat APB (*15*), human LTA_4H (*17*), mouse PSA (*18*), rat TRH-de (*19*), rat vp 165 (*20*), and *E. coli* pepN (*21*). The consensus sequence is underlined, highly conserved domains are boxed, and residues that have been mutated by site-directed mutagenesis are marked ★.

site resembles that of thermolysin with the involvement of Y, R, H, and E/D residues in the hydrolytic activity of the enzyme. Moreover, the development of APN inhibitors has shown that the S_1', S_2' subsites of this peptidase prefer hydrophobic side chains, as do the corresponding subsites in TLN (*30*). However, no structural data on this important class of aminopeptidases is available and, in particular, the element responsible for the "amino-terminal" specificity of these enzymes has not been defined.

Two mechanisms of action have been proposed for aminopeptidases in general. In the first one (*31*), the metallic ion, which at the time was thought to be either a Mn^{2+} or a Mg^{2+} cation, is complexed by both the free amino group and the carbonyl of the peptide bond to be cleaved. In the second mechanism, an ionic binding site recognizing the α-amino group has been suggested (*32*, *33*). Recently, crystallographic studies of complexes between bestatin (*34*, *47*) or amastatin (*35*) and the cytosolic leucine aminopeptidase (LAP) have shown that the free amino group, probably under its unprotonated form in physiological conditions, interacts with both the zinc ion and an aspartate residue of the active site via a hydrogen bond. These data suggest that the two mechanisms proposed could be complementary.

For a better understanding of the mechanism of action of the M1 family of aminopeptidases, it is necessary to verify the presence of an ionic binding site which could be involved in the recognition of the free amino group of substrates or inhibitors and is likely to be conserved in all members of the family. A sequence comparison of these enzymes (Figure 1) showed only two possibilities, D^{220} and E^{350}, both in highly conserved regions, with E^{350} being close to the consensus sequence (Figure 1). Conservative mutations of these residues were therefore carried out (D → N, E → Q/D), as well as the more drastic change E → A, and the enzymatic properties of the mutated enzymes analyzed. In addition, a nonconserved acidic residue E^{265} was mutated to Q as a control. The results reported in this study, in particular the strong reduction in the k_{cat} value for the APN substrate Leu-enkephalin and the 100-fold decrease in IC_{50} values of transition-state inhibitors induced by the conservative mutations of E^{350}, suggest that this residue forms the ionic site in APN and plays a major role in the mechanism of action of this type of exopeptidase.

MATERIALS AND METHODS

Materials. The pTEJ 4-APN plasmid and the APN antiserum were kindly provided by Dr. B. Delmas (INRA, France). $pCDNA_3$ is a eukaryotic expression vector available from Invitrogen Corp. First (UK). Purified pig APN was purchased from Boehringer (France). Restriction and modifying enzymes and the Transformer Site Mutagenesis kit

were, respectively, from New England Biolabs and Clontech products and obtained from Ozyme (France). The sequenase enzyme version 2.0 (United States Biochemical) and [α-^{33}P]-dATP were purchased from Amersham (France). Oligonucleotides were synthesized by Genosys (UK). Cell culture products were from Life Technologies (France). Leucine enkephalin (tyrosyl-glycyl-glycyl-phenylalanyl-leucine) and [*tyrosyl*-3,5-^{3}H]leucine enkephalin (designated [^{3}H]leucine enkephalin) were, respectively, purchased from Bale Biochimie (France) and Izinta (Hungary). Captopril and bestatin were purchased from Sigma (France). Retrothiorphan (*36*), Phe-thiol **1** (*37*), the phosphinopeptides **3** and **4** (unpublished results), and the hydroxamate **5** (RB 38A) (*38*) used in this study were prepared in the laboratory as previously described.

Construction of Expression Plasmid and Site-Directed Mutagenesis. The pTEJ 4-APN plasmid was digested with endonuclease *Xba*I to release a 2889 bp DNA fragment encoding the full-length enzyme. The cDNA fragment was then subcloned in the *Xba*I restriction site of the pCDNA$_3$ vector. The correct orientation was determined by digestion with endonuclease *Sma*I, followed by sequencing to verify the cDNA insertion.

Double-strand mutagenesis was carried out using the Transformer Site Directed Mutagenesis kit following the manufacturer's instructions. Oligonucleotides were for the following replacements: D^{220} → N, E^{265} → Q, and E^{350} → Q, D, or A. The presence of the expected mutations and the absence of nonspecific mutations were confirmed by sequencing the complete coding sequence.

Transfection of COS-7 Cells. COS-7 cells were grown in Dulbecco's modified Eagle's medium complemented with 10% fetal calf serum, and 10 × 10^6 cells were transfected with 50 μg of plasmid by electroporation (250 V and 1000 μF, Biorad electroporator). Each pool of transfected cells was incubated in a 10 cm Petri dish at 37 °C for 48 h. The cells were then washed twice and harvested by scraping in phosphate-buffered saline. After rapid centrifugation at 2000*g*, the cell pellet was resuspended in ice-cold 50 mM Tris-HCl, pH 7.4, and membranes were prepared by homogenizing the cells in a Teflon-glass homogenizer followed by centrifugation at 100000*g* for 30 min at 4 °C. This step was repeated and the final membrane pellet was solubilized in 50 mM Tris-HCl, pH 7.4, containing 1% (w/v) *n*-octyl glucoside for 1 h on ice, before centrifuging at 20000*g* for 15 min. Aliquots of the supernatant were stored at 4 °C and used for Western blot analysis and enzyme assays. Under these conditions, enzyme activity was found to be stable for at least one month.

Western Blotting. Proteins were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (*39*) and electroblotted onto nitrocellulose filters. The blots were rinsed with TBS-Tween 20 buffer, then incubated with superblock blocking buffer (Pierce), antiserum raised against pig APN, and finally with an anti-mouse Ig, horseradish peroxidase-linked antibody from sheep (Amersham Corp.). Peroxidase activity was revealed with a chemioluminescent detection kit from Amersham Corp. Increasing concentrations of purified pig APN were used to construct a calibration curve for the quantification of wild-type and APN mutants.

Enzyme Assay. APN activity and the K_i values of inhibitors were determined using 20 nM [^{3}H]leucine enkephalin as a substrate at 37 °C in a total volume of 100 μL of 50 mM Tris-HCl, pH 7.4 (containing 1 μM captopril and 1 μM retrothiorphan, inhibitors of angiotensin-converting enzyme and neutral endopeptidase, respectively). The reaction was stopped by adding 10 μL of 0.5 M HCl, and the metabolite [^{3}H]tyrosine was separated using porapaq Q beads from Waters (France), as previously described (*40*). In all cases, the reactions were stopped when substrate degradation was around 5%. IC_{50} values were considered to be equal to K_i values, as the concentration of the substrate used was less than its K_m for the enzyme. K_m and k_{cat} values were determined by the isotopic dilution method and calculated using the program ENZFITTER (Biosoft). Addition of the selective APN inhibitor Phe-thiol **1** (10^{-6} M) led to a complete inhibition of [^{3}H]tyrosine production, indicating the absence of contaminant aminopeptidases.

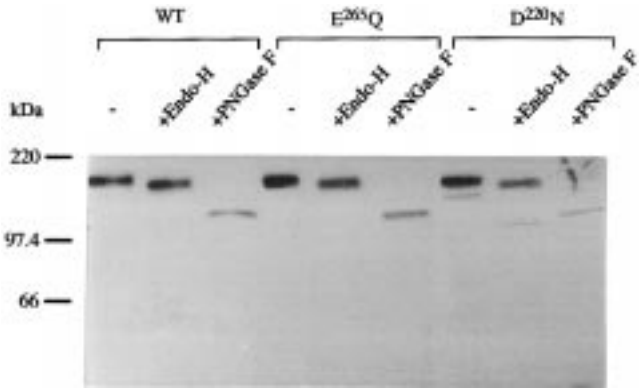


FIGURE 2: Analysis of wild-type and mutated E^{265}Q and D^{220}N APN by Western blots as described in Materials and Methods: enzymes were loaded before or after digestion with endoglycosidase H (endo-H) for 1 h at 37 °C or with *N*-glycosidase F (PNGase F) for 1 h at 37 °C. The positions of molecular mass markers are indicated on the left side.

RESULTS

Expression of Wild-Type and Mutated APN in COS-7 Cells. In order to study the possible role of conserved acidic residues in APN, Asp^{220}, Glu^{350}, and the nonconserved Glu^{265} were mutated using the pcDNA$_3$-APN vector. Wild-type and mutated APN cDNAs were transiently transfected in COS-7 cells. Transfection under the same conditions with control plasmid pcDNA$_3$ was also carried out to determine the background hydrolytic activity. The presence and levels of enzymes in cell extracts were determined by Western blot analysis. Figures 2 and 3 show that the nonmutated and the five mutated enzymes had similar apparent molecular masses of 172 kDa and were expressed at similar levels. Correct glycosylation of the enzymes was verified by digestion with endoglycosidase H and N-glycosidase F (Figures 2 and 3). The apparent molecular masses of wild-type and mutant enzymes were reduced by around 10 kDa after endoglycosidase H digestion and 45 kDa after *N*-glycosidase digestion. A minor band of 140 kDa was also observed in the case of D^{220}N-APN which was endoglycosidase H susceptible and probably corresponds to an immature form of the enzyme.

Enzymatic Activity of Wild-Type and Mutated Enzymes. Initial studies were carried out using the relatively insensitive APN substrate, alanine-*p*-nitroanilide ($K_m = 406 \pm 10$ μM) (*41*). However, due to the decreased activity of three of the mutant enzymes, this compound was replaced by the

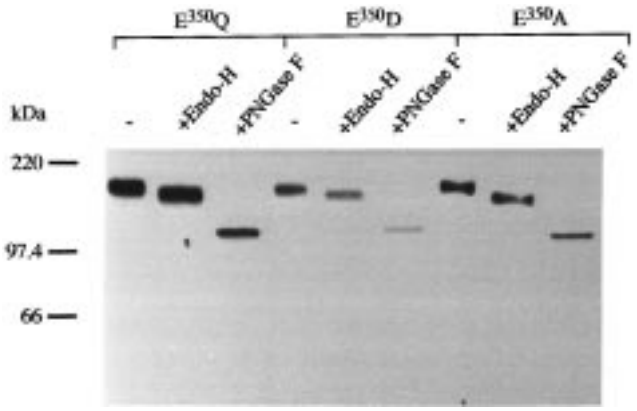


FIGURE 3: Analysis of mutated $E^{350}Q$, $E^{350}D$, and $E^{350}A$ APN by Western blots as described in Materials and Methods: enzymes were loaded before or after digestion with endoglycosidase H (endo-H) for 1 h at 37 °C or with *N*-glycosidase F (PNGase F) for 1 h at 37 °C. The positions of molecular mass markers are indicated on the left side.

Table 1: Kinetic Parameters for the Hydrolysis of [^{3}H]Leucine Enkephalin by Wild-Type and Mutated APNs[a]

enzymes	K_m (M)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
wild-type	56.7 ± 3.9	1989 ± 14	35.1
$D^{220}N$	210 ± 6	410.6 ± 9.4	1.95
$E^{350}Q$	227 ± 38	4.1 ± 1.5	0.018
$E^{350}D$	79.3 ± 4.9	14.4 ± 0.04	0.18
$E^{350}A$	*b*	*b*	*b*
$E^{265}Q$	69.3 ± 7.1	2255 ± 320	32.5

[a] Reactions were carried out as described in Materials and Methods, values are the mean ± SEM from at least three independent determinations. [b] Not measurable.

physiological and more efficient APN substrate, [^{3}H]leucine enkephalin (*40*).

Under the experimental conditions used, there was no detectable formation of [^{3}H]tyrosine from this pentapeptide substrate by membrane preparations of COS-7 cells transfected with the control plasmid $pcDNA_3$. The K_m and k_{cat} values for the degradation of [^{3}H]leucine enkephalin by wild-type and mutated APNs are reported in Table 1. It was not possible to determine the kinetic parameters of the $E^{350}A$ mutant which can be considered as completely inactive. The K_m value observed for the mutant $D^{220}N$-APN was 4-fold higher than that of the wild-type enzyme with a 5-fold decrease in k_{cat} value, leading to a decrease in the specificity factor, k_{cat}/K_m, of 20-fold.

The K_m value of the substrate for $E^{350}Q$-APN was also 4-fold higher than that for the wild-type enzyme. Combined with the k_{cat} value which was significantly reduced (485-fold), the ratio k_{cat}/K_m was thus about 2000-fold lower than that of the wild-type enzyme. For $E^{350}D$-APN, the K_m value was not significantly modified and the k_{cat} value was about 140-fold lower than that for the wild-type enzyme, leading to a decrease in k_{cat}/K_m of nearly 200-fold. The K_m and k_{cat} values for the control mutant $E^{265}Q$-APN were identical to those found for the wild-type enzyme.

Potencies of Various Classes of Inhibitors to Wild-Type and Mutated Enzymes. The inhibitory potencies of several APN inhibitors for the wild-type and mutated enzymes are reported in Table 2. All the molecules tested, which differ by their structure and/or their zinc chelating groups, had similar K_i values for both the control mutant $E^{265}D$-APN and the wild-type enzyme.

For the other three mutations, a decrease in K_i for inhibitor **1** was found in all cases. For bestatin **2** and the phosphinic inhibitor **3**, large modifications were observed with the $E^{350}D$ mutant, resulting in a more than 100-fold increase in their K_i values. For $E^{350}Q$-APN there was only a slight increase (factors of 6 and 5) for compound **3** and bestatin **2**, respectively. For the mutant $D^{220}N$-APN, the inhibitory potency of compounds **2** and **3** was only slightly decreased, by factors of 3 and 5, respectively. No significant change in the inhibitory potency of the phosphonic inhibitor **4** and of the hydroxamate **5** (RB 38A) was found for wild-type and mutated enzymes. As the affinity of these inhibitors is essentially due to the binding of their hydroxamate or phosphinic groups to the zinc atom (*26*), the results obtained with compounds **4** and **5** mean that the various mutations have not significantly modified the coordination of the zinc atom in the catalytic site.

DISCUSSION

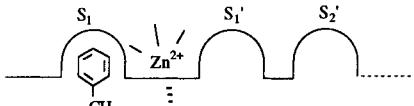
The aim of this work was to investigate, using site-directed mutagenesis, the mechanism of substrate hydrolysis and the molecular components—aspartate or glutamate residues—which ensure a strict exopeptidase action for APN and related enzymes. A sequence alignment of the enzymes belonging to the APN family shows only two possible candidates, E^{350} (close to the consensus sequence) and D^{220}. The mutation $E^{350}A$ led to a nonmeasurable activity, at least 10000-fold lower than that of the wild-type APN. Consequently, conservative mutations of E^{350} and D^{220} were carried out in order to try to maintain some measurable activity. The $E^{350}Q$ mutation removes the negative charge of the glutamate, but the possibility of hydrogen bond formation is conserved, while the $E^{350}D$ mutation displaces the negative charge, due to the shorter side chain of the aspartate. A nonconserved residue, E^{265}, was also mutated to act as a control.

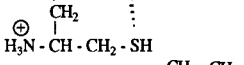
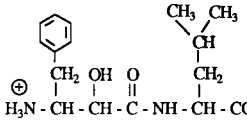
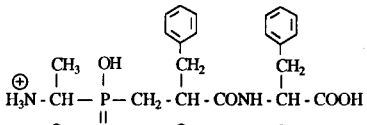
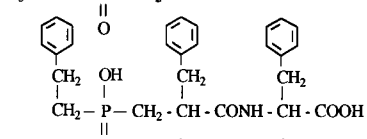
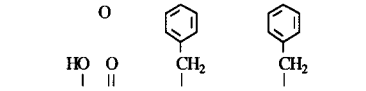
The kinetic constants were measured for the wild-type and mutant enzymes with the pentapeptide [^{3}H]leucine enkephalin as substrate. In addition, the effects of the mutations were also evaluated by studying the potency of various types of selective APN inhibitors, which contain various types of zinc chelating groups. These molecules can be grouped into different categories: (i) the β-aminothiol **1**, which allows both the amine binding site and the S_1 subsite to be investigated; (ii) compounds **2** and **3**, which are transition state analogs and interact with the S_1, S_1' and S_1, S_1', S_2' subsites of the enzymes, respectively; (iii) compound **5**, which recognizes only the S_1' and S_2' subsites (Table 2).

As expected, no modification in activity or inhibitor binding was found by replacing the nonconserved E^{265} by Q. The $D^{220}N$ mutation induced an about 20-fold decrease in k_{cat}/K_m, due to both a small increase in K_m (~4-fold) and a similar decrease in k_{cat} (~5-fold) (Table 1). Furthermore, the affinities of the inhibitors tested (Table 2) were only slightly modified by, at most, a factor of 10. Taken together, these data indicate that D^{220} plays a minor role in the activity of APN. This residue could be located in the vicinity of the active site, but is not directly involved in the catalytic process.

In contrast, mutating E^{350} to either Q or D led to large decreases in k_{cat}/K_m values of 2000-fold and 200-fold,

Table 2: K_i Values (μM) of Inhibitors for Wild-Type and Mutated APN[a]



		WILD-TYPE	D^{220}N	E^{350}Q	E^{350}D	E^{265}D
1		0.039 ± 0.002	0.23 ± 0.06	0.15 ± 0.04	0.74 ± 0.05	0.038 ± 0.003
2		2.1 ± 0.3	7.1 ± 0.6	10 ± 3	> 100 (17% at 10^{-4}M)	2.8 ± 0.5
3		0.0007 ± 0.0002	0.0039 ± 0.0007	0.0044 ± 0.0006	0.12 ± 0.003	0.00036 ± 0.00006
4		19 ± 1	21 ± 1	15 ± 2	26 ± 3	20 ± 4
5		0.29 ± 0.02	0.46 ± 0.04	0.13 ± 0.02	0.34 ± 0.03	0.23 ± 0.02

[a] Legends for compounds are the following: (**1**), Phe-thiol; (**2**), Bestatin; (**3** and **4**), Phosphinopeptides; (**5**), Hydroxamate RB 38A. Data are the mean ± SEM from at least three independent determinations.

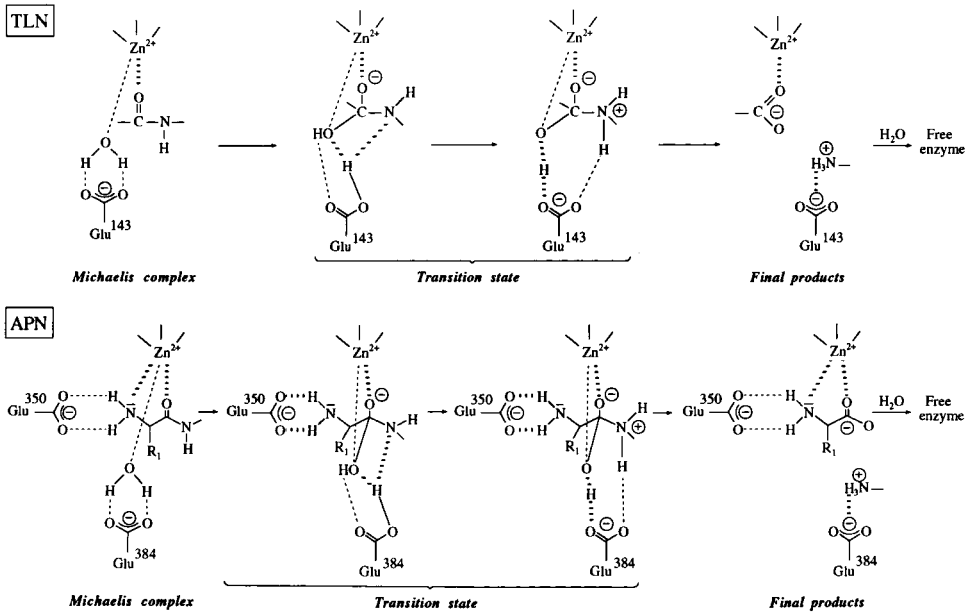


FIGURE 4: Comparison of the mechanism of action of thermolysin (top) as described by Matthews (*26*), and the proposed mechanism of action for aminopeptidases of the M_1 family (bottom).

respectively, essentially due to decreases in k_{cat}. In addition, mutating E^{350} to A reduced dramatically the activity to levels at which it was barely detectable. The results are in favor of E^{350} forming the anionic site in APN, with the presence of the negative charge being essential for enzyme activity. These conclusions were reinforced by evaluating the effects of the mutations on the K_i values of the inhibitors. Only small modifications in the K_i values of **4** and **5** were found with the mutant E^{350}Q and E^{350}D enzymes, implying that E^{350} is not critically involved in their binding. Experimental data show that this type of APN hydroxamate inhibitor very likely interacts with the S_1' and S_2' subsites of the enzyme (*42*), a proposal confirmed by the crystal structure of the complex between *p*-iodo-D-phenylalanine hydroxamate and *Aeromonas proteolytica* aminopeptidase (*43*). In this enzyme, the −CONHOH group binds to the catalytic zinc in a bidentate manner, without forming a hydrogen bond with a glutamate or aspartate residue of the catalytic site. This is supported also by the observation of the synergistic binding of ammoniac and amino acid hydroxamate to the active site of APN (*32*): the hydroxamate chelates the Zn^{2+} ion, leaving the amine binding site free for further occupation by ammoniac.

The E^{350}Q and E^{350}D mutations led to decreases in the inhibitory potencies of the thiol-containing compound **1** of between 4- and 20-fold (Table 2). These relatively small

reductions in affinities suggest that the hydrogen bond between the amino group of this inhibitor and E^{350} in the amine binding site is weak. Furthermore as shown for LAP (*34*, *35*, *47*), the amino group of compound **1** could interact under its deprotonated form, accounting for the limited changes in K_i values of **1** when E^{350} was replaced by Q.

In contrast, the mutation of $E^{350}D$ resulted in a more than 200- and 170-fold reduction in affinities for the inhibitors **2** and **3**, respectively, (Table 2), which are assumed to behave as transition-state analogs. These results are similar to the 130-fold reduction in k_{cat}, observed with $E^{350}D$ mutation (Table 1). An even higher reduction occurred by changing E^{350} to Q, with a 500-fold lower k_{cat} value being observed for this mutant. Taken together, these results seem to indicate that E^{350} plays a more important role in stabilizing the transition state of the substrate, and consequently its rate of degradation, than in ensuring the energetically favorable specific binding of the free amino group of substrates or inhibitors.

Based on these results, a model of the mechanism of action of this TLN-related family of aminopeptidases can be proposed, involving E^{350} in the hydrolysis process. This model is derived from both the structural and mechanistic data available for TLN (*26*) and the crystallographic data on the LAP/inhibitor complex (*34*, *35*, *47*). Indeed the experimental data obtained in this work favor a mode of interaction of APN with its substrate, in the ground state, analogous to that of LAP with its inhibitors (*45*), i.e., an interaction of both the free amino group and the carbonyl of the peptide bond to be cleaved with the zinc ion. Interestingly, the recent crystallographic analysis of the complex between the matrix metalloproteinase stromelysin-1 and its endogenous inhibitor TIMP-1 (*45*) shows a similar mode of enzyme recognition with the coordination of the catalytic zinc ion by the α-amino group and the carbonyl oxygen of the N-terminal cysteine-1 of TIMP-1, in addition to the three histidines of the enzyme. However, the presence in APN of the consensus sequence HEXXH...E, found in TLN and related endopeptidases such as neutral endopeptidase-24.11, angiotensin-converting enzyme, and endothelin-converting enzyme (*44*), is mainly in favor of a hydrolytic mechanism close to that proposed by Matthews (*26*) for TLN. In this mechanism, a simplified model of which is shown in Figure 4 (top), the Zn^{2+} ion in the Michaelis complex is pentacoordinated by the carbonyl group of the scissile bond, a water molecule, and the three protein−zinc ligands. In the transition state, a nucleophilic attack of the peptide bond by the water molecule which is polarized by its substrate-induced proximity to the glutamate, E^{143}, leads to a relatively constrained intermediate in which Zn^{2+} is always pentacoordinated and the carbon of the peptide bond is tetrahedral.

One mechanism of action which could be proposed for APN would involve the bidentate coordination of Zn^{2+} by the free α-amino group and the carbonyl group of the substrate. These two ligands, in addition to the water molecule and the three protein ligands, would lead to a hexacoordinated zinc complex (Figure 4, bottom). There would also be an additional, but slight, interaction between the hydrogen of the α-amino group and E^{350}. However, in the transition state it seems highly improbable, for geometric and energetic reasons, that a constrained intermediate having both the hexa-coordination of the Zn^{2+} ion and the tetracoordination of the peptide-bond carbon would be formed. It seems more probable that the transition state occurs as described for TLN, with the additional creation of stable hydrogen bonds between the free α-amino group and E^{350}, to the detriment of its zinc binding. This model could explain why E^{350} has a more important role in stabilizing the transition state than in favoring the formation of the Michaelis complex. This could also account for the 30000-fold differences in the affinity, for APN, of the transition analogues **3** and **4**, which differ only by the absence of the N-terminal amino group in the latter. The strict exopeptidase action of APN and related peptidases does not seem therefore to be due to a steric blockade of the NH_2 group of the substrate, as occurs for the C-terminal COOH group of substrates in carboxypeptidases (*46*), but to the involvement of the free NH_2 group in the catalytic process (Figure 4). X-ray crystallographic data of zinc aminopeptidase complexed with inhibitor mimicking the transition state will be necessary to give more details on this proposed recognition mechanism.

ACKNOWLEDGMENT

We are grateful to Dr. Bernard Delmas for the gift of the pTEJ4-APN plasmid and the APN antiserum and to Dr. Hui Xiong Chen for providing the phosphinic inhibitors. We also thank Drs. Catherine Llorens-Cortès and Gilles Vazeux for helpful discussions during the course of this work. The authors wish to thank Christine Dupuis for expert manuscript drafting and realization of figures.

REFERENCES

1. Maroux, S., Louvard, D., and Barrati, J. (1973) *Biochim. Biophys. Acta 321*, 282−295.
2. Sanderink, G. J., Artur, Y., and Seist, G. (1988) *J. Clin. Chem. Clin. Biochem. 26*, 795−807.
3. Look, A., Ashmun, R., Shapiro, L., and Peiper, S. (1989) *J. Clin. Invest. 83*, 1299−1307.
4. Delmas, B., Gelfi, J., Kut, E., Sjostrom, H., Noren, O., and Laude, H. (1994) *J. Virol. 68*, 5216−5224.
5. Yeager, C. L., Ashmun, R. A., Williams, R. K., Cardellichio, C. B., Shapiro, L. H., Look, A. T., and Holmes, K. V. (1992) *Nature 357*, 420−422.
6. Waksman, G, Bouboutou, R., Devin, J., Bourgoin, S., Cesselin, F., Hamon, M., Fournié-Zaluski, M. C., and Roques, B. P. (1985) *Eur. J. Pharmacol. 117*, 233−243.
7. Roques, B. P., Noble, F., Daugé, V., Fournié-Zaluski, M. C., and Beaumont, A. (1993) *Pharmacol. Rev. 45*, 87−146.
8. Zini, S., Fournié-Zaluski, M. C., Chauvel, E., Roques, B. P., Corvol, P., and Llorens-Cortes, C. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 11968−11973.
9. Olsen, J., Cowell, G. M., Konigshofer, E., Danielsen, E. M., Moller, J., Laustsen, L., Hansen, O. C., Welinder, K. G., Engberg, J., Hunziker, W., Spiess, M., Sjostrom, H., and Noren, O. (1988) *FEBS Lett. 238*, 307−314.
10. Malfroy, B., Kado-Fong, H., Gros, C., Giros, B., Schwartz, J. C., and Hellmiss, R. (1989) *Biochem. Biophys. Res. Commun. 161*, 236−241.
11. Watt, V. M., and Yip, C. C. (1989) *J. Biol. Chem. 264*, 5480−5487.
12. Yang, X. F., Milhiet, P. E., Gaudoux, F., Crine, P., and Boileau, G. (1993) *Biochem. Cell Biol. 71*, 278−287.
13. Rawlings, N. D., and Barret, A. J. (1995) *Methods Enzymol. 248*, 183−228.
14. Nanus, D. M., Engelstein, D., Gastl, G. A., Gluck, L., Vidal, M. J., Morrison, M., Finstad, C. L., Bander, N. H., and Albino, A. P. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7069−7073.

15. Fukasawa, K. M., Fukasawa, K., Kanai, M., Fugii, S., and Harada, M. (1996) *J. Biol. Chem. 271*, 30731−30735.
16. Cadel, S., Foulon, T., Viron, A., Balogh, A., Midol-Monnet, S., Noël, N., and Cohen, P. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2963−2968.
17. Funk, C. D., Radmark, O., Fu, J. Y., Matsumoto, T., Jornvall, H., Shimizu, T., and Samuelsson, B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6677−6681.
18. Constam, D. B., Tobler, A. R., Rensing-Ehl, A., Kemler, I., Hersh, L. B., and Fontana, A. (1995) *J. Biol. Chem. 270*, 26931−26939.
19. Schauder, B., Schomburg, L., Kohrle, J., and Bauer, K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9534−9538.
20. Keller, S. R., Scott, H. M., Mastick, C. C., Aebersold, R., and Lienhard, G. E. (1995) *J. Biol. Chem. 270*, 23612−23618.
21. McCaman, M. T., and Gabe, J. D. (1986) *Mol. Gen. Genet. 204*, 148−152.
22. Wang, J., and Cooper, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1222−1226.
23. Vazeux, G., Wang, J., Corvol, P., and Llorens-Cortès, C. (1996) *J. Biol. Chem. 271*, 9069−9074.
24. Medina, J. F., Wetterholm, A., Radmark, O., Shapiro, R., Haeggström, J., Vallee, B. L., and Samuelsson, B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7620−7624.
25. Wetterholm, A., Medina, J. F., Radmark, O., Shapiro, R., Haeggström, J., Vallee, B. L., and Samuelsson, B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9141−9145.
26. Matthews, B. W. (1988) *Acc. Chem. Res. 21*, 333−340.
27. Jongeneel, C. V., Bouvier, J., and Bairoch, A. (1989) *FEBS Lett. 242*, 211−214.
28. Toh, H., Minami, M., and Shimizu, T. (1990) *Biochem. Biophys. Res. Commun. 171*, 216−221.
29. Helene, A., Beaumont, A., and Roques, B. P. (1991) *Eur. J. Biochem. 196*, 385−393.
30. Xie, J., Soleilhac, J. M., Schmidt, C., Peyroux, J., Roques, B. P., and Fournié-Zaluski, M. C. (1989) *J. Med. Chem. 32*, 1497−1503.
31. Bryce, G. F., and Rabin, B. R. (1964) *Biochemistry 22*, 3778−3783.
32. DiGregorio, M., Pickering, D. S., and Chan, W. W. C. (1988) *Biochemistry 27*, 3613−3617.
33. Gordon, E. M., Godfrey, J. D., Delaney, N. G., Assad, M. M., von Langen, D., and Cushman, D. W. (1988) *Biochemistry 31*, 2199−2211.
34. Burley, S. K., David, P. R., Sweet, R. M., Taylor, A., and Lipscomb, W. N. (1992) *J. Mol. Biol. 224*, 113−140.
35. Kim, H., and Lipscomb, W. N. (1993) *Biochemistry 32* , 8465−8478.
36. Roques, B. P., Lucas-Soroca, E., Chaillet, P., Costentin, J., and Fournié-Zaluski, M. C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3178−3182.
37. Fournié-Zaluski, M. C., Coric, P., Turcaud, S., Bruetschy, L., Lucas, E., Noble, F., and Roques, B. P. (1992) *J. Med. Chem. 35*, 1259−1266.
38. Schmidt, C., Peyroux, J., Noble, F., Fournié-Zaluski, M. C., and Roques, B. P. (1991) *Eur. J. Pharmacol. 192*, 253−262.
39. Laemmli, U. K. (1970) *Nature 227*, 680−685.
40. Vogel, Z., and Altstein, M. (1977) *FEBS Lett. 80*, 332−335.
41. Xu, Y., Wellner, D., and Scheinberg, D. A. (1995) *Biochem. Biophys. Res. Commun. 208*, 664−674.
42. Fournié-Zaluski, M. C., Coulaud, A., Bouboutou, R., Chaillet, P., Devin, J., Waksman, G., Costentin J., and Roques, B. P. (1985) *J. Med. Chem. 28*, 1158−1169.
43. Chevrier, B., D'Orchymont, H., Schalk, C., Tarnus, C., and Moras, D. (1996) *Eur. J. Biochem. 237*, 393−398.
44. Roques, B. P. (1993) *Biochem. Soc. Trans. 21*, 678−685.
45. Gomis-Rüth, F. X., Maskos, K., Betz, M., Bergner, A., Huber, R., Suzuki, K., Yoshida, N., Nagase, H., Brew, K., Bourenkov, G. P., Bartunik, H., and Bode, W. (1997) *Nature 389*, 77−81.
46. Christianson, D. W., and Lipscomb, W. N. (1989) *Acc. Chem. Res. 33*, 62−69.
47. Kim, H., Burley, S. K., and Lipscomb, W. N. (1993) *J. Mol. Biol. 230*, 722−724.

BI971705P