A glutamate residue contributes to the exopeptidase specificity in aminopeptidase A

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Aminopeptidase A (EC 3.4.11.7, APA) is a 130 kDa membrane-bound aminopeptidase that contains the consensus sequence HEXXH (385–389) found in the zinc metalloprotease family, the zincins. Sequence alignment of the mouse APA with other monozinc-aminopeptidases indicates the presence of a highly conserved glutamate residue (Glu³⁵² in APA) found in the conserved motif GAMEN (349–353). In monozinc-aminopeptidases, the negative charge of the glutamate side-chain carboxylate may constitute the anionic binding site involved in the recognition of the free amino group of substrates or inhibitors. The functional role of Glu³⁵² in APA was investigated by substituting this residue with an aspartate (Asp³⁵²), a glycine (Gly³⁵²), a glutamine (Gln³⁵²) or an arginine (Arg³⁵²) residue by site-directed mutagenesis. Kinetic studies showed that the K_m values of the mutant enzymes were unaffected, whereas k_{cat}

values were decreased 10–250-fold, resulting in a 10-, 30- 260- and 400-fold reduction in cleavage efficiencies for the mutants Asp³⁵², Gly³⁵², Gln³⁵² and Arg³⁵² respectively. The inhibitory potency of two different classes of inhibitors, a thiol and a phosphonate compound, was significantly ($P < 0.05$) decreased by 10- and 4-fold respectively in the mutated enzymes. Moreover, the inhibitory potency of angiotensin I, used as a competitor of the synthetic substrate α -L-glutamyl β -naphthylamide, displayed a 4-fold reduction ($P < 0.01$) in the mutated enzymes, whereas the K_i values of its *N*-acetyl derivative were unchanged. These data strongly suggest that Glu³⁵² is involved in the catalytic process of APA and contributes to the exopeptidase activity of this enzyme through interaction with the N-terminal part of substrates or inhibitors.

INTRODUCTION

Aminopeptidases are a group of exopeptidases that catalyse the hydrolysis of N-terminal amino acid residues of protein and peptide substrates. They belong to a zinc-dependent enzyme family characterized by one or two zinc ions per active site. The aminopeptidase family with two zinc ions has been the most extensively studied, and the X-ray structure resolution of bovine lens leucine aminopeptidase (EC 3.4.11.1, bLAP) in complex with bestatin [1,2] or amastatin [3] has provided insights on the catalytic mechanism of these enzymes. In contrast, structural and mechanistic information on monozinc-aminopeptidases is less extensive. Among these enzymes, aminopeptidase A (EC 3.4.11.7, APA) is a 130 kDa homodimeric zinc-metalloprotease which, *in vitro*, specifically cleaves the N-terminal glutamate or aspartate residue of peptidic substrates such as cholecystokinin-8 or angiotensin (Ang) II [4,5]. APA is strongly expressed in peripheral tissues, such as in intestinal and renal epithelial cells, and in the vascular endothelium [6], but also localized in brain nuclei expressing Ang II type-1 receptors [7]. Recent studies have shown that APA is involved *in vivo* in the conversion of brain Ang II to Ang III, and have led to the identification of Ang III as one of the main effector peptides of the cerebral renin–Ang system in the control of vasopressin release [8]. The determination of the complete amino acid sequence of APA in the mouse [9], man [10,11] and pig [12] shows the presence of the consensus sequence HEXXH (385–389) found in the zinc-metalloprotease family [13,14]. The overall amino acid similarity of APA to other monozinc-aminopeptidases, such as aminopeptidase N (EC 3.4.11.2, APN) [15,16], aminopeptidase B (EC 3.4.11.6, APB) [17,18], thyrotropin-releasing-hormone-degrading enzyme (EC

3.4.19.6, TRH-degrading enzyme) [19], leukotriene A₄ hydrolase (EC 3.3.2.6, LTA₄ hydrolase) [20] and placental leucine aminopeptidase/oxytocinase (EC 3.4.11.3, P-LAP) [21] reaches 22–35%, with a higher conservation in the region flanking the zinc-binding motif. Site-directed-mutagenesis studies of APA have demonstrated that, despite a very limited sequence similarity, the APA active site shares structural similarities with that of the bacterial endopeptidase thermolysin (EC 3.4.24.4, TLN). His³⁸⁹ and Glu⁴⁰⁸ are two of the three zinc ligands in APA, and correspond to His¹⁴⁶ and Glu¹⁶⁶ in thermolysin, whereas Glu³⁸⁶ in APA corresponds to Glu¹⁴³ in thermolysin, acting as the general base in catalysis [22–24]. However, if the hydrolysis of the peptide bond involves similar catalytic steps, the nature of the catalytic effectors can be different: for example, a tyrosine residue (Tyr¹⁷¹) in APA is presumed to be the counterpart of His²³¹ in TLN and would stabilize the enzyme–substrate complex at the transition state [25].

Until now, no structural data were available about the exopeptidase specificity of monozinc-aminopeptidases. Structure–activity studies of various monozinc-aminopeptidases using different peptide substrates [26] or inhibitors, such as bestatin and analogues [27], β -aminothiol [28–30], 3-amino-2-tetralone [31] or α -aminophosphonate [32] derivatives, have shown the role of the free amino group of these compounds in substrate hydrolysis or inhibitor binding.

The N-terminal specificity of aminopeptidases suggested the presence in these enzymes of an anionic binding site which involves a negatively charged carboxylate side chain, expected to be located at the end of the S₁ subsite, allowing recognition of the free amino group of substrates and inhibitors. Sequence alignment of APA with several monozinc-aminopeptidases (Figure 1)

Abbreviations used: APA, aminopeptidase A; bLAP, bovine lens leucine aminopeptidase; APN, aminopeptidase N; APB, aminopeptidase B; TRH, thyrotropin-releasing hormone; LTA₄, leukotriene A₄; P-LAP, placental leucine aminopeptidase; TLN, thermolysin; GluPO₃H₂, glutamate phosphonic acid; GluSH, glutamate thiol; GluNA, α -L-glutamyl β -naphthylamide; ACE, angiotensin (Ang)-converting enzyme.

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Figure 1 Alignment of the mouse APA amino acid sequence with that of other monozinc-aminopeptidases

Alignment of sequences of mouse APA [9], human APN [50], rat APB [17,18], *Escherichia coli* peptidase N [51], human LTA₄H [20], *Saccharomyces cerevisiae* aminopeptidase II [52], rat TRH degrading enzyme [19], human P-LAP [21]. The mutated glutamate residues are indicated in **bold** letters. The consensus sequences are underlined and the conserved residues are marked with an asterisk (*).

reveals the presence of a strictly conserved glutamate in the conserved motif GXMEN located 32 residues from the zinc-binding motif HEXXH.

We therefore hypothesized that Glu³⁵², located in the GAMEN motif (349–353) of APA, may belong to the anionic binding site, accounting for the exopeptidase specificity of APA. To test this hypothesis, we investigated the functional role of this residue by replacing Glu³⁵² with an aspartate, a glycine, a glutamine or an arginine residue by site-directed mutagenesis. In addition, a non-conserved glutamate residue (Glu⁴²³ in APA) was replaced with an alanine residue and used as an internal control. The biochemical and kinetic characterizations of the recombinant enzymes are reported.

EXPERIMENTAL

Site-directed mutagenesis and construction of expression plasmids

Site-directed mutagenesis was performed on the cDNA- encoding mouse APA cloned in the expression vector SR α BP1 [33], using PCR as previously described [34]. The region of the cDNA to be mutated was amplified using for the Glu³⁵² mutants, an upstream oligonucleotide which contained the unique restriction site *EcoRV*, a downstream oligonucleotide which contained the modified codon and the unique restriction site *NarI*. and, for the

Glu⁴²³ mutant, an upstream oligonucleotide which contained the modified codon and the unique restriction site *Alw*NI, and a downstream oligonucleotide which contained the restriction site *Nar*I. *Pfu* *Taq* polymerase (1 unit) was used (30 cycles: 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min). After purification of the resulting fragment from a 2 %-(w/v)-agarose gel (Gene Clean 2, Bio101), the 368 bp or 250 bp PCR DNA insert containing the mutation was substituted for the corresponding non mutated region (*Nar*I–*Eco*RV or *Alw*NI–*Nar*I) of the full-length APA cDNA. The presence of the mutations and the absence of non-specific mutations was confirmed by sequencing the 368 bp and 250 bp mutated region by the dideoxy-chain-termination method (Sequenase 2; U.S. Biochemical Corp.).

The glutamate residue at position 352 was replaced with either an aspartate, a glycine, a glutamine or an arginine residue. The glutamate residue at position 423 was replaced by an alanine residue.

The mutated primers used for constructing the mutant cDNA species, were:

5'-GGCACCGGCGCCATGGATAATTGG-3' for Asp³⁵²

5'-GGCACCGGCGCCATGGGAAATTGG-3' for Gly³⁵²

5'-GGCACCGGCGCCATGCAAAAATTGG-3' for Gln³⁵²

5'-GGCACCGGCGCCATGAGAAATTGG-3' for Arg³⁵²

5'-ctgactgagcatctgccagtcttttgctgcg-3' (anti-sense) for Ala⁴²³

The underlined bases encode the new amino acid at positions 352 and 423. Amino acid numbering corresponds to that of the APA sequence deposited in the GenBank database (accession number M29961).

Transfection of COS-7 cells

COS-7 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) with 10% (v/v) foetal-calf serum, and were transfected with 30 µg of plasmid by electroporation (250 V, 1800 µF; Eurogentec electroporator). Each pool of transfected cells was divided between two 35 mm-diameter Petri dishes, one for enzyme and protein assays and one for metabolic labelling.

Metabolic labelling and immunoprecipitation

At 48 h after transfection, transfected COS-7 cells were incubated for 5 h in methionine-and-serum-free medium (Ham's F12) containing 50 µCi/ml of [³⁵S]methionine/cysteine. After discarding the cell medium, proteins of the cell pellet were solubilized for 2 h in 1 ml of 50 mM Tris/HCl buffer (pH 7.4)/150 mM NaCl/10 mM EDTA/1% (v/v) Triton X-100, then centrifuged at 15000 *g* for 5 min at 4 °C. The supernatants were recovered, and immunoprecipitation was performed by incubating these solubilized cell extracts overnight at 4 °C with 50 µl of Protein A-Sepharose (Pharmacia-LKB Biotechnology) [50% (w/v) suspension in solubilization buffer] and 3 µl of a polyclonal rabbit anti-(rat APA) serum [35] (final dilution 1:200). The immune complexes were collected by centrifugation and washed three times with 1 ml of solubilization buffer and once with 1 ml of 20 mM Tris/HCl buffer, pH 6.8. Proteins were eluted [by boiling in 30 µl of 20 mM Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 2% (w/v) SDS] and resolved by SDS/7.5%-(w/v)-PAGE as described by Laemmli [36]. The dried gel was then exposed for autoradiography.

Enzyme assay

Transfected COS-7 cells in a 35 mm-diameter Petri dish were washed twice and harvested by scraping them off in 1 ml of PBS. After centrifugation at 2000 *g* for 10 min at 4 °C, the cell pellet was resuspended in 1 ml of ice-cold 50 mM Tris/HCl buffer, pH 7.4, then sonicated for 30 s. Membranes were recovered by ultracentrifugation at 30000 *g* for 30 min, resuspended by a 30 s sonication in 1 ml of ice-cold 50 mM Tris/HCl buffer, pH 7.4, and dispensed in aliquots for enzyme assay. APA activity was determined in a microtitre plate by monitoring the rate of hydrolysis of a synthetic substrate, α-L-glutamyl β-naphthylamide (GluNA; Bachem), in the presence or absence of 1 µM of the specific APA inhibitor glutamate phosphonic acid (GluPO₃H₂), a concentration that abolishes APA activity [25]. Bestatin, a non-specific aminopeptidase inhibitor, was used at 1 µM, a concentration that did not inhibit APA activity but prevented the degradation of the substrate by aminopeptidases such as B-, N-, W- and cytosolic aminopeptidases [31,37]. To estimate the specific APA activity, the absorbance from the assay with GluPO₃H₂ was subtracted from the absorbance without the inhibitor.

The kinetic parameters (initial velocity, *k*_{cat} and *K*_m) were determined from Lineweaver-Burk plots using final concentration of GluNA ranging from 0.025 to 0.4 mM. The quantification of the amount of APA was performed by microdensitometric scanning of the immunoprecipitated fractions, as described previously [24]. To determine the activities of the mutants with equivalent amounts of enzyme, the substrate

hydrolysis rates were reported for an equivalent level of expression of each mutant. For this purpose, we scanned a low exposure of the autoradiogram of immunoprecipitated wild type and mutant proteins. We have verified that the quantity of immunoprecipitated protein was proportional to the concentration of antigen in the cell extract. An internal standard curve was constructed with different dilutions of immunoprecipitated wild-type protein and different exposure times of the autoradiogram were performed to optimize conditions so that a linear relationship was observed between the amount of protein and the mean density. The specific activity of the wild-type enzyme (45000 nmol/min per mg), established by immunoblotting using purified rat kidney APA [38] as standard, served as a reference.

The sensitivity of wild-type and mutated APAs and some of the mutants to glutamate-thiol (D-, L-) (GluSH), (L-)GluPO₃H₂ and captotril [39] was determined by establishing dose-dependent inhibition curves and calculating their *K*_i values with the Graph Pad Prism 2 software. Statistical comparisons were performed using the Student's unpaired *t*-test (Graph Pad Prism 2). Differences were considered to be significant if *P* was less than 0.05.

RESULTS

Site-directed mutagenesis of APA cDNA

Sequence alignment in the region flanking the zinc-binding motif of several monozinc-aminopeptidases showed a conserved sequence located upstream of this motif, including a glutamate residue in position 352 in APA (Figure 1). To study the functional role of this amino acid in APA, Glu³⁵² was replaced by an aspartate, a glutamine, a glycine or an arginine residue by site-directed mutagenesis. Equivalent amounts of these mutated APA cDNAs were transiently transfected into COS-7 cells.

Metabolic labelling of the wild-type APA and mutants show the presence of a major band migrating with an apparent molecular mass of 130 kDa (Figure 2).

Figure 2 Metabolic labelling of recombinant APAs

Transfected COS-7 cells were labelled for 5 h with [³⁵S]methionine/cysteine mix, and cell-lysate proteins were immunoprecipitated with an anti-APA polyclonal antiserum, resolved by SDS/7.5%-PAGE and identified by autoradiography. The first lane corresponds to the cells transfected with the wild-type APA. The mutants have been named according to the position of the wild-type residues and the amino acid to which they have been mutated. The molecular mass of recombinant APAs is indicated by an arrow. Abbreviation: pSV neo, selection vector that confers neomycin resistance (Clontech).

Table 1 Kinetic parameters of wild-type (WT) and mutated APAs

K_m and k_{cat} values are the mean $\pm$ S.E.M. for three independent experiments with duplicate determinations.

Enzyme	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
WT Glu ^{352/423}	68 $\pm$ 5	109 $\pm$ 8	1600
Asp ³⁵²	106 $\pm$ 30	12.3 $\pm$ 3	115
Gly ³⁵²	70 $\pm$ 6	3.5 $\pm$ 0.8	50
Gln ³⁵²	60 $\pm$ 5	0.4 $\pm$ 0.05	6.4
Arg ³⁵²	100 $\pm$ 12	0.4 $\pm$ 0.06	4
Ala ⁴²³	108 $\pm$ 41	162 $\pm$ 33	1500

Enzymic activity of recombinant APAs

The measurements of enzymic activity using the synthetic substrate GluNA indicated that all substitutions in position 352 led to a large decrease in substrate hydrolysis when compared with the wild-type enzyme. The conservation of the negative charge with an aspartate residue appeared to be more conservative towards enzymic activity than the glycine and glutamine substitutions. The most drastic effects were obtained when the charge was inverted with an arginine residue. To analyse more precisely the effects of the mutations on enzymic activity, K_m and k_{cat} values were measured for the recombinant enzymes and

reported in Table 1. The K_m values of the mutants were in the same range as that of the wild-type APA. In contrast, the k_{cat} values were reduced, leading to a k_{cat}/K_m decrease of 10-, 30-, 250- and 400-fold for Asp³⁵², Gly³⁵², Gln³⁵² and Arg³⁵² respectively. The kinetic parameters of the Ala⁴²³ mutant were not significantly different from those found for the wild-type enzyme.

As previously shown, APA activity is dependent on the presence of Ca²⁺ [40]. At 3 mM Ca²⁺, the activation of enzymic activity was 50 % of maximal level for the mutants as well as for the wild-type enzyme, thus suggesting that the mutation does not modify the sensitivity to Ca²⁺ (results not shown).

Inhibitory potencies of various classes of inhibitors on recombinant APA enzymes

To characterize further the role of Glu³⁵² in substrate and inhibitor binding, the inhibition of GluNA hydrolysis by the wild-type and mutant enzymes was compared using different classes of APA inhibitors which differ by the length of the pseudopeptide and the nature of the zinc-chelating group. Results are summarized in Table 2. The inhibitory potency of the substrate analogue GluSH for the mutants is decreased by one order of magnitude as compared with the wild type enzyme, the strongest effects being obtained with the glutamine and arginine substitutions (Table 2).

GluPO₃H₂, a pseudoanalogue of the transition state, was shown to be a potent inhibitor of APA (K_i : 8.7 $\pm$ 0.6 nM)

Table 2 K_i values (μ M) of several compounds on wild-type and mutated APAs

K_i values are the means $\pm$ S.E.M. for six or seven independent experiments with duplicate determinations: * P < 0.05; ** P < 0.01; *** P < 0.001; n.s., no significance versus wild-type.

	Wild Type	Asp-352	Gly-352	Gln-352	Arg-352
<p>Angiotensin I</p> <p>H₂N - CH - C - NH - CH - CONH - CH - CO - Tyr - Ile - His - Pro - Phe - His - Leu - COOH</p>	4.3 $\pm$ 0.5	9 $\pm$ 0.5**	7.9 $\pm$ 0.4*	13.9 $\pm$ 0.7**	16.7 $\pm$ 0.9**
<p>N-Acetyl-Angiotensin I</p> <p>CH₃COHN - CH - C - NH - CH - CONH - CH - CO - Tyr - Ile - His - Pro - Phe - His - Leu - COOH</p>	109.9 $\pm$ 7.3	95.8 $\pm$ 2.9 n.s.	69.3 $\pm$ 0.7 n.s.	133.9 $\pm$ 3.3 n.s.	163.9 $\pm$ 14.5 n.s.
<p>Glutamate-Thiol</p> <p>H₂N - CH - CH₂ - SH</p>	0.069 $\pm$ 0.007	0.49 $\pm$ 0.20 *	0.49 $\pm$ 0.15**	0.65 $\pm$ 0.24*	0.66 $\pm$ 0.28*
<p>Glutamate phosphonic acid</p> <p>H₂N - CH - CH₂ - P(=O)(OH)₂</p>	0.008 $\pm$ 0.0007	0.030 $\pm$ 0.006 **	0.037 $\pm$ 0.004***	0.030 $\pm$ 0.011 n.s.	0.016 $\pm$ 0.003 *
<p>Captopril</p> <p>HS - CH₂ - CH - C(=O) - N - CH - COOH</p>	10 $\pm$ 1.0	14 $\pm$ 1.3 n.s.	12 $\pm$ 1.3 n.s.	14 $\pm$ 1.2 n.s.	13 $\pm$ 1.4 n.s.

Figure 3 Effect of Ang I and *N*-acetyl-Ang I on the enzymic activity of wild-type and mutated APAs

APA activity in the particulate fraction of transfected COS-7 cells was estimated by measuring the hydrolysis of 0.2 mM of GluNA in the presence or absence of different concentrations of Ang I or *N*-acetyl-Ang I. Each point represents the mean $\pm$ S.E.M. from two to three independent experiments with duplicate determinations.

through interaction of one of its phosphoryl oxygen with the zinc ion and the phenolic oxygen of Tyr⁴⁷¹ [25]. The potency of this inhibitor towards the mutant enzymes (Asp³⁵², Gly³⁵² and Arg³⁵²) was significantly decreased (2–5-fold).

The third compound, captopril, is a potent inhibitor of Ang-converting enzyme (ACE) [39]. Captopril was also shown to inhibit LTA₄ hydrolase [41], despite the absence of a free amino group in its structure. As shown in Table 2, this compound is a weak inhibitor of APA (K_i 10 μ M) and similarly inhibits the mutated and wild-type enzymes.

Competition of GluNA hydrolysis with Ang I or *N*-acetyl-Ang I

To evaluate the importance of the free amino group in substrate or inhibitor binding to APA, enzymic activities of the recombinant enzymes were estimated by measuring GluNA hydrolysis in the presence of Ang I or its *N*-acetylated derivative (*N*-acetyl-Ang I). The K_i values (Table 2) indicate first that the compound Ang I is 25-fold more efficient than its *N*-acetylated derivative to inhibit GluNA hydrolysis by the wild-type enzyme. In the case of the mutated enzymes, this difference is decreased to 10-fold. Secondly, the affinity of Ang I is between two and four times decreased for the mutated enzymes as compared with the wild-type APA, the weakest binding affinity being observed when the negatively charged glutamate residue is substituted with an arginine. In contrast, *N*-acetyl-Ang I similarly binds the wild-type and mutated APAs (Figure 3).

DISCUSSION

The active sites of zinc-metallopeptidases such as thermolysin or carboxypeptidase A are similar in many aspects, although they have unique features which contribute to their different specificities. The specificity of carboxypeptidase A as an exopeptidase can be attributed to Arg¹⁴⁵, which binds the free C-terminal group of the substrate, thus preventing the binding of an extended polypeptide. In the case of the endopeptidase thermolysin, Arg²⁰³ interacting with the amide carbonyl group of the P'₁ residue is located on the side of the substrate so that a substrate of any length can be accommodated [22]. The dipeptidyl-carboxypeptidase specificity of neutral endopeptidase (EC 3.4.24.11) has also been attributed to the presence of a functional arginine (Arg¹⁰²) in its active site [42], whereas the corresponding

arginine residue (Arg¹²⁹) in the closely related endothelin-converting enzyme was shown to have a different function, in agreement with the strictly endopeptidase specificity of this enzyme [43]. By analogy to carboxypeptidase A, the presence of a glutamate or an aspartate carboxylic side chain in monozinc-aminopeptidases may constitute the negative charge of the anionic subsite that would contribute to the exopeptidase specificity of these enzymes through interaction with the free N-terminus of peptidic substrates.

Sequence alignment of APA with several monozinc-aminopeptidases in the region surrounding the zinc-binding motif revealed the presence of a strictly conserved glutamate in a consensus sequence GXMEN which may account for such a function.

The functional role of Glu³⁵² in the APA GAMEN (349–353) motif was investigated by replacing this residue by site-directed mutagenesis with either an aspartate residue, which conserves the negative charge, a glycine or a glutamine residue, which suppress it, or an arginine residue, which reverses it. Each of these substitutions led to large decreases in enzymic activities of the recombinant enzymes. The specificity of the effect was demonstrated by the lack of change in enzymic activity observed when the non-conserved glutamate residue in position 423 (Glu⁴²³) is replaced with an alanine residue. Kinetic studies showed that the K_m values of the Glu³⁵² mutants were similar to that determined for the wild-type enzyme. By contrast, k_{cat} values of the mutant enzymes were widely decreased, leading to 10-, 30-, 260- and 400-fold reductions in cleavage efficiency of the mutants Asp³⁵², Gly³⁵², Gln³⁵² and Arg³⁵² respectively. The results indicate that Glu³⁵² is involved in the catalytic process of APA, and in addition to the charge, the length of the side chain of Glu³⁵² is crucial for the catalysis.

To characterize further the role of Glu³⁵² in the active site of APA, the inhibitory potencies of various classes of inhibitors were evaluated on the mutant and wild-type enzymes: (i) a substrate analogue, the β -aminothiol inhibitor GluSH, which is presumed to interact with the amine-binding site, the S₁ subsite and the zinc ion via its strong chelating thiol group; (ii) a pseudoanalogue of the transition state, the α -aminophosphonate inhibitor GluPO₃H₂, which is presumed to interact with the amine-binding site, the S₁ subsite and the zinc ion via one of its phosphoryl oxygens; (iii) the inhibitor captopril, a pseudo-dipeptide where the free amino group is replaced by a thiol group, is assumed to interact in APA with the S'₁ and S'₂ subsites and the zinc ion via its thiol group. Mutating Glu³⁵² to either aspartate, glycine, glutamine or arginine led to a 7–10-fold decrease in affinities of the mutant enzymes for the GluSH compound and to only a 4-fold reduction for the GluPO₃H₂ compound. These changes in inhibitory potencies suggest that an interaction occurs between the negative charge of the side-chain carboxylate of Glu³⁵² and the amino group of the inhibitors. In addition, no modification was observed in the K_i values of captopril, suggesting that it does not interact with Glu³⁵², an observation which can be correlated with the absence of the free amino group in this inhibitor. In order to characterize further the importance of the free amino group in the recognition of substrates (or inhibitors) by APA, enzymic activities of the recombinant enzymes were estimated in the presence of Ang I or its *N*-acetyl derivative (*N*-acetyl-Ang I). Ang I has been shown to be, *in vitro*, a substrate for APA [44]. The very weak hydrolysis efficiency of APA toward N-terminally blocked substrates and the absence of specific N-terminal blocked inhibitors led us to use Ang I and *N*-acetyl-Ang I as competitors of the substrate GluNA. The most marked effects were obtained with the mutants Gln³⁵² and Arg³⁵² (Figure 3): (i) the K_i values of Ang I were

Figure 4 Putative interaction of the N-terminal part of the peptide substrate with Glu³⁵² in the active site of APA

increased by 2–4-fold in mutants when compared with the wild-type; (ii) similar K_i values of *N*-acetyl-Ang I were obtained for the mutant and wild-type enzymes; (iii) the ratio between K_i values of *N*-acetyl-Ang I versus Ang I, is decreased from 25 in the wild-type enzyme to 10 in mutant enzymes. These results indicate that the carboxylate side chain of Glu³⁵² interacts with the free amino group of Ang I, as shown by the decreased Ang I binding and the weaker sensitivity to its N-terminally blocked derivative observed in the mutants as compared with the wild-type enzyme.

The variable difference observed between Ang I and *N*-acetyl-Ang I binding among the mutated enzymes probably results from a severe steric hindrance around the weakly accessible negatively charged side chain of Glu³⁵² in the active site of the enzyme. This explains the greatest affinity of the *N*-acetylated ligand for the small residues Asp³⁵² and Gly³⁵² as compared with the bulkier side chains of Gln³⁵², Arg³⁵² and Glu³⁵².

The important effect on the k_{cat} values and the slight effect on the K_m values induced by the mutation of Glu³⁵² corroborates with the findings that, in aminopeptidases such as APA or bLAP, the loss or the N-substitution of the free amino group in enzyme ligands has a more important effect on substrate hydrolysis (substrates with blocked N-terminal amino acid are not hydrolysed) as compared with inhibitor binding [30,32,45]. These results suggest that the interaction between the free N-terminus of the substrate and the enzyme does not favour the stabilization of the Michaelis complex, but rather ensures an energetically favourable state for the cleavage of the scissile peptide bond.

Under these conditions the exopeptidase specificity of APA cannot easily be explained by an interaction of the free amino group of the substrate with the carboxy group of Glu³⁵², ensuring substrate recognition and stabilization, as suggested for the C-terminus of substrates in other enzymes such as neutral endopeptidase 24.11 [42] or by a blockade of the amino group into a 'dead-end' pocket, as occurs for the C-terminal specificity of carboxypeptidase A [46]. Despite the lack of sequence similarity in monozinc- and two zinc-containing aminopeptidases sequences, these results are more consistent with the molecular basis of the exopeptidase specificity of APA sharing similarities with that proposed for bLAP. In this two-metal-ion model, the α -amino group (probably unprotonated under physiological conditions) of the substrate is co-ordinated to one of the two zinc ions (Zn⁴⁸⁹) and interacts with an aspartate residue of the active

site (Asp²⁷³) via hydrogen bonding [1,47,49]. The co-ordination of the zinc ion to the free N-terminus is essential to bind and preorient the peptide substrate. Upon hydrolysis, the increased pK_a of the α -amino group of the newly formed free amino acid would result in protonation and release of the product away from the positively charged zinc ion. Such a mechanism can serve as a basis for a structural model accounting for the exopeptidase specificity of APA (Figure 4): the free amino group may be located in the vicinity of the positively charged zinc ion so that the unprotonated form of the free amino group of substrates or inhibitors should be in a more favourable state for binding. The unprotonated α -amino group may interact with the zinc ion, and a negatively charged ligand, such as the carboxylate of Glu³⁵², which should facilitate binding of the free amino group to the zinc. According to this model, the small difference in the K_i values of GluPO₃H₂ between mutated and wild-type enzymes can be explained by the strong acidity of the phosphonate group, which would raise the pK_a of the amino group of GluPO₃H₂ compared with that of GluSH, therefore disfavoring the binding of this inhibitor at physiological pH.

However, Glu³⁵² could also act directly in the catalytic process of APA as a nucleophilic catalyst via the free amino function of peptidic substrates.

Removing or displacing the carboxylate side chain of Glu³⁵² would alter such interactions and would result in the observed decreased k_{cat} values towards GluNA.

The position of Glu³⁵² in the active site of APA and direct confirmation of these findings should be provided by X-ray structure analysis of the protein.

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