A tyrosine residue essential for catalytic activity in aminopeptidase A

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Aminopeptidase A (EC 3.4.11.7; APA) is a 130 kDa membrane-bound zinc enzyme that contains the consensus sequence HEXXH (residues 385–389) conserved among the zinc metalloprotease family. In this motif, both histidine residues and the glutamic residue were shown to be involved respectively in zinc co-ordination and catalytic activity. Treatment of APA with *N*-acetylimidazole results in a loss of enzymic activity; this is prevented by the competitive aminopeptidase inhibitor amastatin, suggesting the presence of an important tyrosine, lysine or cysteine residue at the active site of APA. A tyrosine residue was previously proposed to be involved in the enzymic activity of aminopeptidase N. Furthermore sequence alignment of mouse APA with other monozinc aminopeptidases indicates the presence of a conserved tyrosine (Tyr-471 in APA). The functional role of Tyr-471 in APA was investigated by replacing this residue

with a phenylalanine (Phe-471) or a histidine (His-471) residue by site-directed mutagenesis. Kinetic studies showed that the K_m values of both mutants were similar to that of the wild-type enzyme, whereas k_{cat} values were decreased by three orders of magnitude and corresponded to a variation in free energy of the rate-limiting step by 4.0 and 4.2 kcal/mol (0.96 and 1.00 kJ/mol) for the Phe-471 and His-471 mutants respectively. The mutation did not modify the inhibitory potency of a thiol-containing inhibitor that strongly chelates the active-site zinc ion, whereas that of a putative analogue of the transition state presumed to mimic the reaction intermediate was reduced. Taken together, these results strongly suggest that the Tyr-471 hydroxy group participates in catalysis by stabilizing the transition state complex through interaction with the oxyanion.

INTRODUCTION

Aminopeptidase A (EC 3.4.11.7; APA) is a homodimeric membrane-bound protease that specifically cleaves N-terminal acid residues *in vitro* from peptides such as cholecystokinin 8 or angiotensin II [1,2]. APA is predominantly expressed in the brush borders of intestinal and renal epithelium as well as in the vascular endothelium [3], but is also present in other tissues including brain [4,5]. Until now, the absence of specific APA inhibitors has made it difficult to identify the physiological role of this enzyme. Such molecules have been recently developed [6,7] and APA was shown to be involved *in vivo* in the metabolism of brain angiotensin II [8] and cholecystokinin [9]. The determination of the complete amino acid sequence of APA in the mouse [10], and more recently in man [11,12], predicts a type II integral membrane protein of 946 residues, composed of a small N-terminal cytoplasmic domain (17 residues), a 22-residue transmembrane domain and a large extracellular C-terminal domain that contains the active site, including the consensus sequence HEXXH found in the zinc metalloprotease family: the zincins [13,14].

In the absence of the three-dimensional structure of APA, little is known about the molecular mechanisms of action of this enzyme. Site-directed mutagenesis studies have identified His-385 and His-389 of the zinc-binding motif and Glu-408 as the three zinc ligands, indicating that APA, like thermolysin and neutral endopeptidase (EC 3.4.24.11), belongs to the gluzincin family [15,16]. Furthermore Glu-386 was shown to be involved in catalysis [16]. These results led us to propose a general base mechanism for the peptide hydrolysis, similar to that proposed for thermolysin, deduced from X-ray diffraction studies [17]. In

thermolysin (EC 3.4.24.27), a glutamic residue (Glu-143) promotes the attack of a water molecule on the carbonyl carbon of the scissile peptide bond, the resulting tetrahedral intermediate being stabilized by hydrogen bonds formed with both His-231 and Tyr-157 [17]. Site-directed mutagenesis of residues involved in the stabilization of the transition state, as with His-231 of thermolysin, which resulted in a drastic decrease in catalytic activity as well as binding affinities of transition-state analogue inhibitors, emphasized the role of such residues in this crucial catalytic step [18]. In the zinc metalloendopeptidase neutral endopeptidase, His-711 has been proposed to have a similar role to that of His-231 of thermolysin [19].

Co-crystallization of astacin, a member of the metzincin family, with a phosphinic inhibitor reveals that a tyrosine residue (Tyr-149) seems to be the counterpart of His-231 of thermolysin in both position and function [20].

Interestingly, sequence alignment of several members of the gluzincin family (thermolysin, neutral endopeptidase and several zinc aminopeptidases) in the region surrounding the zinc-binding motif (Figure 1) shows that the presence of a tyrosine residue in APA (Tyr-471), which aligns with Tyr-476 in aminopeptidase N (EC 3.4.11.2; APN) and Tyr-383 in leukotriene A4 (LTA4) hydrolase (EC 3.3.2.6). The distance separating this residue from the zinc-co-ordinating glutamic residue (63 amino acids) is similar to that observed between His-231 in thermolysin and His-711 in neutral endopeptidase with their respective third zinc ligands (64 residues in both cases).

In the present study we examined the presence of an active-site tyrosine residue involved in the enzymic activity of APA by chemical modification of the enzyme with *N*-acetylimidazole. The specific role of Tyr-471 in the enzymic activity of APA was

Abbreviations used: APA, aminopeptidase A; APN, aminopeptidase N; GluPO₃H₂, glutamate phosphonic acid; GluSH, glutamate thiol; GluNA, α -L-glutamyl- β -naphthylamide; LTA4, leukotriene A4.

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Figure 1 Alignment of amino acid sequences in the active sites of different zinc metallopeptidases

The putative active-site tyrosine residues in APA and its homologous residues in the other sequences are indicated in bold letters. The number refers to the position of the mutated residue in the APA sequence. APN, APII, neutral aminopeptidase; TRH degrading enzyme, thyrotropin-degrading enzyme; NEP, neutral endopeptidase.

further investigated by replacing the tyrosine residue with a phenylalanine or histidine residue and studying the activity of the recombinant enzymes. Changes observed in the kinetic parameters, as well as the binding affinity of a putative transition-state analogue, suggest that Tyr-471 participates in catalysis via transition-state stabilization.

EXPERIMENTAL

Inactivation of APA with *N*-acetylimidazole

Recombinant APA was obtained after transfection of COS-7 cells with the expression vector SR α BP1 encoding APA [21]. Total protein (250 μ g) from a membrane preparation of transfected COS-7 cells was diluted in 50 mM Hepes, pH 7.4, and treated with 50 or 100 mM *N*-acetylimidazole at 25 °C for 30 min, in a total volume of 100 μ l. The reaction was stopped by a 1:50 dilution of the samples in 50 mM Hepes buffer, pH 7.4. Ultracentrifugation of the membranes was done at 30000 *g* for 30 min at 4 °C. The pellet was rinsed once with 1 ml of 50 mM Tris/HCl buffer, pH 7.4, then resuspended in 0.2 ml of the same buffer and assayed for enzyme activity. To evaluate the recovery of the membrane pellets, total protein content was estimated in each sample by the procedure of Lowry et al. [22].

Protection from inactivation was performed by preincubating the membrane fraction for 3 h at 4 °C with 0.1 mM glutamate thiol (GluSH), an inhibitor of APA [23], or 0.5 mM amastatin, a pseudotetrapeptide that also inhibits APA [24].

Site-directed mutagenesis and construction of expression plasmids

Site-directed mutagenesis was performed on the cDNA encoding mouse APA, contained in the expression vector SR α BP1 [21], with the PCR as described previously [25]. The region of the cDNA to be mutated was amplified by using an upstream oligonucleotide containing the modified codon and the unique restriction enzyme site *EcoRV*, and a downstream oligonucleo-

tide containing the restriction site *AgeI*. *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 (GeneClean 2; Bio101), the 528 bp PCR DNA insert containing the mutation was substituted for the corresponding non-mutated region (*EcoRV*–*AgeI*) of the full-length APA cDNA. The presence of the mutations and the absence of non-specific mutations was confirmed by sequencing the 528 bp mutated region by the dideoxy-chain-termination method (Sequenase 2; U.S. Biochemical Corp.).

The tyrosine residue at position 471 was replaced with either a phenylalanine (Phe-471) or a histidine (His-471) residue. The mutated primers used for constructing the mutant cDNA species were 5'-GATGGGATATCATTCAGCAAGG-3' for Phe-471, and 5'-GATGGGATATCACACAGCAAGG-3' for His-471. The underlined bases encode the new amino acid residue at position 471. Amino acid numbering corresponds to that of the APA sequence deposited in the GenBank database (accession no. M29961).

Transfection of COS-7 cells

COS-7 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) with 10% (v/v) fetal calf serum, and were transfected with 30 μ g of plasmid by electroporation (250 V, 1800 μ F; Eurogentec electroporator). Each pool of transfected cells was divided between two 35 mm 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 μ Ci/ml [³⁵S]methionine. After the cell medium had been discarded, 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 μ l of Protein A-Sepharose (Pharmacia LKB Biotechnology) [50% (w/v) suspension in solubilization buffer], and 3 ml of a polyclonal rabbit anti-(rat APA) antiserum (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 μ l of 20 mM Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS] and resolved by SDS/PAGE [7.5% (w/v) gel] as described by Laemmli [26]. The dried gel was then exposed for autoradiography.

Enzyme assay

Transfected COS-7 cells in a 35 mm Petri dish were washed twice and harvested by scraping into 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 and dispensed in aliquots for enzyme assay. APA activity was determined in a microtitre plate by following the rate of hydrolysis of a synthetic substrate, α -L-glutamyl- β -naphthylamide (GluNA; Bachem), by the method of Lojda et al. [3] with minor modifications as previously described [6]. The background levels of substrate hydrolysis, owing to the presence of contaminating endogenous proteases in COS-7 cells, were estimated by measuring the enzymic activity in the presence or absence of

the APA inhibitor GluSH at 5 μ M, a concentration that abolishes APA activity. Bestatin, a non-specific aminopeptidase inhibitor, was used at 1 μ M, 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 [27,28]. To estimate the specific APA activity, the absorbance from the assay with GluSH was subtracted from the absorbance without the inhibitor.

The kinetic parameters (initial velocity, k_{cat} and K_m) were determined from Lineweaver–Burk plots with a 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 [16]. 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 exposures 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 (45 000 nmol/min per mg), established by immunoblotting with purified rat kidney as standard, served as a reference.

The sensitivity of APA and some of the mutants to (D-,L-) (GluSH) and glutamate phosphonic acid (L-)(GluPO₃H₂) [29] was determined by establishing dose-dependent inhibition curves and calculating their K_i values.

RESULTS

Chemical modification of recombinant APA with *N*-acetylimidazole

The presence of a tyrosine residue in the active site of APA was investigated by testing the sensitivity of the enzyme to *N*-acetylimidazole, a chemical reagent that acetylates tyrosine, lysine and cysteine residues [30]. Treatment of a membrane fraction of transfected cells overexpressing APA with 50 or 100 mM *N*-acetylimidazole for 30 min decreased the enzymic activity to respectively 38% and 17% of the initial activity (Table 1), indicating that inactivation of APA is proportional to the dose of reagent used.

To determine which subsite of the APA active site contains the amino acid(s) modified by the chemical reagent, protection

experiments with two competitive APA inhibitors were performed (Figure 2). Incubation with 0.1 mM GluSH, an inhibitor that interacts with the APA S1 subsite [23], does not protect APA from acetylation by 50 mM *N*-acetylimidazole for 30 min (Table 1). In contrast, incubation with amastatin, a pseudo-tetrapeptide (0.5 mM) that is supposed to interact with S1, S'1, S'2 and S'3 subsites, totally protects the enzyme from the same treatment. This differential protection indicates the presence of an important tyrosine, lysine, or cysteine residue in the active site of APA, but not in the S1 subsite. Further experiments were necessary to elucidate the presence of an essential tyrosine residue at the active site of APA.

Site-directed mutagenesis of APA cDNA

The sequence alignment in the region surrounding the zinc-binding motif of several aminopeptidases showed a conserved sequence downstream of this motif including a tyrosine residue in position 471 in APA (Figure 1). To study the functional role of this amino acid in APA, Tyr-471 was replaced by a phenylalanine or histidine residue by site-directed mutagenesis. Equivalent amounts of mutated APA cDNA species were transiently transfected into COS-7 cells.

Metabolic labelling of the wild-type APA and the two mutants showed the presence of a major band migrating with an apparent molecular mass of 130 kDa (Figure 3).

Enzymic activity of recombinant APAs

The enzymic activities of the Phe-471 and His-471 mutants were largely decreased compared with that of the wild-type enzyme. To analyse more precisely the effects of the mutations on enzymic activity, K_m and k_{cat} values were measured for the wild-type APA and both mutants (Table 2), using the conditions described in the Experimental section. The K_m values were equivalent for the wild-type APA and the Phe-471 and His-471 mutants. In contrast, the k_{cat} values were greatly decreased, leading to a decrease in k_{cat}/K_m to 1/850 and 1/1200 for the Phe-471 and His-471 mutants respectively, equivalent to a change in free energy of 4.0 and 4.2 kcal/mol (0.96 and 1.00 kJ/mol) respectively. We verified that the basal activity (COS-7 cells transfected with the control plasmid pSVneo) represented less than 10% of the activity of the mutants [0.07 ± 0.01 compared with 0.8 ± 0.1 (Phe-471) nmol of GluNA hydrolysis/h per μ g of total protein]. This basal activity exhibits a different profile of sensitivity to Ca²⁺ and GluSH from that of the wild-type APA (1.5-fold activation compared with 4-fold at 4 mM Ca²⁺, and 30% inhibition compared with 100% at

Table 1 Inactivation of aminopeptidase A by *N*-acetylimidazole

A membrane fraction of transfected cells was treated with 50 or 100 mM *N*-acetylimidazole for 30 min in the presence or absence of 0.1 mM GluSH or 0.5 mM amastatin; the reaction was stopped by dilution and centrifugation. The residual APA activity was assayed and the percentage inhibition was calculated from the activity of control APA (treated as described above in the absence of *N*-acetylimidazole). Results are means $\pm$ S.D. for three independent experiments with duplicate determinations.

Treatment	APA activity (nmol/min per mg of total protein)	Inhibition (%)
Control	10 $\pm$ 1	0
<i>N</i> -Acetylimidazole (50 mM)	3.8 $\pm$ 0.4	62 $\pm$ 7
<i>N</i> -Acetylimidazole (100 mM)	1.7 $\pm$ 0.4	83 $\pm$ 20
<i>N</i> -Acetylimidazole (50 mM) + GluSH (0.1 mM)	3.7 $\pm$ 0.2	63 $\pm$ 3
<i>N</i> -Acetylimidazole (50 mM) + amastatin (0.5 mM)	9.7 $\pm$ 0.3	3 $\pm$ 0.1

Figure 2 Schematic representation of the active site of APA binding a natural substrate (angiotensin II) or competitive inhibitors (GluSH or amastatin).

Figure 3 Metabolic labelling of recombinant APAs

Transfected COS-7 cells were labelled for 5 h with [³⁵S]methionine, and the cell lysate proteins were immunoprecipitated with an anti-APA polyclonal antiserum, resolved by SDS/PAGE [7.5% (w/v) gel] and identified by autoradiography. The two first lanes correspond to the cells transfected with pSVneo and the wild-type APA respectively. The molecular mass of recombinant APAs is indicated by an arrow.

Table 2 Kinetic parameters of non-mutated and mutated APAs

K_m and k_{cat} values shown are means $\pm$ S.D. for three separate determinations, with GluNA as the substrate. $\Delta\Delta G$ is the difference in free energy for mutated enzymes and is calculated from the equation:

$$\Delta\Delta G = RT \ln(k_{cat}/K_m)_{wildtype} / (k_{cat}/K_m)_{mutant}$$

APA form	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	$\Delta\Delta G$ (kcal/mol)
Wild-type	63 $\pm$ 5	103.0 $\pm$ 9.0	1700	—
Phe-471	73 $\pm$ 5	0.15 $\pm$ 0.004	2	4.0
His-471	60 $\pm$ 2	0.084 $\pm$ 0.013	1.4	4.2

Table 3 K_i values of inhibitors for wild-type and mutated APAs

APA form	K_i (GluSH) (μ M)	K_i (GluPO ₃ H ₂) (nM)
Wild-type	0.07 $\pm$ 0.01	8.7 $\pm$ 0.6
Phe-471	0.10 $\pm$ 0.01	116.0 $\pm$ 18.0

5 μ M GluSH). It results mostly from the presence of several endogenous aminopeptidases in COS cells.

The Phe-471 mutant is activated by Ca²⁺ similarly to the wild-type enzyme (30 μ M; 50 % of maximal activation) (results not shown).

Inhibition of the recombinant APAs enzymic activities by GluSH and GluPO₃H₂

The inhibition of GluNA hydrolysis by the wild-type APA and the Phe-471 mutant was compared by using different classes of inhibitor of APA. GluSH, a substrate analogue that fits in the S1 subsite and possesses a strong zinc-chelating group [23], has a K_i value similar for the wild-type enzyme and the Phe-471 mutant (Table 3), showing that this inhibitor binds similarly in the mutated active site. GluPO₃H₂, previously shown to be a modest competitive inhibitor of leucine aminopeptidase (K_i 80 μ M), and practically inactive towards APN [29], is clearly more active towards recombinant APA (K_i 8.7 $\pm$ 0.6 nM). This inhibitor, which is presumed to be an analogue of the transition state in APA, exhibits a K_i for the Phe-471 mutant that is smaller by one order of magnitude.

DISCUSSION

In the absence of structural data on monozinc aminopeptidases, site-directed mutagenesis studies remain important tools for probing the organization of their active sites and will facilitate the design of drugs directed at such targets.

A sequence comparison of APA with other aminopeptidases revealed the presence of a short homologous peptide segment

Figure 4 Putative interactions of the inhibitors GluSH (A), GluPO₃H₂ (B) and a peptide substrate (C) with the Zn²⁺ ion and Tyr-471 in the active site of APA

containing a conserved tyrosine residue (Tyr-471 in APA). Chemical modifications by *N*-acetylimidazole treatment of APN and LTA4 hydrolase, which has an aminopeptidase activity in addition to its epoxide hydrolase activity [31,32], revealed the presence of an essential tyrosine residue at the active site [33,34]. Recently, site-directed mutagenesis of Tyr-383 in LTA4 hydrolase resulted in inactive mutants towards peptidase activity, but consequently no conclusions could be drawn as to its role in catalysis [35].

In a preliminary experiment, treatment of APA with *N*-acetylimidazole resulted in a loss of enzymic activity. In contrast with the inhibitor GluSH, preincubation with the inhibitor amastatin protected the enzyme from inactivation, indicating that the modified residue, important for enzymic activity, is localized at the active site of APA (not in the S1 subsite).

The functional role of Tyr-471 in APA was investigated by replacing this residue by either a Phe or a His residue by site-directed mutagenesis. The choice of a Phe was dictated to alter as little as possible the structure of the active site of APA. This substitution led to a marked loss of activity, as observed in a similar experiment conducted on LTA4 hydrolase [35]. However, because of the high peptidase efficiency of APA compared with LTA4 hydrolase towards substrates smaller than tripeptides [36], the kinetic parameters could be determined. The similar K_m values of the substrate GluNA for the mutant and the wild-type enzyme indicates that Tyr-471 is not involved in substrate binding. Furthermore the similar K_i for GluSH shows that the mutation does not change the interaction of the thiol group with the zinc atom. The behaviour of the Phe-471 mutant and the

wild-type enzyme towards Ca²⁺ activation is identical, showing that the Tyr-471 mutant does not co-ordinate this ion. In contrast, the k_{cat} value of the Phe-471 mutant decreases drastically and lowers the k_{cat}/K_m by three orders of magnitude, indicating that the phenolic oxygen of Tyr-471 has a crucial role in the cleavage of the scissile peptide bond. This decrease in k_{cat} corresponds to a variation of binding energy ($\Delta\Delta G$) of 4.0 and 4.2 kcal/mol (0.96 and 1.00 kJ/mol) for Phe-471 and His-471 respectively. This indicates that Tyr-471 in APA is not the counterpart of Tyr-248 in carboxypeptidase A, because the mutation of that residue affected the K_m weakly and not the k_{cat} towards peptide substrates [37]. In contrast, these kinetic measurements are consistent with those observed for the mutation of the residues proposed to stabilize the transition state in carboxypeptidase A (Arg-127) and thermolysin (His-231) [18,38]. It was calculated that the loss of binding energy $\Delta\Delta G$ of the transition state due to the mutations ranged from 3.7 to 3.8 kcal/mol (15.48 to 15.90 kJ/mol) for His-231 in thermolysin [18], and from 4.1 to 6.0 kcal/mol (17.15 to 25.10 kJ/mol) for Arg-127 in carboxypeptidase A [38]. In these enzymes, hydrolysis is believed to proceed by the attack of a Zn²⁺-bound water molecule on the carbonyl carbon of the peptide bond to form a tetrahedral intermediate, the negative charge induced on the oxygen of the carbonyl group being stabilized by His-231 and Tyr-157 in thermolysin [17], and by Arg-127 in carboxypeptidase A [39]. The similar $\Delta\Delta G$ values are suggestive of a similar role for Tyr-471 in APA. These variations in free energy are consistent with the loss of a strong hydrogen bond that occurs between an uncharged side chain of the enzyme and a charged group of the substrate [40]. This could correspond to hydrogen bonding from the hydroxy group of Tyr-471 and the

negatively charged carboxylic oxygen of the tetrahedral intermediate (Figure 4C). His-711 in neutral endopeptidase, whose replacement by a phenylalanine residue also led to a variation in free energy, was previously identified to behave like His-231 in thermolysin [19].

The involvement of a tyrosine residue in the stabilization of the transition state in peptide catalysis has not previously been demonstrated by site-directed mutagenesis studies in zinc metallopeptidases. Structural studies proposed that a tyrosine residue, Tyr-157, in thermolysin interacts in concert with His-231 with one of the oxygen atoms of the hydrated peptide via hydrogen bonding [17]. Furthermore the structure of the zinc peptidase astacin in complex with a phosphinic inhibitor indicates that a tyrosine residue (Tyr-149) forms a hydrogen bond to the phosphinyl group that mimics the carboxyanion of the transition state [20]. This tyrosine residue was suggested to be analogous to His-231 of thermolysin and Arg-127 of carboxypeptidase A [20].

To ensure the functional role of Tyr-471 in the stabilization of the transition state, we compared the binding modes of two different classes of inhibitor to the active site of wild-type and mutant APAs. Thiol inhibitors are potent inhibitors of zinc metalloproteases, the thiol group chelating the zinc ion strongly [41,42]. The inhibitory potency of the thiol-containing inhibitor GluSH is equivalent for the wild-type APA and Phe-471, as previously observed with thiorphan on wild-type (His-231) and mutated (Phe-231) thermolysins [18]. It was suggested that the histidine residue in position 231 does not interact with the thiol group because the distance between the sulphur atom of the inhibitor and the imidazole of His-231 is too great to allow a hydrogen bond [17,43]. This result suggests a similar spatial positioning of Tyr-471 in APA and His-231 in thermolysin towards metallopeptidase thiol inhibitors (Figure 4A).

Phosphonate and phosphoramidate transition-state analogues in zinc metallopeptidases interact with the amino acids involved in transition-state binding, and usually show displaced K_i values when these residues are mutated [18,38]. The phosphonate inhibitor GluPO_3H_2 binds particularly strongly to APA and constitutes the first compound to inhibit this enzyme in the nanomolar range. It was proposed to behave as a putative transition-state analogue, in which the replacement of the substrate scissile amide bond with a phosphonic acid group mimics the tetrahedral transition state [29], with one of the phosphoryl oxygens ligating the zinc in a tetrahedral complex and forming one or more hydrogen bonds with the residue(s) involved in transition-state stabilization (Figure 4B). The mutation of Tyr-471 to Phe-471 significantly decreases the affinity of the phosphonic acid inhibitor. This demonstrates that the inhibitor interacts with the tyrosine hydroxy group of Tyr-471, strengthening the hypothesis that this residue stabilizes the transition state in APA. This interaction (shown in Figure 4B) is presumed to be similar to that observed between Tyr-149 of astacin and a phosphinic transition-state analogue inhibitor, suggesting that Tyr-471 in APA might be the counterpart of Tyr-149 in astacin [20].

The decrease (to 1/13) in the K_i of the inhibitor GluPO_3H_2 for the mutant is small compared with the three orders of magnitude by which k_{cat}/K_m decreases. This result can be explained by the fact that the structure of the phosphonate group is not, like a phosphonamide group, analogous with the peptide amide bond, so that its geometry is not strictly identical with that of the tetrahedral intermediate. The non-optimized positioning of the oxygen of the inhibitor compared with the carbonyl oxygen of the substrate towards the hydroxy group of Tyr-471 would result in different binding energies that are reflected in the differences in the kinetic measurements.

In comparison with the thermolysin, carboxypeptidase A and astacin active-site models, the interaction of Tyr-471 with the tetrahedral intermediate of a peptide substrate in the active site of APA is proposed in Figure 4(C). The free amino group and the side-chain carboxylate of the N-terminal glutamic residue of the substrate are complexed with residues of the S1 pocket, and the zinc atom is co-ordinated to its three ligands (His-385, His-389 and Glu-408) and a water molecule. The negative charge of Glu-386 polarizes the zinc-co-ordinated water molecule and promotes the nucleophilic attack of the carbonyl carbon of the peptide bond. The resulting tetrahedral intermediate might be then stabilized by an electrostatic interaction with the Zn^{2+} ion and hydrogen bonds with Glu-386 and Tyr-471.

In conclusion, Tyr-471 in APA seems to stabilize the transition state of the catalytic process by 4.0 kcal/mol (0.96 kJ/mol), acting as an electrophilic catalyst through interaction of the tyrosine hydroxy group with the oxyanion. Direct confirmation of these observations will be provided by crystallographic analysis of the protein.

This work emphasizes the possibility of using transition-state analogues in the design of specific and potent inhibitors of APA, tools that are essential for exploring the physiological role of this enzyme.

We thank Dr. V. Dive and Professor B. P. Roques for their critical reading of the manuscript, Dr. S. Wilk for providing the APA antiserum and purified APA, Professor M. C. Fournié-Zaluski for providing the inhibitor GluSH, and Dr. B. Lejczak for the gift of the inhibitor GluPO_3H_2 . We also thank N. de Mota for technical assistance and Dr. K. Curnow for revising the manuscript. G.V. was supported in part by *Naturalia & Biologia*.

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