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Catalytic site studies on tuna (*Thunnus albacares*) pyloric caeca aminopeptidase

Mustapha Hajjou^{*}, Yves Le Gal

Laboratoire de Biologie Marine, BP 225, 29182 Concarneau Cedex, France

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Abstract

Tuna pyloric caeca aminopeptidase (tAP) is a glycosylated zinc-metalloenzyme containing apparently two identical subunits. The enzyme is reversibly inhibited in a time-dependent manner by amastatin. Slow development of tAP inhibition by this inhibitor could be demonstrated. Dissociation of the complex of tAP with amastatin is also slow. Two molar equivalents of the inhibitor are bound by the enzyme suggesting the presence of one catalytic site in each subunit. Chemical modification of tAP with 1-cyclohexyl-3-(2-morpholinoethyl)carbonyl-metho-*p*-toluene sulfonate and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinone revealed the presence of essential acidic amino acid residues probably located at the active site. Compatible with the presence of arginine and tyrosine residues at the catalytic site of most metalloproteinases, tAP is reversibly inhibited by phenylglyoxal and inactivated by tetranitromethane in a time-dependent fashion. The rate of inhibition by these modifiers could be significantly decreased if the enzyme was previously treated with amastatin suggesting that the modified amino acid residues are located at the catalytic site. Diethylpyrocarbonate did not affect the activity of both native and zinc-depleted tAP suggesting that histidine is not involved in the zinc-ligand formation.

Keywords: Aminopeptidase; Aminopeptidase; Inhibition; Amastatin; Catalytic site; (Tuna)

1. Introduction

Aminopeptidases (APs) are hydrolases which split off the N-terminal amino acid residues from peptides and proteins. These enzymes are widely distributed in vertebrate, invertebrate and plant tissues, and in microorganisms. Many functions of APs have been suggested. They participate in the breakdown of peptides in the gut, and maturation of hormones. They have been associated with some pathological conditions [1]. Aminopeptidase N (EC 3.4.11.2) has been shown to be involved in Na⁺-dependent amino acid transport [2]. It is involved in human cy-

tomegalovirus infection [3], and it is also a specific receptor for human coronavirus 229E [4], and the enteropathogenic coronavirus TGEV [5]. It has been suggested that aminopeptidases regulate, via amino terminal processing, the susceptibility of some proteins to degradation by ubiquitin-dependent proteolytic system [6].

Many APs have been purified and characterized. They are classified with respect to certain physical and biochemical properties such as metal ion content, kinetic characteristics including specificity and relative efficiency with which amino acid residues are removed, and susceptibility to inhibition by bestatin, amastatin, and puromycin [7–9]. They can be distinguished by the location of the enzyme which can be cytosolic, membraneous or a secreted form. Specificity of APs are broad and it has been suggested that an aminopeptidase should be assayed with a range of peptide substrates before choosing a name [10].

Amastatin is one of the potent inhibitors of aminopeptidases. It behaves as a competitive slow and tight-binding inhibitor [11]. Its complexation with bovine lens leucine aminopeptidase (b1LAP) has been recently used to eluci-

Abbreviations: tAP, Tuna aminopeptidase; LPNA, L-leucine-*para*-nitroanilide; GPNA, glycine-*p*-nitroanilide; IDA, iminodiacetic acid; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-metho-*p*-toluene sulfonate; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinone; TNM, tetranitromethane; DEPC, diethylpyrocarbonate.

^{*} Corresponding author. Present address: Meharry Medical College, School of Medicine, Department of Microbiology, 1005, D.B. Todd, Jr. Blvd., Nashville, TN 37208, USA. Fax: +1 (615) 3276072.

date the catalytic mechanism of this enzyme [12]. It is also a good tool for titration of aminopeptidases to determine the number of active sites [11].

In a previous published study, we reported several physical, chemical and kinetic properties of an aminopeptidase purified from tuna (*Thunnus albacares*) pyloric caeca [13]. The enzyme appears to be distinct from leucine aminopeptidase (EC 3.4.11.1) and AP M (EC 3.4.11.2). In our attempt to identify tAP we decided to investigate the composition of the catalytic site of this enzyme. In this paper we report some additional properties of tuna aminopeptidase (tAP) relating to its inhibition by amastatin and to the identification of some essential amino acid residues of the catalytic site.

2. Materials and methods

2.1. Chemicals

Leucine-*p*-nitroanilide, glycine-*p*-nitroanilide, amastatin, phenylglyoxal, *N*-ethoxycarbonyl-3-(2-morpho-*liethyl*)-1-2-dihydroquinone, tetranitromethane, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-metho-*p*-toluene sulfonate were obtained from Sigma (USA). IDA-Sephadex 6B and Sephadex G-25 were obtained from Pharmacia (Sweden). Reagents for electrophoresis were obtained from Serva (Germany). All common reagents were from Prolabo (France).

2.2. Aminopeptidase assay

Aminopeptidase activity against LPNA or GPNA was determined spectrophotometrically as described previously.

2.3. Protein determination

The amount of protein was determined by the method of Bradford [14] using bovine serum albumin as the standard.

2.4. Reactivation of amastatin-treated tAP

For the experiments involving chemical modifications of APs, free amastatin and or excess of the modifying agent were removed by gel filtration using small columns (1.5 ml) of Sephadex G-25. The reaction mixture of amastatin and AP or amastatin, AP, and or modifying agent in 50 to 100 μ l of total volume was chromatographed on a Sephadex column and fractions of 100 μ l were collected.

2.5. Stoichiometry of binding of amastatin to tAP

Tuna AP (0.4 nM) was titrated with 0, 0.24, 0.4, 0.64, and 0.8 nM amastatin and the activity was plotted as percentage activity vs. equivalent of aminopeptidase [11].

2.6. Modification by CMC and EEDQ

Enzyme samples (50 μ l) were diluted with 150 μ l of 50 mM Mes buffer (pH 6.0) containing 0.1 M CMC or 5 mM EEDQ. Some samples were treated with amastatin (0.2 mM final concentration) for 30 min at 35°C and some reaction mixtures contained 1 mM glycine methyl ester. After incubation at 35°C for the time periods indicated in the text, aminopeptidase activity was measured and plotted as percent of the control which corresponds to the enzyme incubated in the same conditions but in the absence of the modifying reagent.

Reactivation of CMC-treated tAP was assayed by addition of 1 M hydroxylamine after incubation of the enzyme with the modifying reagent for 5 h at 35°C. The reaction mixture containing hydroxylamine (0.5 M final concentration) was incubated for an additional hour at the same temperature.

2.7. Treatment of tAP and zinc-depleted tAP with DEPC

Apo-tAP was prepared by incubating the enzyme in the presence of 10 mM EDTA for 3 h at 35°C. Then, EDTA-treated tAP and untreated enzyme were chromatographed on small columns of Sephadex G-25 (1 ml) previously equilibrated with 0.1 M potassium phosphate buffer (pH 6.0) which was used for protein elution. 10 μ l of 25 mM DEPC were added to 1 ml of each enzyme preparation in the same buffer and incubated for 30 min at room temperature. Then, DEPC-treated tAP activity was directly measured, whereas DEPC-treated apo-tAP preparations were subjected to a chromatography step on an IDA(Co²⁺)-Sephadex 6B column (0.5 ml) prior to the measurement of the activity. All enzyme samples were used in duplicate and the experiment was independently repeated twice.

3. Results

3.1. Inhibition by amastatin

In order to avoid significant substrate depletion that could interfere with inhibition development [15,16], GPNA, which has a lower k_{cat} and higher K_m compared to LPNA, was used as substrate. Two approaches were used to examine the inhibition of tAP by amastatin. (1) Enzyme was added to a solution containing GPNA and amastatin. (2) The hydrolysis reaction of GPNA was initiated by addition of a mixture of tAP and amastatin, previously incubated for 30 min at 35°C, to a solution of substrate. The former showed a slow development of tAP inhibition while the later indicated that this inhibition was reversible (Fig. 1). Total activity of amastatin-treated tAP could be restored after diluting 1000-fold in 50 mM phosphate buffer (pH 8.0) and incubation for 2 h at room temperature (Fig. 2). We took advantage of this property and used

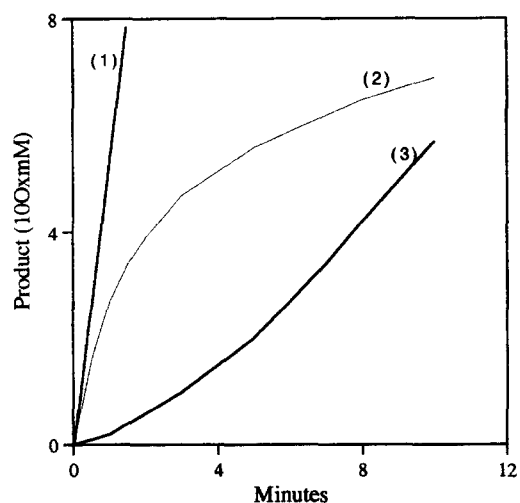


Fig. 1. Time-course of inhibition of tAP by amastatin. Curve (1): hydrolysis of substrate in the absence of amastatin. Curve (2): hydrolysis reaction was initiated by addition of 0.4 nM tAP (final concentration) to a solution containing 2 mM GPNA and 4 nM amastatin. Curve (3): tAP was incubated with amastatin for 30 min at 35°C prior to addition of a substrate solution (2 mM).

amastatin as a protective agent of tAP active sites in the enzyme chemical modification experiments. Furthermore, titration of tAP active sites with amastatin was achieved. The results revealed the binding of two molar equivalents of amastatin to the enzyme (Fig. 3).

3.2. Modification by CMC and EEDQ

Carbodiimide reagents are used for determination of carboxyl groups in protein and have been used for specific modification of acidic amino acids in enzymes [17].

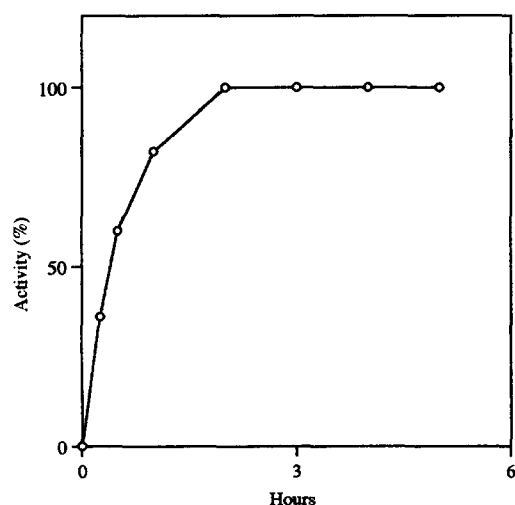


Fig. 2. Reversibility of tAP inhibition by amastatin. Duplicate samples of a mixture of tAP and amastatin (4 nM and 0.2 mM, respectively) were incubated for 30 min at 35°C. These solutions were then diluted 2000-fold with 10 mM phosphate buffer (pH 8). Aliquots (50 μ l) were withdrawn at the time periods indicated and added to 2 mM LPNA in the same buffer. The activity is plotted as percent of the control value (activity of tAP in the absence of amastatin).

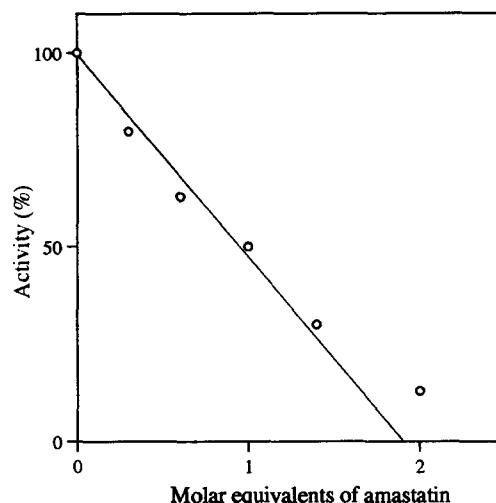


Fig. 3. Stoichiometry of binding of amastatin per dimer. Plot of percentage activity vs. molar equivalent of amastatin. Initial velocities for the hydrolysis of GPNA were obtained for assays containing 0.4 nM tAP and 0.12, 0.2, 0.4, 0.56, or 0.8 nM amastatin. The enzyme was preincubated with the inhibitor prior to addition of substrate. Each assay was carried out in duplicate. The experiment was carried out three times and representative values are plotted.

EEDQ also react with carboxyl groups. This reaction leads to the formation of mixed anhydride. It has been used as a condensation agent in ligand immobilization on solid support [18].

More than 80% of tAP activity was lost after incubation of the enzyme in the presence of CMC. As shown in Fig. 4, strong inactivation of tAP (more than 80%) was observed after incubation of the enzyme with CMC for 2 h at 35°C. This inactivation is irreversible and tAP activity

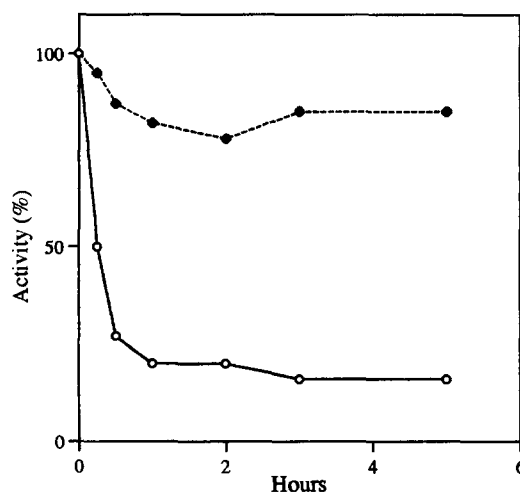


Fig. 4. Inactivation of tAP by CMC. Duplicate samples of tAP (○—○) were incubated at 35°C for the time periods indicated with 25 mM CMC in 50 mM Mes buffer (pH 6). Samples of amastatin-treated tAP (◆—◆) were incubated in the same conditions with the modifying reagent. Excess of modifying reagent and amastatin was then removed by gel filtration of Sephadex G-25 as described in Materials and methods and tAP activity was determined toward LPNA and expressed as percentage of untreated-tAP.

could not be restored by incubation with 0.5 M hydroxylamine. The presence of glycine methyl ester as a nucleophile did not influence the rate of inactivation by CMC. These results indicate that the inactivation is probably caused by the modification of a carboxyl group.

Preincubation of tAP with amastatin decreased the inactivation effect of CMC. Only 20% of the enzyme activity was lost after incubation for 5 h at 35°C with the modifying reagent. Treatment of tAP with EEDQ resulted in a strong inactivation of the enzyme. More than 90% of tAP activity was lost after incubation for 1 h with EEDQ. A protective effect of the enzyme with amastatin was also observed. Amastatin-treated tAP lost 64% of activity after incubation for 1 h at 35°C with this modifying reagent (Fig. 5).

3.3. The presence of an arginine residue at the catalytic site

Phenylglyoxal is a chemical modifying reagent which reacts specifically with arginine residue at alkaline pH values [19]. Treatment of tAP with phenylglyoxal resulted in a decrease in the enzyme activity. As shown in Fig. 6, the inhibition of tAP is time dependent. Modification of arginine residues by phenylglyoxal is reversible at pH 8.0. Phenylglyoxal-treated tAP activity can be restored after dilution in 50 mM phosphate buffer (pH 8.0) and incubation for 2 h at room temperature (Table 1). Partial reactivation of the inhibited enzyme was obtained after dilution with 0.65 M ammonium acetate whereas very low reactivation occurred at pH 6. Since the modified arginine residue is stable at this pH, we used 50 mM Mes buffer (pH 6) to elute amastatin-treated tAP from Sephadex G-25 column,

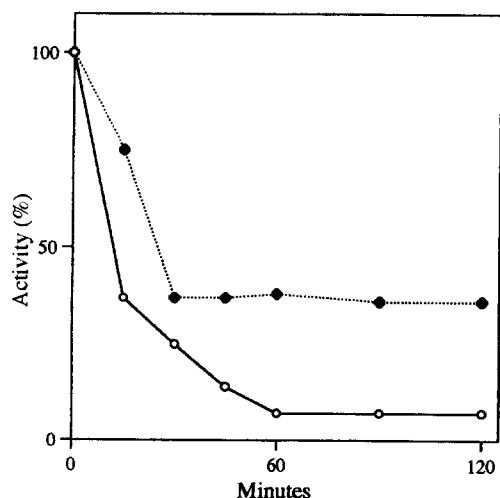


Fig. 5. Treatment of tAP with EEDQ (○—○). The enzyme samples were incubated at 35°C for the time periods indicated with 5 mM EEDQ in 50 mM Mes buffer (pH 6). Amastatin-treated tAP (◆—◆) was incubated in the same conditions with the modifier. Excess of amastatin and EEDQ was removed by gel filtration and tAP activity towards LPNA was measured and expressed as percentage of the enzyme activity in the absence of EEDQ.

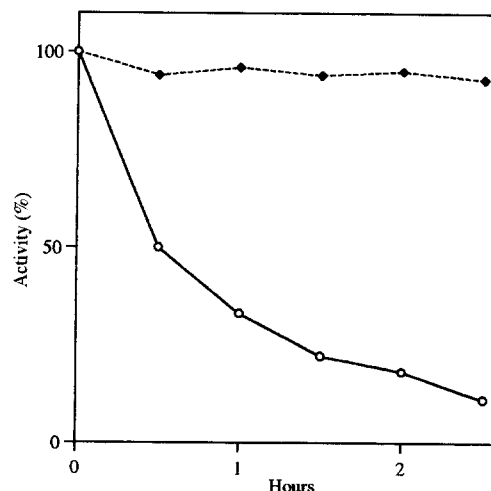


Fig. 6. Inhibition of tAP by phenylglyoxal. The enzyme samples (tAP (○—○) and amastatin-treated tAP (◆—◆)) were incubated at 35°C for the time periods indicated with 0.1 M phenylglyoxal in 10 mM phosphate buffer (pH 8). Excess of modifying reagent and amastatin was then removed by gel filtration and tAP activity was determined toward LPNA and expressed in percentage of untreated-tAP.

and then the complex tAP-amastatin was diluted in the same buffer to permit its dissociation. Amastatin-treated tAP is not affected by phenylglyoxal treatment since the remaining activity was more than 95%. All these results indicate the presence of an essential arginine residue probably located at the active site of tAP.

3.4. Inactivation with TNM

TNM is a suitable mild reagent for the nitration of tyrosyl residues at pH 8 [20]. The effect of this modifying reagent on tAP activity was examined. Treatment of the enzyme with 50 mM TNM resulted in the inactivation of

Table 1
Reactivation of phenylglyoxal-treated tAP

Sample	Reactivation %		
	ammonium acetate	phosphate buffer	Mes buffer
Phenylglyoxal-treated tAP	37	81	17
Phenylglyoxal-treated tAP Pretreated with amastatin	-	94	95

Reactivation was assayed with 0.65 M ammonium acetate (pH 7.5) 10 mM phosphate buffer (pH 8), and 10 mM Mes buffer (pH 6). After incubation of tAP with 0.1 M phenylglyoxal for 3 h at 35°C, excess of modifier and amastatin was removed by gel filtration on a mini-column of Sephadex G-25 previously equilibrated with a suitable buffer. Fractions containing the enzyme were pooled. Reactivation of phenylglyoxal-treated tAP by phosphate buffer was achieved by diluting the enzyme 100-fold with this buffer and incubating for 20 h at room temperature. Pooled fractions eluted with ammonium acetate were incubated in the same conditions. Pooled fractions eluted with Mes buffer were diluted 500-fold in the same buffer and incubated for 2 h at room temperature. Duplicate samples were used for each reactivation assay. The experiments were carried out twice.

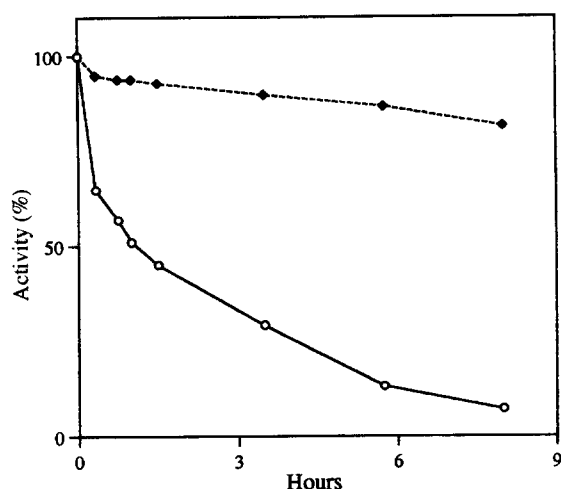


Fig. 7. Inactivation of tAP by TNM (○—○). Duplicate samples of tAP were incubated at 25°C for the time periods indicated with 50 mM TNM in Tris-HCl buffer (pH 8). Amastatin-treated tAP (◆—◆) was incubated in the same conditions with the modifier. Excess of modifying reagent and amastatin was then removed and the enzyme activity was determined with LPNA as substrate and expressed as a percentage of untreated-tAP activity.

the aminopeptidase. The enzyme is inactivated in a time-dependent manner (Fig. 7). When tAP was preincubated with amastatin, less than 20% of the enzyme activity was lost after incubation with TNM for 6 h at 20°C. These results reveal the presence of an essential tyrosine residue in tAP.

3.5. Treatment of tAP with diethylpyrocarbonate

DEPC is an acylation agent which can react with histidine at pH 6.0. This reaction usually leads to the formation of carboethoxyhistidine [21].

Incubation of DEPC with tAP at pH 6.0 did not affect the enzyme activity (results not shown). Incubation of zinc-depleted tAP with DEPC also had no effect on the enzyme activity. Indeed, full activity of apo-tAP pretreated with the modifying agent was restored by Co^{2+} using an IDA (Co^{2+})-Sephrose 6B column.

4. Discussion

Tuna aminopeptidase is reversibly inhibited by amastatin which acts as a competitive slow and tight-binding inhibitor of many aminopeptidases such as bovine lens leucine aminopeptidase, aminopeptidase M and *Aeromonas* aminopeptidase [11,22]. The slow formation of tAP-amastatin complexes can be demonstrated and dissociation of these complexes was also shown to be slow suggesting a tight binding behaviour. This property allows amastatin to be used as a catalytic site titrant; tAP binds two molar equivalents of the inhibitor. Because amastatin has been assumed to be a transition-state analogue, only catalytically active enzyme is expected to bind the inhibitor with

high affinity. The result obtained in this study suggests the presence of one catalytic site per tAP subunit. This is consistent with the presence of one zinc-atom per tAP subunit [13]. However, the possibility that species other than catalytically active tAP might bind the inhibitor cannot be ruled out. Bestatin, another transition-state analogue, was used for titration of catalytic site of bILAP [23]. The enzyme binds six equivalents of the inhibitor per hexamer. This result is in agreement with X-ray crystallographic studies [24,25]. As for AP-A tAP is not inhibited by bestatin. This characteristic distinguishes markedly tAP from LAP.

Chemical modification of tAP with some modifying reagents allowed us to identify some amino acid residues essential for the enzyme activity.

CMC reversibly inhibits the enzyme in a time-dependent way. More marked inhibition of tAP was obtained with EEDQ. Treatment of tAP with amastatin prior to addition of one of these modifiers results in a protective effect on the enzyme.

The reaction of carboxyl groups with carbodiimide reagents leads to the formation of an intermediate *O*-acylisourea, and the product is stabilized by addition of a nucleophile [17]. Addition of glycine methyl ester as a nucleophile did not affect the rate of tAP inactivation by CMC. This has been already observed for some other enzymes [26,27].

Since carbodiimide reagents can react with tyrosine residues, treatment of CMC-treated tAP with 0.5 M hydroxylamine was carried out. The failure of hydroxylamine to reactivate the enzyme suggests that inactivation of tAP is mainly caused by the modification of carboxyl groups located at the active site. Actually CMC should have reacted with tyrosine residues present in the active site (see below). The absence of any partial reactivation by hydroxylamine suggests that all functional carboxyl groups were modified by CMC. These results reveal the presence of essential carboxyl groups in tAP catalytic site. Similar studies have been reported for *Aeromonas* aminopeptidase [28], angiotensin converting enzyme [27], *Clostridium histolyticum* collagenase [29], and human neutrophil collagenase [30]. Our results are consistent with the presence of His-Glu-Xaa-Xaa-His consensus sequence in most zinc-containing metalloproteases [26]. In addition, Glu and both Asp and Glu are involved in zinc binding in AP-M and LAP, respectively [31].

Treatment of tAP with TNM led to inactivation of the enzyme. Amastatin-treated tAP is not affected by this modifying reagent. TNM reacts with tyrosine residues and can also oxidate SH groups [20]. Since tAP contains no essential cysteine residues [13] our results indicate the presence of a tyrosine residue essential for the activity of tAP. The protective effect of amastatin suggests that the nitrated tyrosine is part of the active site. No tyrosine residue has been shown to be present in LAP catalytic site [12,24,25].

The active sites of metalloproteinases usually contain glutamic acid, tyrosine, and basic amino acids essential for their activity [26,32].

Chemical modification of tAP with phenylglyoxal, a mild and specific modifier of arginine, results in a marked decrease of the enzyme activity. Pretreatment of tAP with amastatin prior to addition of the modifying reagent led to a significant decrease of the inhibition rate. These results reveal the presence of an arginine residue located at tAP catalytic site.

X-ray crystallographic studies of bLAP revealed the presence of an arginine residue (Arg-336) at the active site [24,25]. Kim and Lipscomb [12] have recently proposed a catalytic mechanism for the same enzyme based on X-ray crystallographic studies of bLAP complex with amastatin. The mechanism invokes a role for Arg-336 as an electrophilic substrate activator and transition state stabilizer. This amino acid residue seems to be conserved in other aminopeptidases and in carboxypeptidase A [33]. The presence of a catalytically important arginine residue in thyrotropin-releasing-hormone-degrading ectoenzyme was demonstrated by O'Connor and O'Cuinn [34].

The failure of diethylpyrocarbonate to affect both native tAP and zinc-depleted enzyme indicates that histidine residues are neither involved in the zinc-ligand formation nor in the catalysis mechanism. These results are in agreement with those obtained for *Aeromonas* aminopeptidase [28,35].

As APs include a number of apparently distinct hydrolases, utilisation of various criteria for their classification is required. Otherwise nonexclusive names may be concluded [10]. For this purpose, investigation of the active site composition is an asset.

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