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Kinetics of Nitroanilide Cleavage by Astacin

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Summary: The investigation of the catalytic properties of astacin, a zinc-endopeptidase from the crayfish *Astacus astacus* L., has gained importance, because the enzyme represents a novel, structurally distinct family of metalloproteinases which also includes a human bone morphogenetic protein (BMP1). Astacin releases nitroaniline from succinyl-alanyl-alanyl-alanyl-4-nitroanilide (Suc-Ala-Ala-Ala-pNA), a substrate originally designed for pancreatic elastase. This activity was unexpected since only few metalloproteinases cleave small nitroanilide substrates, and, moreover, the primary specificity of astacin toward protein substrates is determined by short, uncharged amino-acid sidechains in the P_1'-position, i.e. the new N-terminus after cleavage. The specificity constants, k_{cat}/K_m, for the release of nitroaniline from substrates of the general structure Suc-Ala_n-pNA (n = 2, 3, 5) and Ala_n-pNA (n = 1, 2, 3) increase with the number of alanine residues. The longest peptide, Suc-Ala-Ala-Ala-Ala-Ala-pNA, is the only one out of eleven substrates used in this study, which is cleaved at two positions by astacin. The first cleavage yields Suc-Ala-Ala and Ala-Ala-Ala-pNA. From the resulting C-terminal fragment, Ala-Ala-Ala-pNA, a second cut releases nitroaniline. The 1200-fold higher specificity constant observed for the first as compared to the second cleavage in Suc-Ala-Ala-Ala-Ala-Ala-pNA reflects the preference of astacin for true peptide bonds and also the importance of a minimum length of the substrate. Amino-acid substitutions in peptides of the structure Suc-Ala-Ala-X-pNA (X = Ala, Phe, Pro, Val) cause a drop in activity following the order Phe > Ala >> Pro > Val. This is the same order seen for the frequency of these residues to occur in the P_1-position of the pattern, that had been derived previously from the analysis of 71 cleavage sites of astacin in α- and β-tubulin. The kinetic data obtained for peptides of the composition Suc-(Ala, Ala, Pro)-pNA also can be interpreted in accordance with this pattern, since a proline residue is well accepted in S_3, but less tolerated in S_2 and excluded from S_1. The data suggest that the peptide moiety is in contact with the enzyme subsites on the N-terminal side of the cleavage point, i.e. the S-subsites, and that the nitroanilide moiety is facing the S′-subsites. Hence, astacin binds Suc-Ala-Ala-Ala-pNA in a similar orientation as elastase, although the primary specificity of elastase is determined by the P_1-position and of astacin by the P_1'-position.

Enzymes:
Astacin (EC 3.4.24.–), former designations: EC 3.4.99.6, Crayfish small-molecule proteinase, *Astacus* protease);
Bovine pancreatic trypsin (EC 3.4.21.4);
Human aminopeptidase N (EC 3.4.11.2);
Porcine pancreatic elastase (EC 3.4.21.36);
Snake venom metalloproteinase (EC 3.4.24.1);
Thermolysin (EC 3.4.24.4).

Abbreviations:
Ampso, 3-(1,1-dimethyl-2-hydroxyethylamino)-2-hydroxypropanesulfonic acid; BMP, bone morphogenetic protein; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; Dns, Dansyl; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid); HPLC, high-pressure liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; pNA, 4-nitroanilide; Suc, succinyl; TFA, trifluoroacetic acid; Tris, Tris(hydroxymethyl)-aminomethane.

Kinetik der Spaltung von Nitroaniliden durch Astacin

Zusammenfassung: Die Untersuchung der katalytischen Eigenschaften des Astacins, einer Zink-Endopeptidase aus dem Verdauungstrakt des Flußkrebses *Astacus astacus* L., hat an Bedeutung gewonnen, weil dieses Enzym eine neue, strukturell distinkte Familie von Metalloproteinasen repräsentiert, die auch ein Knochenwachstum-induzierendes, menschliches Protein (BMP1 = bone morphogenetic protein) einschließt. Astacin setzt aus dem ursprünglich für pankreatische Elastase entwickelten Substrat Succinyl-alanyl-alanyl-alanyl-4-nitroanilid (Suc-Ala-Ala-Ala--pNA) Nitroanilin frei. Diese Aktivität war unerwartet, denn nur wenige Metalloproteinasen spalten kleine Nitroanilidsubstrate. Darüberhinaus wird die primäre Spezifität des Astacins gegenüber Proteinsubstraten durch Aminosäurereste mit kurzen, ungeladenen Seitenketten in der Position P_1' bestimmt, d.h. dem neuen N-Terminus nach der Spaltung. Die Spezifitätskonstanten, k_{cat}/K_m, für die Freisetzung von Nitroanilin aus Peptiden der Struktur Suc-Ala_n--pNA ($n = 2, 3, 5$) und Ala_n-pNA ($n = 3, 2, 1$) steigen mit der Zahl der Alaninreste. Das längste Peptid, Suc-Ala-Ala-Ala-Ala-Ala-pNA, ist das einzige der elf untersuchten Substrate, welches von Astacin an zwei Positionen gespalten wird. Bei der ersten Spaltung entstehen Suc-Ala-Ala und Ala-Ala-Ala-pNA. Aus dem C-terminalen Fragment, Ala-Ala-Ala-pNA, setzt ein zweiter Schnitt Nitroanilin frei. Die 1200mal höhere Spezifitätskonstante für die erste gegenüber der zweiten Spaltung in Suc-Ala-Ala-Ala-Ala-Ala--pNA spiegelt die Präferenz des Astacins für echte Peptidbindungen wider und ebenso die Bedeutung einer Mindestlänge des Substrates. Aminosäuresubstitutionen in Peptiden der Struktur Suc-Ala-Ala-X--pNA (X = Ala, Phe, Pro, Val) bedingen eine Abnahme der Aktivität in der Reihenfolge Phe> Ala>>Pro>Val. Dies steht in Übereinstimmung mit der relativen Häufigkeit dieser Reste in der P_1-Position des Spaltungsmusters, welches durch die Analyse der 71 Spaltstellen des Astacins in α- und β-Tubulin ermittelt worden war. Nach diesem Muster können auch die kinetischen Daten für die Substrate der Zusammensetzung Suc-(Ala, Ala, Pro)-pNA interpretiert werden: Ein Prolinrest wird gut akzeptiert in S_3, weniger gut in S_2 und ausgeschlossen von S_1. Diese Ergebnisse sprechen dafür, daß die Nitroanilidsubstrate mit ihrem Peptidanteil an die S-Positionen des Enzyms binden, also auf der N-terminalen Seite der Spaltstelle, während die Nitroaniliddomäne den S'-Positionen zugewandt ist. Somit bindet das Astacin Suc-Ala-Ala-Ala-pNA in der gleichen Orientierung wie Elastase, obwohl die primäre Spezifität der Elastase durch den Rest in P_1-Position, die des Astacins dagegen durch den Rest in P_1'-Position bestimmt wird.

Key terms: Astacin, *Astacus protease*, metalloproteinase, nitroanilide substrates, kinetics.

Astacin, a digestive endopeptidase of M_r 22614, is synthesized in the hepatopancreas of the crayfish *Astacus astacus*[1,2]. The enzyme contains one gram-atom of tightly-bound zinc per mol which is essential for catalysis[3]. Metal binding agents like 1,10-phenanthroline, EDTA, amino-acid hydroxamates and certain thiol-containing compounds reversibly inhibit astacin[3–5]. The enzyme is also inhibited by specific peptide inhibitors recently discovered in plants of the families Cucurbitaceae[6] and Solanaceae[7], and by the unspecific proteinase inhibitor α_2-macroglobulin[8].

The amino-acid sequence of astacin is not related to those of other enzymes[9]. Therefore, this enzyme can be considered as prototype of a novel family of metalloproteinases abundant in the digestive tract of decapod crustaceans. As has been demonstrated recently, this protein family also includes a human bone morphogenetic protein (BMP1) whose domain A and astacin exhibit 36% sequence identity[10]. This observation poses new questions as to the tertiary structure, to the evolution and to the function of the crayfish enzyme.

The present report is concerned with the catalytic function of astacin in the hydrolysis of small synthetic peptide-4-nitroanilide substrates.

The cleavage specificity of astacin towards protein substrates has been deduced from the hydrolysis of α- and β-tubulin. The sequences around the 71 cleavage sites observed in these substrates revealed that the primary specificity is determined by short, uncharged amino-acid sidechains in position P_1'[11] (nomenclature according to Schechter and Berger (1967)[12]), i.e. the enzyme liberates peptides with Ala, Thr, Ser and Gly in N-terminal position. There is also a high frequency of proline in P_2', which already had been noticed earlier[13]. Statistical analysis of the tubulin cleavage sites taking into account the subsites P_4–P_4'

resulted in a cleavage pattern which demonstrated that in addition to the residues in P_1' and P_2', adjacent side-chains significantly influence substrate binding and turnover, indicating an extended substrate-binding site. The enzyme prefers proline in P_3, basic and/or large uncharged side-chains in P_2 and P_1 and hydrophobic residues in P_3' and P_4'[14].

Due to this uncommon but distinct cleavage specificity, at the outset no chromogenic peptide substrates had been available for astacin. The first convenient substrate found was Suc-Ala-Ala-Ala-pNA[15], which originally had been designed for pancreatic elastase[16]. So far, nitroanilides have been known as substrates of serine and cysteine proteinases, e.g. cf.[17,18], whose specificity is governed by the residue in P_1-position. By contrast, the primary specificity of the majority of the known metalloproteinases is determined by the residue in P_1'. These enzymes rarely cleave off chromophores C-terminally attached by amide bonds to amino acids or peptides[19] but are rather restricted to true peptide bonds and substrate binding involves interactions with a minimum number of subsites on both sides of the cleavage point[20]. Hence, the activity of astacin versus Suc-Ala-Ala-Ala-pNA is in apparent contradiction to its specificity toward protein substrates. In contrast, nitroanilides are known as reasonable substrates for a different group of metalloproteases, namely metalloaminopeptidases[21,22].

In an attempt to clarify the mechanism of the unexpected cleavage of Suc-Ala-Ala-Ala-pNA we have kinetically analysed a series of peptide-nitroanilides of varying length and composition. HPLC analysis of the cleavage products indicates that astacin does hydrolyse the terminal amide-bond in these substrates. It is seen that the specificity constants k_{cat}/K_m increase with the length of the substrates and that the position of a proline residue in subsites P_3, P_2, or P_1 is critical in the rate-limiting step of substrate hydrolysis. The specificity of astacin toward nitroanilide substrates is compared to fluorescent peptide substrates analysed recently[14,23].

Materials and Methods

All chemicals were of p.A. quality and purchased from Sigma (Deisenhofen), Riedel de Haen (Seelze), Serva (Heidelberg), Merck (Darmstadt) and Roth (Karlsruhe). Suc-Ala-Ala-Ala-Ala-pNA, Suc-Ala-Ala-Ala-pNA, Suc-Ala-Ala-pNA, Suc-Ala-Ala-Val-pNA, Suc-Ala-Ala-Phe-pNA, Ala-Ala-Ala-pNA, Ala-Ala-pNA and Ala-pNA were obtained from Bachem (Heidelberg). Suc-Pro-Ala-Ala-pNA, Suc-Ala-Pro-Ala-pNA and Suc-Ala-Ala-Pro-pNA were kindly provided by Dr. Dieter Brömme (Halle).

Astacin, prepared according to Zwilling and Neurath[1], was dissolved in 0.02M Tris/HCl buffer, pH 8.0 and further purified by anion-exchange chromatography on a HR 5/5 Mono Q column (Pharmacia, Freiburg) using a Dupont Model 8800 HPLC instrument. Elution at 1 ml min^{-1} was performed in 0.02M Tris/HCl, pH 8.0, with a linear gradient of NaCl (0.2–0.35M in 20 min). Purified astacin was desalted on a Sephadex G-10 column (20 cm × 4 cm; Pharmacia) equilibrated with 0.05M NH_4HCO_3, pH 8.0, flow rate 100 ml min^{-1}, and lyophilized. Astacin concentrations are based on the absorbance coefficient $\varepsilon_{280nm} = 42\,800 M^{-1} cm^{-1}$[3].

Polyacrylamide gel electrophoresis was performed according to Laemmli (1970)[24], omitting sodium dodecyl sulfate and reducing agents.

To avoid metal contamination, metal-free glassware, polypropylen containers and pipette tips were used and buffer stock solutions (1M) of Mes, Hepes, Ampso and Caps were extracted with dithizone[25].

Kinetic assays based on the release of nitroaniline were conducted in plastic cuvettes containing a total volume of 750 µl, buffered by 0.05M Hepes, pH 8.0. Release of nitroaniline product was monitored at 405 nm in a LKB Ultrospec 4050 spectrophotometer thermostated to 25 °C and interfaced with an Apple IIe computer. The concentration of product is based on the absorbance coefficient $\varepsilon_{405nm} = 10200 M^{-1} cm^{-1}$[16]. Substrate concentrations were determined by weight and, eventually, residual substrate in assay solutions was hydrolysed completely by addition of 100 µl of concentrated NaOH and the final product concentration determined spectrophotometrically. Assays were run at 4 to 10 different substrate concentrations. Kinetic parameters were calculated by non-linear regression analysis[26] using the software package ENZPACK 3 (Biosoft, Cambridge, UK). Standard errors are indicated for k_{cat} and K_m.

Reversed phase HPLC of assay mixtures was performed on a Dupont 8800 Instrument equipped with an Beckman Ultrasphere PTH column (5 µm, 100 Å, 4.6 × 250 mm; Beckman, München). Samples were eluted with a linear gradient of acetonitrile (0–56% in 40 min; 1.4% min^{-1}; flowrate 1 ml min^{-1}) in 0.1% trifluoroacetic acid and detected spectrophotometrically at 220 nm.

For kinetic analysis by HPLC of the astacin (2.5×10^{-8}M) catalysed hydrolysis of Suc-Ala-Ala-Ala-Ala-Ala-pNA (0.16–4.94mM), assays were conducted as described[14]. A linear gradient of acetonitrile (14–35% in 15 min; 1.4% min^{-1}) was used to resolve the products.

Peptide fragments were hydrolysed in vacuo at 110 °C in 200 µl 6M HCl for 24 h and finally identified by amino-acid analysis on a Biotronics LC 2000 instrument according to the manufacturer's recommendations.

Assays revealing the pH dependence of the hydrolysis of Suc-Ala-Ala-Ala-pNA and Ala-Ala-Ala-pNA were performed at 25 °C in 0.05M sodium acetate (pH 3.5–5.0), 0.05M Mes (pH 5–7), 0.05M Hepes (pH 7–8), 0.05M Ampso (pH 8–10) and 0.05M Caps (pH 9–11). The pH was determined immediately after the assay (10 min). Kinetic parameters for Ala-Ala-Ala-pNA were determined at varying pH and four different substrate concentrations (0.52, 0.39, 0.26, and 0.13mM) as described above. For Suc-Ala-Ala-Ala-pNA, k_{cat}/K_m was calculated from the slope of Lineweaver-Burk plots using 1mM and 2mM substrate.

Results and Discussion

The release of nitroaniline from Suc-Ala-Ala-Ala-pNA by astacin has been helpful to monitor the activity of the enzyme in previous studies[3–5,15]. However, this activity was unexpected and not easy to interpret since astacin is a metalloproteinase[3] and its primary specificity requires amino-acid residues with short, uncharged side chains in P_1'-position. There are only few reports on amidase activity of metalloprotein-

ases. Examples are *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic-acid hydrolase from human small intestine mucosa[27] and two fibrinolytic metalloproteinases from snake venom hydrolysing Pro-Phe-Arg-pNA and Suc-Ala-Ala-Ala-pNA, respectively[28]. On the other hand, a metalloproteinase found in human serum[29] reported to cleave Suc-Ala-Ala-Ala-pNA turned out to hydrolyse the peptide bond between the second and third alanyl residue and the release of nitroaniline was due to the auxiliary action of an aminopeptidase. This principle is used in aminopeptidase-assisted assays elaborated to analyse metalloproteinases which cleave peptide-pNAs but do not release nitroaniline[30].

Astacin, purified according to the method of Zwilling and Neurath[1] consists of immunologically identical isozymes. The multiple enzyme forms were resolved further by anion exchange chromatography on Mono Q (Fig. 1). Elution by a linear salt gradient yielded three peaks, which refer to isozymes termed *a*, *b*, and *c*, comprising approximately 75%, 22%, and 3% of the total protein, respectively. Kinetic analysis of material of peaks *a* and *b* with Suc-Ala-Ala-Ala-pNA as substrate gave nearly identical values for k_{cat} and K_m (Table 1). Besides the metalloproteinase astacin, the stomach of *Astacus* contains several endopeptidases and exopeptidases[1] including a recently detected aminopeptidase (unpublished work of this laboratory). In order to exclude trace contaminations by this aminopeptidase, which cleaves N-terminally unblocked substrates, HPLC-purified astacin isozyme *a* was used for the present study.

HPLC analyses, shown for Suc-Ala-Ala-Ala-Ala-Ala-pNA as an example (Fig. 2), demonstrated that all peptide-4-nitroanilides used in this study are cleaved by astacin at the terminal amide bond thereby releasing nitroaniline which can be monitored spectrophotometrically at 405 nm. For all peptides tested strict Michaelis-Menten kinetics have been obtained.

The specificity constants for the release of nitroaniline from peptides of the general structure Suc-Ala_n-pNA ($n = 2, 3, 5$) and Ala_n-pNA ($n = 1, 2, 3$) increase with the number of alanyl residues supporting previous evidence for an extended substrate binding site (Table 1)[11,14]. In the peptides devoid of a succinyl group, Ala-Ala-Ala-pNA ($k_{cat}/K_m = 53.1 M^{-1}s^{-1}$) is a 7 times better substrate than Ala-Ala-pNA ($k_{cat}/K_m = 7.4 M^{-1}s^{-1}$) and a more than 5000-times better substrate than the mono-alanyl-derivative Ala-pNA (estimated value for $k_{cat}/K_m = 0.01 M^{-1}s^{-1}$). In this context it may be noted that there is presumably an accumulation of negative charges in or near the active site of astacin[3,9] which might be responsible for the binding of the protonated α-amino group supplied by unblocked substrates. Furthermore, astacin contains the sequence His-Glu-Leu-Met-His[3,9,14] which in analogy to thermolysin[32] is believed to play a role in catalysis and zinc binding. This typical feature which is found in the active sites of virtually all zinc endopeptidases[33] has been also found in some zinc exopeptidases such as human aminopeptidase N (His-Glu-Leu-Ala-His)[34]. Thus, despite the lack of overall homology, there are structural similarities in the active sites of both zinc-endopeptidases and zinc-exopeptidases which could implicate also functional analogies.

Among the N-succinylated peptides Suc-Ala-Ala-Ala-Ala-Ala-pNA yields the highest value for k_{cat}/K_m ($46.4 M^{-1}s^{-1}$). However, this substrate comprising a chain of five alanyl residues in an exception since it is the only peptide that is cleaved at two positions by astacin. As shown by HPLC (Fig. 2), the first bond hy-

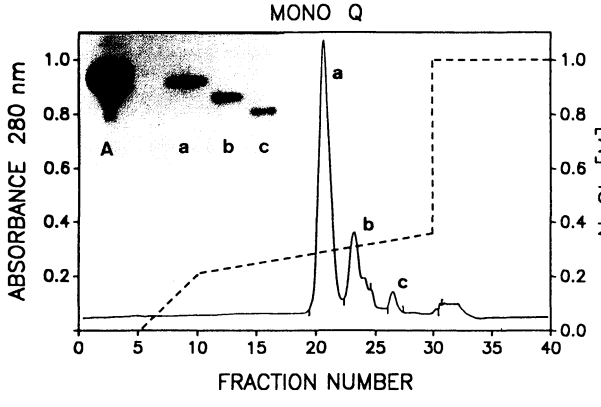

Fig. 1. Separation of astacin isozymes by anion-exchange chromatography.

Mono Q separation of astacin isoenzymes from 4 mg of material prepared according to Zwilling and Neurath[1] dissolved in 20mM Tris/HCl, pH 8.0. Elution was performed by a linear gradient of 0.2–0.35M NaCl within 20 min at a flow rate of 1 ml/min. Inset: Native PAGE at pH 8.8 in the absence of reducing agents and SDS; acrylamide concentration 12.5%; anode at the bottom. A = astacin starting material. *a*, *b*, *c* = peak fractions from the Mono Q separation. In the presence of SDS and reducing agents *a*, *b* and *c* have the same electrophoretic mobility (not shown).

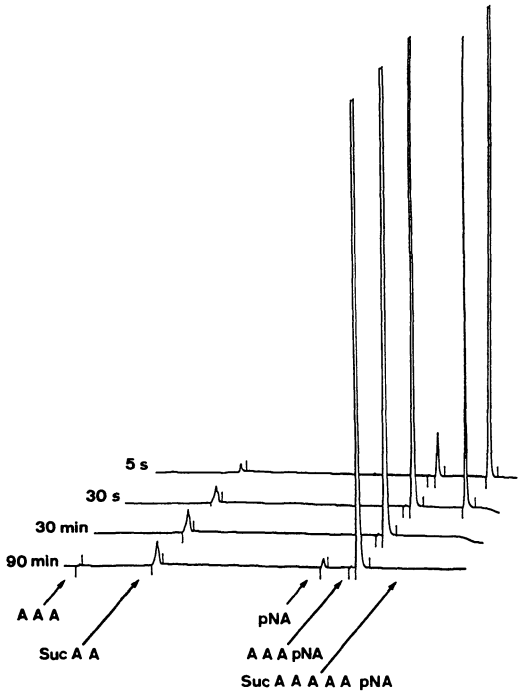

Fig. 2. HPLC-recorded cleavage of Suc-Ala-Ala-Ala--Ala-Ala-pNA by astacin.

HPLC analysis of the astacin-catalysed (5.6×10^{8}M) hydrolysis of Suc-Ala-Ala-Ala-Ala-Ala-pNA (1.3mM). The reaction is monitored after 5 s, 30 s, 30 min, and 90 min respectively. After 30 s the breakdown of Suc-Ala--Ala-Ala-Ala-Ala-pNA to Ala-Ala-Ala-pNA and Suc--Ala-Ala is almost complete. Nitroaniline product from amide-bond cleavage in Ala-Ala-Ala-pNA is seen after 90 min. Gradient: 0–56% acetonitrile in 0.1% TFA in 40 min, wavelength 220 nm.

Table 1. Kinetic constants for the hydrolysis of peptide-nitroanilide substrates of varying length by astacin.

Substrate P_6 P_5 P_4 P_3 P_2 P_1 P_1'	Peptide no.	Concentr. range [mM]	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m [$M^{-1}s^{-1}$]
Ala-Ala-Ala-pNA	I	0.2– 5.3	0.028 ± 0.0006	0.52 ± 0.04	53.8
Ala-Ala-Ala-pNA	II	1.2–12.0	0.172 ± 0.03	23.2 ± 0.7	7.4
Ala-pNA	III	3.1			0.01[a]
Suc-Ala-Ala-Ala-Ala-Ala-pNA (↓[b] ↓[c])	IV[b]	0.2– 5.0	118.0 ± 0.3	2.1 ± 0.18	56190.0
	IV[c]	0.2– 2.5	0.026 ± 0.0006	0.56 ± 0.04	46.4
Suc-Ala-Ala-Ala-Ala-pNA	V[d]	0.5–16.0	0.25 ± 0.01	9.7 ± 0.8	25.8[f]
	V[e]	0.5–10.0	0.28 ± 0.02	12.5 ± 0.5	22.4
Suc-Ala-Ala-pNA	VI	2.2–22.2	0.012 ± 0.0003	7.1 ± 0.5	1.69

[a] Estimate, calculated from the rate of product appearance divided by the concentrations of enzyme (7μM) and substrate (3.14mM).
[b] Peptid-bond cleavage monitored by HPLC using 2.5×10^{-8}M astacin in 0.05M Hepes, pH 8.25 and Suc-Ala-Ala-Ala-Ala-Ala-pNA ranging from 0.16–4.95mM.
[c] Amide-bond cleavage by 1.1μM astacin monitored spectrophotometrically at 405 nm.
[d] Astacin isoenzyme *a*.
[e] Astacin isoenzyme *b*.
[f] Previous determinations in 0.2M triethanolamine/HCl, pH 7.8, buffer yielded k_{cat}/K_m values of 20.8$M^{-1}s^{-1}$[31] and 21.2$M^{-1}s^{-1}$[5], respectively. Enzyme concentration: 1.1μM if not indicated otherwise.

drolysed in this substrate is not the terminal amide bond, but the peptide bond between the second and the third alanyl residue. The two products released are Suc-Ala-Ala and Ala-Ala-Ala-pNA. The presence of alanine in peak fractions was monitored by amino-acid analysis (Fig. 2). Kinetic analysis by HPLC revealed $k_{cat}/K_m = 56200 M^{-1}s^{-1}$ for the first cleavage (Table 1, Fig. 2), which is 1200 times faster than the cleavage of the amide bond of Ala-Ala-Ala-pNA ($k_{cat}/K_m = 46.4 M^{-1}s^{-1}$). Therefore, an accumulation of Ala-Ala-Ala-pNA is observed, which subsequently is broken down to Ala-Ala-Ala and 4-nitroaniline (Fig. 2). Hence, Ala-Ala-Ala-pNA has been analysed as both an individual substrate and an intermediate during the breakdown of Suc-Ala-Ala-Ala-Ala-Ala-pNA. The kinetic parameters based on the release of nitroaniline from free Ala-Ala-Ala-pNA ($k_{cat} = 0.028 s^{-1}$; $K_m = 0.52 M$; $k_{cat}/K_m = 53.8 M^{-1}s^{-1}$) and Suc-Ala-Ala-Ala-Ala-Ala-pNA-derived Ala-Ala-Ala-pNA ($k_{cat} = 0.026 s^{-1}$; $K_m = 0.56 M$; $k_{cat}/K_m = 46.4 M^{-1}s^{-1}$) are therefore very similar, the slight differences in K_m and k_{cat} are within the range of experimental error (see Table 1).

Two sets of nitroanilide substrates allowed to test the subsite specificity of astacin, which then was compared to the tubulin pattern. By this approach it was possible to determine the orientation of the substrates in the active site.

In the first set the amino-acid residue adjacent to the scissile bond is varied in substrates of the general structure Suc-Ala-Ala-X-pNA, where X is Ala, Phe, Pro or Val (Table 2). In these peptides the k_{cat}/K_m value drops by 50% if Phe ($k_{cat}/K_m = 45.6 M^{-1}s^{-1}$) is replaced by Ala ($k_{cat}/K_m = 25.5 M^{-1}s^{-1}$). The most drastic decrease in activity by over 300- and 1000-fold, respectively, is seen if Pro ($k_{cat}/K_m = 0.08 M^{-1}s^{-1}$) and Val ($k_{cat}/K_m = 0.02 M^{-1}s^{-1}$), occupy this position. Interestingly, the drastic change between Suc-Ala-Ala-Ala-pNA and Suc-Ala-Ala-Pro-pNA is solely due to a 300-fold drop in k_{cat}, whereas K_m stays the same (Table 2), suggesting that the replacement of Ala by Pro is unfavourable for the rate-determining step rather than it has an effect on substrate binding. The decrease in k_{cat}/K_m follows the same order, Phe > Ala > Pro > Val, as does the frequency of these residues in the P_1-Position of the tubulin cleavage pattern (Table 2)[14]. On the contrary, to match the tubulin pattern in position P_1', the order should be completely different, namely Ala > Val > Pro, Phe. This is a strong argument that the peptide moiety, but not the nitroanilide domain, binds to the S-positions in the active site of astacin.

A further argument is provided by three substrates of the general composition Suc-(Ala, Ala, Pro)-pNA which reveal the effect of a proline residue in different distances from the cleavage point (Table 3). As already seen above, Pro in P_1 essentially abolishes activity ($k_{cat}/K_m = 0.08 M^{-1}s^{-1}$) and also if Pro is in P_2 the specific activity toward astacin is very low ($k_{cat}/K_m = 1.9 M^{-1}s^{-1}$). In contrast, Pro in P_3 yields k_{cat}/K_m in the

Table 2. Kinetic constants for the hydrolysis of tripeptide-nitroanilides of varying composition by astacin.

Substrate P_4 P_3 P_2 P_1 P_1'	Peptide no.	Concentr. range [mM]	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m [$M^{-1}s^{-1}$]
Suc-Ala-Ala-**Phe**-pNA[a]	VII	0.8–9.0	0.19 ± 0.009	4.2 ± 0.42	45.2
Suc-Ala-Ala-**Ala**-pNA[a]	V	0.5–16.0	0.25 ± 0.01	9.7 ± 0.8	25.8
Suc-Ala-Ala-**Pro**-pNA[b]	VIII	0.9–10.6	0.0008 ± 0.00003	9.7 ± 0.8	0.08
Suc-Ala-Ala-**Val**-pNA[b]	IX	1.7–13.0	0.0003 ± 0.00008	11.4 ± 0.3	0.03

Enzyme concentration: [a] 1.0 μM; [b] 19 μM.

Table 3. Kinetic constants for the hydrolysis of proline-containing tripeptide-nitroanilides.

Substrate P_4 P_3 P_2 P_1 P_1'	Peptide no.	Concentr. range [mM]	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m [$M^{-1}s^{-1}$]
Suc-**Pro**-Ala-Ala-pNA[a]	X	0.6–13.0	0.05 ± 0.0009	1.3 ± 0.1	38.5
Suc-Ala-**Pro**-Ala-pNA[a]	XI	1.7–12.5	0.008 ± 0.0003	4.1 ± 0.4	1.95
Suc-Ala-Ala-**Pro**-pNA[b]	VIII	0.9–10.6	0.0008 ± 0.00003	9.7 ± 0.8	0.08

Enzyme concentration: [a] 1.0 μM; [b] 19 μM.

range of the best pNA substrates tested (k_{cat}/K_m = 39.2M^{-1}s^{-1}) (Table 3). Thus the data on these substrates corroborate the tubulin pattern, e.g. Pro is not tolerated in S_1 but well accepted in S_3. As it was seen for the substitutions Phe>Ala>Pro>Val in P_1, the position of the proline residue affects k_{cat} rather than K_m. Thus in Suc-Ala-Ala-Pro-pNA and Suc-Pro-Ala-Ala-pNA the k_{cat} values differ by a factor of 60, but the K_m's vary only by a factor of seven (Table 3).

If Suc-Ala-Ala-Ala-pNA is considered as substrate for pancreatic elastase, a serine proteinase, the P_1-residue, alanine, binds to the S_1-subsite and the pNA-moiety binds to S_1'[16]. In contrast, in the case of astacin, alanine is the most preferred amino-acid sidechain in the S_1'-position followed by threonine, serine, glycine and valine. Furthermore, astacin can accommodate aromatic sidechains of Tyr or Phe in P_1 and exhibits a high preference for Pro in P_2'[14]. For these reasons, and also stimulated by the observed inverse binding of L-Leu-hydroxamate to thermolysin[35], speculations have been put forward that astacin might bind pNA substrates in an inverse orientation, i.e. the aromatic nitroanilide moiety might bind to the S_1-subsite and the alanine-peptide moiety might be in contact with the S'-subsites[5]. If this were the case, Suc-Ala-Pro-Ala-pNA should be a relatively good substrate, because in the inverse mode the pNA-moiety in S_1, Ala in S_1' and Pro in S_2' would match excellently the requirements of astacin (see above). However, this substrate is even less active than Suc-Ala-Ala-Ala-pNA (Table 3). In conclusion, the kinetic data on the two sets of substrates shown in Tables 2 and 3 render inverse binding unlikely, which is less difficult to interpret from a stereochemical point of view since enzyme substrate complexes with inversely bound substrates should be non-productive.

It has to be noted that the specific activity of astacin toward the nitroanilides studied is at least by four orders of magnitude lower as compared to a series of highly sensitive, fluorescent dansyl-heptapeptide substrates which were synthesized recently based on the tubulin cleavage pattern[14]. This indicates the preference of astacin for substrates which span a minimum number of subsites on both sides of the scissile bond. However, the nitroanilides interact only with the S-subsites and, therefore, are less reactive. It fits into the scheme that the kinetic parameters for the first (peptide) bond cleaved between Ala-2 and Ala-3 in Suc-Ala-Ala-Ala-Ala-Ala-pNA indeed fall into the range of the tubulin-based substrates (k_{cat}/K_m = 56200M^{-1}s^{-1}).

The space available for amino-acid sidechains in the S_1'-subsite of astacin seems to be restricted, since replacement of Ala for Leu in P_1' of the highly active Dns-Leu-Lys-Arg-Ala-Pro-Trp-Val (k_{cat}/K_m = 4.5 × 10^{5}M^{-1}s^{-1}) to Dns-Leu-Lys-Arg-Leu-Pro-Trp-Val (k_{cat}/K_m = 0.9M^{-1}s^{-1}) almost abolishes activity[14]. However, the data presented above suggest that in the nitroanilide substrates the bulky pNA group is facing the S_1'-subsite. This should pose sterical strain on the binding of these substrates. However, there are probably hydrophobic and/or aromatic interactions that facilitate the binding of the nitroanilide moiety. An additional binding site had already been proposed in the context of the observed inhibition of astacin by 1,7-phenanthroline[3], a non-chelating isomer of 1,10-phenanthroline. This site was suggested to explain the cooperative interaction of two 1,10-phenanthroline molecules during metal removal[4,5].

The pH-dependence of the hydrolysis of Ala-Ala-Ala-pNA and Suc-Ala-Ala-Ala-pNA reveals the optimum activity of astacin in the neutral to slightly alkaline region (Fig. 3), which is typical for metalloproteinases. However, the optimum depends upon the chemical nature of the substrate. Ala-Ala-Ala-pNA (optimum near pH 6) has a free N-terminal alanyl residue whereas in Suc-Ala-Ala-Ala-pNA (optimum near pH 8) the N-terminus is blocked. The catalytic activity in terms of k_{cat}/K_m for Ala-Ala-Ala-pNA at pH 8 is twice as high as for Suc-Ala-Ala-Ala-pNA. It should be noted that the values for k_{cat}/K_m at pH 8 from the pH profile match those presented in Table 1 (25.3M^{-1}s^{-1} and 53.1M^{-1}s^{-1}) although for the pH profile a smaller number of substrate concentrations was used. Below pH 4.5 there is a drop of activity toward Ala-Ala-Ala-pNA which results in an asymmetric pH profile. It

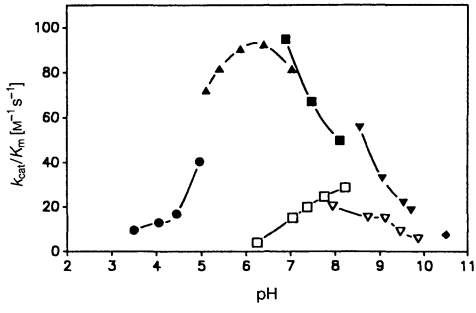

Fig. 3. pH-activity profile of astacin.

pH-activity profile of astacin for Ala-Ala-Ala-pNA (0.13–0.52mM; solid symbols) and Suc-Ala-Ala-Ala-pNA (1.0–4.0mM; open symbols) at 25 °C. Activity is indicated as k_{cat}/K_m. Buffers used are 0.05M sodium acetate (circles), 0.05M Mes (triangles), 0.05M Hepes (squares), 0.05M Ampso (inverse triangles) and 0.05M Caps (diamonds).

had been observed that astacin is irreversibly inactivated at acid pH[1]. This is frequently observed in metalloenzymes and is probably due to loss of zinc.

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