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Activation of snake venom metalloproteinases by a cysteine switch-like mechanism

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The cDNAs of several snake venom zinc endopeptidases code for a putative propeptide, which includes the conserved cysteine-containing sequence PKMCGVT. It has been suggested that binding of the cysteine thiol function to the active-site zinc, resulting in inactivation of the catalytic domain, occurs in a mode similar to the 'cysteine switch' mechanism proposed for matrix metalloproteinases. In order to confirm this hypothesis, inhibition kinetics have been performed on the metalloproteinase adamalysin II of the venom of the snake *Crotalus adamanteus* using several cysteine peptides. Among these the synthetic hexapeptide PKMCGV-NH_2, corresponding to the conserved sequence portion of the known propeptides, was found to be by far the strongest inhibitor of this proteinase with a K_i of 3.4 μM. The inhibitory potencies of an equivalent peptide with the L-Cys replaced by a D-Cys or by an L-Ser as well as of reduced glutathione, cysteine and two unrelated cysteine peptides were by one to two orders of magnitudes lower. These findings strongly support a cysteine switch-like mechanism even for activation of the snake venom metalloproteinases.

Cysteine switch; Matrix metalloproteinase; Snake venom metalloproteinase; Adamalysin; Proteinase activation; Stability of cysteine peptide

1. INTRODUCTION

The venom of poisonous snakes contains several pharmacologically active enzymes, among them zinc endopeptidases. The low-molecular weight zinc proteinases exhibit a highly conserved catalytic domain [1], which apparently is derived from larger precursors via post-translational proteolytic processing. The high-molecular weight snake venom metalloproteinases consist of an N-terminal putative prodomain, a catalytic domain including the active-site zinc, a disintegrin domain and a cysteine-rich domain [2–7]. Almost all of these metalloproteinases show proteolytic activity against matrix proteins. They are most likely stored as inactive zymogens in order to prevent autodigestion, and are then activated by suitable proteolytic processing. A conserved PKMCGVT sequence located between the proposed signal sequence and the catalytic domain has been identified in the cDNA of the snake venom proteinases Ht-e [2], Jararhagin [5], Trigramin [4] and Rhodostamin [8], and an activation mechanism has been suggested [2] similar to the cysteine switch as proposed for matrix metalloproteinases [9]. The cysteine thiol function in the precursor sequence should bind to the active-site zinc in the inactive zymogen, thereby blocking it.

In the present study we have analyzed this hypothetical mechanism for adamalysin II, formerly called proteinase II [10], a metalloproteinase of the venom of the eastern diamond rattlesnake *Crotalus adamanteus*, the only snake venom metalloproteinase for which the tertiary structure is available [1]. Although the precursor sequence of this protein is not known, we assume a similar or even identical sequence around the Cys residue as that found in the four known propeptides of snake venom metalloproteinases, i.e. the conserved PKMCGV region. Correspondingly, the peptide PKMCGV-NH_2 as well as other cysteine peptides were synthesized and analyzed comparatively for their inhibitory potency.

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Abbreviations: standard abbreviations for amino acids and peptide derivatives are used as recommended by the IUPAC-IUB commission on biochemical nomenclature. The amino acids are of L-configuration unless stated otherwise. HPLC, high-pressure liquid chromatography; FAB-MS, fast atom bombardment mass spectroscopy; Abz, 2-aminobenzoyl.

2. MATERIALS AND METHODS

2.1. *Synthetic peptides*

The peptides used in the present study, i.e. H-Pro-Lys-Met-Cys-Gly-Val-NH_2, H-Pro-Lys-Met-Ser-Gly-Val-NH_2 and H-Lys-Pro-Arg-Cys-Gly-Val-NH_2, were synthesized in high-pressure continuous-flow reactors according to the Fmoc/tBu strategy [11] using the modified Rink-linker on Tentagel S RAM resin (Rapp, Tübingen). Fmoc-Gly, Fmoc-Ser(tBu), Fmoc-Leu and Fmoc-Lys(Boc) were coupled as NCA derivatives [12], and Fmoc-Pro-OH, Fmoc-Val-OH, Fmoc-Cys(StBu)-OH and Fmoc-Arg(Pmc)-OH via TBTU/HOBt/DIEA (1:1:2). Intermediate Fmoc removal and final acidolytic cleavage steps were performed as described previously [13]. Upon tributylphosphine-mediated reductive cleavage of the the S-tert-butylthio group, the crude products were purified by preparative HPLC on Lichroprep C18 (15–

25 μm) by linear gradient elution with CH_3CN/0.1% trifluoroacetic acid. The products were isolated as lyophilized materials and their homogeneity was determined by analytical HPLC on Nucleosil 300–5 C8 (linear gradient elution with CH_3CN/2% phosphoric acid), quantitative amino acid analysis of the acid hydrolysates (6 M HCl containing 2.5% thioglycolic acid, 110°C, 24 h), FAB-MS, and quantitative determination of the cysteine thiol group according to Grassetti and Murray [14]. H-Pro-Lys-Met-D-Cys-Gly-Val-NH_2 was obtained as byproduct in the synthesis of the L-diastereomer, since coupling of Fmoc-Cys(StBu)-OH via the TBTU method is known to provoke racemization in the range of 20–30% [13]. H-Lys-Thr-Phe-Thr-Ser-Cys-OH and Ac-Trp-Cys-Gly-Pro-NH_2 were synthesized previously by classical methods in solution (unpublished results). Cysteine was purchased from Fluka, AG (Buchs, Switzerland) and reduced glutathione from Sigma Chemie GmbH (Deisenhofen, Germany).

Assay solutions of the peptides were prepared in the enzyme assay borate buffer (pH 8.0). Peptide concentrations were determined by weight and peptide content as derived from quantitative amino acid analysis or thiol content. The stability of the cysteine peptides in the assay buffer has been analyzed on two representative compounds, i.e. reduced glutathione and KPRCGV-NH_2, as follows: a 2 mM glutathione and a 0.8 mM KPRCGV-NH_2 solution, in air- and argon-saturated assay buffer, were prepared and stored at 25°C and on ice, respectively. Aliquots taken over a time period of 10 h were analyzed for the thiol content by uv measurements [14].

2.2. *Enzyme assay*

The assay was performed at 25°C in a freshly prepared 50 mM borate buffer (pH 8.0) treated with dithizone to remove traces of heavy metals [15]; 4 mM $CaCl_2$ was added and the buffer saturated with argon. Stock solutions of adamalysin II were prepared as previously described [10,16] by centrifugation of the protein from an ammonium sulfate suspension and subsequent dissolution in the assay buffer. Enzyme concentrations were determined by uv measurements ($\varepsilon_{288} = 2.2 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) and the stock solutions were stored in the cold. This solution was diluted 1:100 to obtain the final 16 nM assay concentration. The fluorogenic substrate Abz-Pro-Ser-Phe-Leu- Tyr(NO_2)-Gly-OH with a K_m of 52 μM (MSc thesis, J.Lutz, (1993) LMU München, in preparation) was used at a concentration of 21.4 μM; for the K_i determination according to Dixon [17] a 12.8 μM concentration has also been used. Substrate fluorescence was measured at an excitation and emission wavelength of λ = 320 and 420 nm, respectively, on a spectrofluorimeter (Perkin Elmer, Model 650–40) equipped with a thermostated cell holder. Substrate hydrolysis was monitored for 10 min immediately after adding the enzyme. All reactions were performed at least in triplicate. The K_i values of the inhibitors were calculated from the intersection point of the straight lines obtained by the plots of v_o/v_i vs. [I], whereas IC_{50} values were calculated from plots of v_i/v_o vs. [I] by non-linear regression with simple robust weighting. Errors for IC_{50} values were inferred from the reduced χ^2 of the curve fittings. All calculations were carried out with the program GraFit [18].

3. RESULTS

3.1. *Alignments of precursor sequences*

The known sequences of snake venom metalloproteinases were all derived from the corresponding cDNAs. Sequence alignments of the known four propeptides of snake venom metalloproteinases were done visually. As shown in Fig. 1, this alignment requires only two single residue deletions/insertions for the common range. The sequence identity is between 76% for the pair Rhodostamin/Trigramin and 87% for Ht-e/Trigramin, and the overall shared identity is still 66%. Each propeptide sequence contains three cysteine residues, which are located in the homologous regions VEDHCYY, STASISAC and PKMCGVT, respectively. Even if the cysteines are involved in disulfide bridging, at least one free thiol group which could interact with the active-site zinc of the protein is therefore

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Trigramin:                                          Signal-Peptide-SSIILESGNLNDYEVVYPEKVT
Ht-e:                                               Signal-Peptide-SSIILESGNVNDYEVIYPRKVT
Chou&Fasman:                                                      HHHHHHTTT     BBB
Garnier:                                                          BBBBBB      BBBBBB

Trigramin:   A-LPKGAVQQKYEDAMQYEFKVNGEPVVLHLEKNK-LFSEDYSEIHYSPDGGREITAYPSVEDHCYYHGRIENDAD
Ht-e:        A-LPKGAVQPKYEDTMQYELKVNGEPVVLHLEKNKGLFSKDYSETHYSFDG-RKITTNPSVEDHCYYHGRIENDAD
Jararhagin:  ATRPKGAVQPKYEDAMQYEFKVNQEPVVLH-EKNKGLFSKDYSEIHYSPDGGREITTYPPVEDHCYYHGRIENDAD
Rhodostamin:                                                FAKNYSETHYSPDGGYRITTYPSVEDHCYYGGRIHDGAN
Chou&Fasman:              HHHHHHHHHH          HH
Garnier:        TT        HHHHHHHH      HHHHHHHHH          TTT   TT          TTTTTTTTT

Trigramin:   STASISACDGLKGHFKLQGEMYLIEPWELSDSEAHAVFKYENVEKEDEPPKMCGVT-QNWESYESTKKASQLNVTP
Ht-e:        STASISACNGLKGHFKLQGEMYLIEPLKLSDSEAHAVFKLKNVEKEDEAPKMCGVT-QNWESYEPIKKASDLNL
Jararhagin:  STASISACNGLKGYFKLGRETYFIEPLKLPDSEAHAVFKYENVEKEDEAPKMCGVTG-NWKSYEPIKKASQLAFTA
Rhodostamin: STASISACNGLKGHFKLGGETYFIEPMKLPDSEAMAVFKYENIEKEDESPKMCGVTEQNWESDEPIKKVSQLNLNH
Chou&Fasman:                                        HHHHHH
Garnier:                                      HHHH  HHHHHHHHHHHH
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Fig. 1. Sequence alignment and secondary structure prediction according to Chou and Fasman [11,12] and Garnier et al. [13] for the propeptides of Jararhagin [5], Trigramin [4], Rhodostamin [8] and Hte [2] deduced from cDNAs. Homologies in the primary structure are underlined. Identity of the Chou & Fasman and Garnier et al. prediction is underlined only if it is the same for the compared sequences.

present. The cysteine residue closest to the N-terminus of the mature protein is located in the PKMCGVT homologous sequence portion and therefore 21 (Ht-e), 23 (Jararhagin, Trigramin) or 24 (Rhodostamin) residues apart from the presumed starting residue of the mature protein sequence. This conserved cysteine sequence displays homology to the peptide RCGVD, which has been proposed to play a role in the activation of the matrix metalloproteinases [9] and may therefore be involved in a cysteine switch-like mechanism in the activation of the snake venom metalloproteinases, too.

3.2. *Secondary structure prediction*

Preferred secondary structures were predicted using programs based on statistical probability factors according to Chou and Fasman [19,20] and Garnier et al. [21]. Whilst the region immediately preceeding the conserved KPMCGV segment shows a clear preference for α-helix in all the propeptide segments, these programs predict neither a common structure for the Cys-portion itself nor for the subsequent sequence portion, i.e. for the connecting loop. Using both the sequence and conformational homologies of the known propeptides as well as the 3D structure of the catalytic domain, a model for the unprocessed metalloproteinases was constructed as shown in Fig. 2.

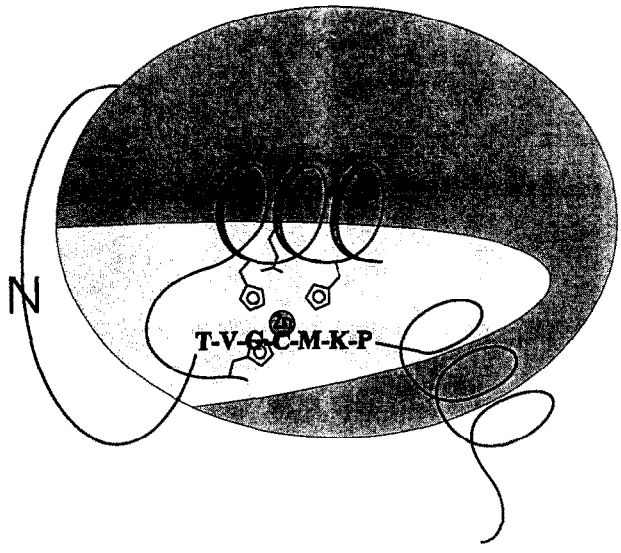

Fig. 2. Model of the adamalysin II zymogen. The catalytic domain of the proteinase, which consists of a regulary folded 'upper' domain and a less regular 'lower' subdomain subdivided by the zinc-binding active site cleft (horizontal incision) [1], is schematically shown as an ellipsoid. The catalytic zinc is bound by three His residues contained in the zinc binding consensus sequence HEXXHXXGXXH forming an 'active-site helix' and the succeeding extended strand. The X-ray structure shows that the N-terminus of the mature proteinase starts on the 'left hand side' of the molecule. In the zymogen model it is elongated by a propeptide which surrounds the C-terminal helix (indicated only, by the left hand edge of the incision), binds with the thiol function of a cysteine to the zinc and leaves the cleft as a helix.

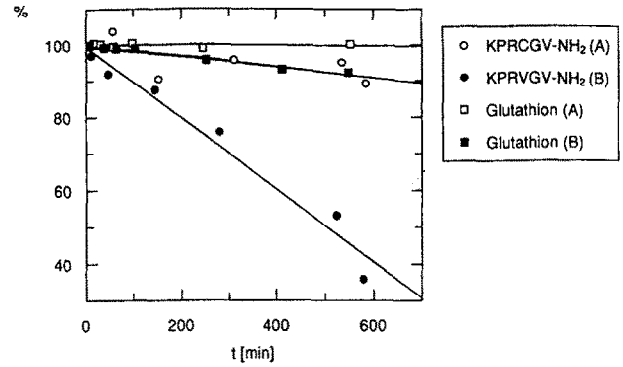

Fig. 3. Stability of 0.8 mM KPRCGV-NH_2 and 2 mM reduced glutathione in 50 mM borate buffer (pH 8.0): (A) argon-saturated on ice; (B) air-saturated at 25°C.

3.3. *Enzyme inhibition*

As optimal assay conditions require alkaline media (pH 8.0), kinetic data of enzyme inhibition with cysteine peptides can be affected by concomitant oxidation of the thiol function. Correspondingly, reduced glutathione and the peptide KPRCGV-NH_2 were analyzed as model compounds for their stability in aerated assay buffer at 25°C and in argon-saturated assay buffer on ice (Fig. 3). Practically no loss of thiol concentration was observed in argon-saturated buffer, whereas ca 8% of the thiol groups of glutathione and 60% of KPRCGV-NH_2 were oxidized in the aerated buffer over a period of 10 h.

Among the peptides analyzed for their inhibitory potency, the proposed 'cysteine switch' peptide PKMCGV-NH_2 was found to represent the most powerful inhibitor, with an IC_{50} of 3.2 μM and a K_i of 3.4 μM. As shown in Fig. 4, the straight lines of the Dixon plot intersect in the second quadrant of the coordinate system supporting a predominantly competitive inhibition mechanism. In order to assess the role of the cysteine thiol function as well as of its configuration, the related Ser-analog and the D-Cys diastereomer of PKMCGV-NH_2 were examined, respectively. With the Ser-peptide no inhibition could be observed up to a concentration of 1.6 mM. For the D-Cys-peptide, an about 20 times lower inhibition was determined in comparison to the L-Cys-analog (Table I). The hexapeptide KPRCGV-NH_2, corresponding to the cysteine switch sequence of the human PMN-collagenase, was also analyzed for its inhibition of adamalysin II. It was found to exhibit inhibitory activity, although of significantly lower potency (IC_{50} = 67 μM) than PKMCGV-NH_2, the cysteine peptide of the snake venom metalloproteinases. On the other hand, neither cysteine nor reduced glutathione, the most abundant thiol peptide in tissues, nor cysteine peptides with randomly selected sequences such as KTFTSC or Ac-WCGP-NH_2 were capable of inhibiting adamalysin II to extents comparable to PKMCGV-NH_2, as evidenced by the IC_{50} values listed in Table I.

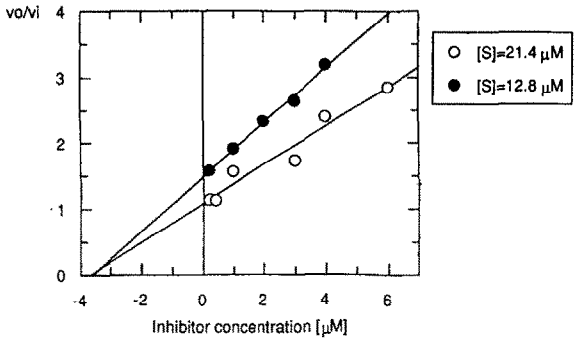

Fig. 4. Dixon plot of the inhibition of adamalysin II by PKMCGV-NH_2.

4. DISCUSSION

The kinetic data clearly indicate that the proposed cysteine switch-peptide, i.e. PKMCGV-NH_2, represents among the peptides analyzed the most efficient inhibitor of the snake venom metalloproteinase adamalysin II, with a K_i of 3.4 μM (IC_{50} = 3.2 μM). It is about 20 times more potent than its D-Cys-analogue and at least 20 times more inhibiting than the other Cys-peptides examined. L-cysteine itself shows some inhibitory effect, but of much weaker intensity (IC_{50} = 117 μM). Conversely, reduced glutathione exhibits a rather strong binding to the metalloproteinase.

As PKMCGV-NH_2 binds most likely to the active-site zinc, it can be assumed that the whole propeptide would exhibit a similar behaviour, although the binding affinities might be slightly different. Endogeneous conformations stabilized by the rest of the propeptide could account for a more or less favourable binding, and therefore the question arises as to whether the affinity of the switch peptide is sufficiently high to protect the producing tissue against the collagenolytic activity, yet low enough to allow disruption of the thiol-zinc bond in the activation process of the enzyme. A too strong binding constant of the propeptide-derived Cys-peptide would prevent its release upon proteolytic cleavage at the connecting loop and thus, proteinase activation. On the other hand, in the proenzyme the Cys-peptide is covalently linked and correspondingly its effective concentration should be sufficiently high to fully inactivate the enzyme, despite the moderate K_i value of this peptide as separate entity.

The number of residues separating the cysteine residue of the potential switch-peptide region from the N-terminus of the mature proteinase amounts to 21 (HT-e), 23 (Jararhagin, Trigramin) or 24 (Rhodostamin). An inspection of the tertiary structure of adamalysin-II, which is well defined from the 3rd residue onwards, indicates that in the hypothetical non-activated protein the N-terminal propeptide must bend around the C-terminal helix, indicated by the left edge of the incision in Fig. 2. This would require a chain length of at least 30 Å, corresponding to eight residues. The 21 to 24 propeptide residues are certainly sufficient to build up this connective segment with a pronounced surface-exposed loop well accessible to activation proteinases. The propeptide would then bind into the cleft with the thiol group ligating the zinc atom. However, this binding mode would be different from that proposed for the substrate binding, as for the structurally related enzyme astacin a substrate binding from left to the right along the active-site edge was revealed [22,23].

Immediately N-terminal to the switch-peptide region, the secondary structure prediction (Fig. 1) suggests a helical stretch which presumably protrudes from the binding cleft. Thus the resulting zymogen model of adamalysin II, outlined in Fig. 2, resembles a hot dog-like structural motif.

This structural model might also be valid for matrix metalloproteinases. Several ways of activation have been reported for these enzymes which may also hold for the snake venom metalloproteinases, i.e. activation by proteinases [24], by interaction or modification of the cysteine residue with organomercurials [25], metal ions [26,27], disulfide reagents [28], oxidants such as NaOCl [29] and thiol alkylating agents such as *N*-ethylmaleimide [27], or by conformational changes of the polypeptide backbone as induced by chaotropic salts [30] and detergents [31].

Evidence exists that activation by proteinases of at least some of the matrixin-zymogens takes place in two steps [32]. The cysteine switch defines activation via the switch peptide with a pre-existing equilibrium between a zinc-bound and -unbound thiol function. This equilibrium is shifted by the above mentioned activation methods. In the zinc-unbound form the matrixins do not exhibit full activity which is achieved only upon autodigestion or proteolysis by other proteinases.

In this context the results of the present study confirm the role of a switch-peptide in the activation of snake venom metalloproteinases, but cannot determine whether a preceeding disruption of the thiol-zinc bond is necessary or whether a proteolytic cleavage is suffcient for activation.

Table I

IC_{50}-values of tested compound

Peptide	IC_{50} (μM)
PKMSGV-NH_2	> 1600
KTFTSC	170 (± 20)
KPRCGV-NH_2	67 (± 7)
Ac-WCGP-NH_2	58 (± 11)
PKM(L-)CGV-NH_2	3.2# (±0.6)
PKM(D-)CGV-NH_2	74 (± 6)
L-Cysteine	117# (± 11)
Red. glutathione	50# (± 8)

The # indicates the average of two measurements at different substrate concentrations

As described previously [33], the superfamily of metalloproteinases with the consensus sequence HEXXHXXGXXH, which have been called Metzincins due to a conserved methionine turn that forms a hydrophobic basis for the zinc ion and the three liganding histidine residues, can be divided into the families of the matrix metalloproteinases ('matrixins'), the snake venom metalloproteinases ('adamalysins'), the serratia proteases ('serralysins') and the members of the astacin family ('astacins'). No cysteine or cysteine switch peptide sequence have been found in the propeptide of the serralysin of *Erwinia chrysantemi* [34] or in the propeptide sequence of astacin (W. Stöcker, personal communication). Members of these families are probably activated by a different mechanism. In fact, a tyrosine is found as additional fifth zinc ligand in these two families [35,1] which is absent in the two other families. The presence of a tyrosine correlates well with the absence of the cysteine-switch region because of possible sterical interferences of the tyrosine residue with a cysteine supplied by a potential propeptide.

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