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FRAGMENTATION OF CERULOPLASMIN BY THROMBIN

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Copper containing proteolytic fragments of ceruloplasmin (CP), a 135 KD α_2-glycoprotein, have been shown to induce angiogenesis in the rabbit cornea assay. Neovascularisation plays an important role in different biological phenomena including chronic inflammation, wound healing, recanalisation of occluded blood vessels, tumor growth etc. Most of these events are associated with elevated CP levels. Neovascularisation occurs in diabetic macro- and microangiopathies as well. Serum CP concentrations in 92 diabetics were measured and compored to 50 healthy blood donors and 50 unselected hospitalized patients without malignancies. A marked CP elevation was observed in diabetics, especially in those with vascular complications. There was no correlation between the CP and the actual blood glucose concentrations, duration of the disease or the type of the treatment. Activation of the haemostatic processes has been found in the majority of diabetic patients with vascular diseases. In case CP is a substrate for thrombin, the generation of this specific serine protease may lead to the release of angiogenic peptides from CP on the site of vascular occlusion. For this reason, purified human CP was incubated with thrombin at pH 7.4, 37°C, and samples were removed at 0, 30, 60, 120, 240, min., and after 24 hours for CP oxidase activity measurements and for SDS PAGE. Thrombin treatment did not affect the enzymic activity of CP. On SDS PAGE the band corresponding to the parent molecule eventually disappeared, and a M_r 116 KD fragment together with three smaller peptides has been produced, with molecular masses 61, 41 and 20 KD, respectively. Based on these experiments, CP is to be considered as a new protein substrate for thrombin. The physiological relevance of this phenomenon needs further examination.

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ACTIVE SITE-SELECTIVE LABELING OF THROMBIN WITH FLUORESCENCE PROBES USING THIOESTER DERIVATIVES OF PEPTIDE-CHLOROMETHYL KETONES.

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Active site-directed inactivation of a serine protease with a thioester derivative of a peptide-chloromethyl ketone followed by reaction of the unique thiol group generated in the presence of hydroxylamine with a fluorophore-iodoacetamide has been investigated as a new method for covalent incorporation of extrinsic fluorescence probes into the active sites of blood coagulation proteases. The specificity of labeling by this method was evaluated by quantitation of the reactions between human thrombin, acetylthioacetyl-D-Phe-Pro-ArgCH_2Cl (ATA-FPRCK) and 5-iodoacetamidofluorescein (IAF). ATA-FPRCK was synthesized by reaction of FPRCK with succinimidyl acetylthioacetate and purified by chromatography on SP-Sephadex and Sephadex G10. Titrations of the loss of thrombin chromogenic substrate activity with ATA-FPRCK were linear, with end points of 1.1-1.2 mol ATA-FPRCK added/mol active sites, consistent with a reaction stoichiometry of 1 and the ~90% purity of the compound estimated by reverse-phase HPLC. Inactivation of thrombin was quantitatively correlated with incorporation of the thioester, with a maximum of 1.04 mol/mol active sites. IAF labeling of ATA-FPR-thrombin in the presence of 0.1M NH_2OH yielded a maximum of 0.96 mol IAF incorporated/mol active sites in a reaction accompanied by loss of the thiol group. Incorporation of ATA-FPRCK was dependent on the functional thrombin active site, as demonstrated by less than 4% thioester or IAF incorporation for the enzyme previously inactivated with FPRCK. I conclude that active site-selective fluorescence labeling can be achieved by the method described here with the advantage of a wide choice in the properties of the probe incorporated. In addition, a 2.3-fold difference in fluorescence intensity was observed for 2,6-ANS derivatives of ATA-FPR-thrombin and ATA-D-Phe-Phe-Arg-thrombin, indicating that the spectral properties of environmentally sensitive fluorescence probes are influenced by the structure of the peptide inhibitor.

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FUNCTIONAL CHARACTERIZATION OF HUMAN β-THROMBIN.

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Autolysis or tryptic hydrolysis converts α-thrombin (α-T) to β-thrombin (β-T), and subsequently β-T to γ-thrombin (γ-T). Human β-T differs from native α-T by the loss of a unique 11-residues peptide arising from the B chain. Unlike its bovine counterpart, human β-T is a transient intermediate and its enzymatic properties had not yet been investigated using purified material.

After 3 min incubation of human α-T with trypsin-sepharose, the resulting β-T was separated from α- and γ-T by chromatography on Biorex 70 with a gradient from 10 mM to 500 mM phosphate at pH 8. No major differences were found between human α- and β-T regarding the kinetic parameters (Km, kcat, kcat/Km) on S 2238, nor the rate of inactivation by TLCK. In contrast, inhibition of β-T by DFP was slower ($k = 426 \pm 10.8\ M^{-1}\ min^{-1}$) compared to α-T ($764.5 \pm 19.5\ M^{-1}\ min^{-1}$) and the inhibition constant for benzamidine was higher with β-T ($Ki = 11.2 \pm 0.2\ 10^{-4}$ M) compared to α-T ($Ki = 2.86 \pm 0.06\ 10^{-4}$ M). The drastic reduction in the clotting activity of β-T (25 u mg^{-1} versus 3000 u mg^{-1} for α-T) was further explored by measuring the affinity of β-T for fibrinogen and fibrin. Human fibrinogen was used as a competitor in the inactivation of thrombin by DFP : 10 µM fibrinogen prevented the inhibition of α-T by DFP but failed to modify the inactivation rate of β-T. Binding of thrombin to fibrin was studied using fibrin monomers covalently linked to sepharose 4B, equilibrated in 50mM Tris, pH 7.5, 50 mM NaCl : β-T did not bind to the resin, whereas α-T was retained and eluted upon application of a NaCl gradient.

In conclusion, the loss of the peptide extending from Ile (63) to Arg (73) in the thrombin B chain is responsible for multiple defects in thrombin enzymatic activity. Although, the three active site residues Ser (205), His (43), Asp (99) remain in an active configuration, subtle changes are induced in the microenvironment of the catalytic Ser (205), and in particular, in the primary binding pocket. In addition, the results presented in this study indicate that the loss of clotting activity is mainly the result of a decreased affinity for fibrinogen and fibrin, suggesting that the structural changes affect both the fibrinopeptide groove and the anionic binding site involved in fibrinogen/fibrin recognition.

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EVIDENCE FOR MULTIPLE BINDING SITES OF HIRUDIN IN THROMBIN.

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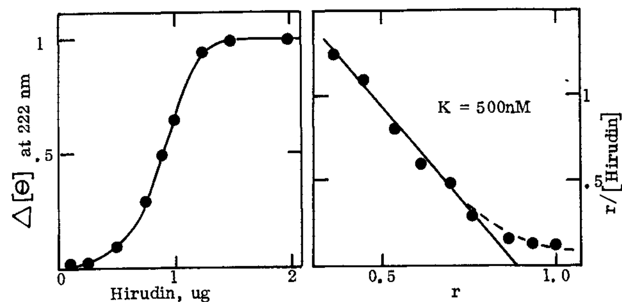
A highly purified hirudin with a specific activity of 13,950 AT units/mg was used in these studies. Investigation of the circular dichroism of hirudin and thrombin showed that the CD spectrum of the thrombin-hirudin complex deviates significantly from additivity towards a less organized structure (i.e. loss of α-helix). A sigmoidal curve, rather than a hyperbolic curve, is generated when the deviation from additivity is plotted against hirudin concentration. This suggests cooperativity of the binding process. At low concentation, a Scatchard plot of the data fits into a straight line clearly indicating one binding site per mole of thrombin. This site binds hirudin with a dissociation constant of 500 nM. However, the data cannot be fitted to a straight line at higher concentration of hirudin suggesting that hirudin binds also to another site with a different affinity. These results agree with the findings of Stone and Hofsteenge (Biochemistry 25, 4622-4628, 1986) and support the idea that initially hirudin binds at a site distinct from the active site, which then rearranges through a conformational change (detected by CD) to form a tigher complex in which hirudin is also bound to the active site.



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