Distinct Binding Sites of Ala$^{48}$-Hirudin$^{1-47}$ and Ala$^{48}$-Hirudin$^{48-65}$ on α-Thrombin*

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The interaction of α-thrombin with Ala$^{48}$-hirudin, Ala$^{48}$-hirudin$^{1-47}$, and Ala$^{48}$-hirudin$^{48-65}$ was analyzed. Mutations at Pro$^{48}$ were found to cause only slight changes in the $k_{on}$ (human: $3.1 \pm 0.3 \times 10^{6} \text{ M}^{-1} \text{s}^{-1}$; bovine: $1.03 \pm 0.3 \times 10^{6} \text{ M}^{-1} \text{s}^{-1}$) and $k_{off}$ (human: $0.4 \pm 0.2 \times 10^{3} \text{ s}^{-1}$; bovine: $2.9 \pm 0.4 \times 10^{3} \text{ s}^{-1}$) rate constants for the formation of the thrombin-hirudin complex. The amino-terminal fragment Ala$^{48}$-hirudin$^{1-47}$ containing three disulfide bridges and the carboxyl-terminal fragment Ala$^{48}$-hirudin$^{48-65}$ were derived from the Ala$^{48}$ mutant by proteolysis with endoproteinase Lys-C. These fragments inhibit bovine α-thrombin clotting activity with $K_i$ values of 0.6 and 0.2 $\times 10^{-3} \text{ M}$, respectively (2.4 $\times 10^{-3} \text{ M}$ for r-hirudin). By mapping the interaction of Ala$^{48}$-hirudin-derived fragments with bovine α-thrombin by limited proteolysis with trypsin and pancreatic elastase distinct binding sites for each fragment were determined. The carboxyl-terminal fragment was found to bind to the proposed anion-binding exosite in the region B62-74, whereas the amino-terminal fragment binds to a region around the elastase cleavage site at residues 150-151 of the α-thrombin B-chain.

The thrombin-specific inhibitor hirudin is a polypeptide of 65 or 66 amino acid residues isolated from the leech Hirudo medicinalis (Markwardt, 1970; Bagdy et al., 1976; Dodt et al., 1986a; Tripier, 1986). Sequence analysis of different naturally occurring forms of hirudin revealed homologies of about 80%. The 6 cysteine residues were assigned to three disulfide bonds occurring forms of hirudin revealed homologies of about 80%. The 6 cysteine residues were assigned to three disulfide bonds connecting Cys$^{4}$ with Cys$^{4+}$, Cys$^{16}$ with Cys$^{26}$, and Cys$^{32}$ with Cys$^{39}$ (Dodt et al., 1985). The three-dimensional structure of hirudin in solution has been determined using $^1$H two-dimensional NMR (Clore et al., 1987; Haruyama and Wuthrich, 1989) and has been refined by analyzing r-hirudin and the Ala$^{48}$ mutant (Folkers et al., 1989). These studies indicate that hirudin consists of an amino-terminal compact domain (residues 1-49) held together by the three disulfide linkages and a distorted carboxyl-terminal tail (residues 50-65) which does not fold back on the rest of the protein. Hirudin reacts rapidly with α-thrombin ($k_{on} = 1.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$) forming tight, noncovalent complexes ($K_d = 10^{-14} \text{ M}$ with human α-thrombin, Sneath and Hofsteenge, 1986; $K_d = 10^{-12} \text{ M}$ with bovine α-thrombin, Dodt et al., 1988). The specificity of the interaction of α-thrombin with macromolecular substrates is assumed to reside in interactions at three distinct regions: the primary binding pocket for the P$_1$ residue (Magnusson, 1971), an apolar binding site adjacent to the catalytic site (Berliner and Shen, 1977; Sonder and Fenton, 1984), and an anion-binding region (Fenton et al., 1989) which may be responsible for the specific interaction of thrombin with fibrinogen (Fenton et al., 1989, Heurisch and Mann, 1988). As thrombin usually cleaves adjacent to arginine it was believed that a basic amino acid residue in hirudin binds in the primary binding pocket. The region around the Lys-Pro peptide bond at positions 47-48 displays strong homology with the α-thrombin cleavage site in prothrombin (Petersen et al., 1976). As other proteinase inhibitors of serine proteinases become bound in a substrate-like manner to the active site of their target proteinase, the prothrombin-like region of residues 40-48 could perform substrate-like interactions with thrombin. Attempts have been made by site-directed mutagenesis of a hirudin gene to determine the basic amino acid residue which interacts with the primary binding pocket (Braun et al., 1988; Dodt et al., 1988). The results indicate, however, that hirudin does not require interactions in the primary specificity pocket to form tight complexes with thrombin. Nevertheless, the observed minor differences in $K_d$ of complexes of Lys$^{47}$ hirudin mutants with α-thrombin correlate with the decreased in vivo antithrombotic efficiency of these mutants (Degryse et al., 1989). As the carboxyl-terminal region of hirudin is especially rich in acidic amino acid residues it may therefore be involved in the interaction with the anion-binding exosite of the enzyme. The importance of an intact carboxyl-terminal region for inhibitory activity has been demonstrated by successive enzymatic removal of the acidic tail (Chang, 1983; Dodt et al., 1987). Dependence of the association rate constant for thrombin-hirudin complex formation on the acidic nature of the carboxyl-terminal region was observed with site-specific hirudin mutants (Braun et al., 1988). Peptide synthesis of hirudin$^{48-65}$ confirmed the binding of the carboxyl-terminal region to a noncatalytic site of α-thrombin and inhibition of thrombin-mediated clotting, albeit with a specific anticoagulant activity 3-4 orders of magnitude lower than intact hirudin. Amino acid residues 56-65 and 53-64 were found to be minimally required for this action (Krstenansky and Mao, 1987; Mao et al., 1988; Maraganore et al., 1989). These studies as well as kinetic analysis of hirudin-thrombin interaction have revealed two or more binding sites in complex formation including at least one that involves substrate competition (Stone and Hofsteenge, 1986; Degryse et al., 1989).

In this paper we describe the generation of Ala$^{48}$-hirudin$^{1-47}$ containing three disulfide bonds and Ala$^{48}$-hirudin$^{48-65}$ by changing Pro$^{48}$ to alanine by site-directed mutagenesis. The experiments were performed (a) to examine the importance of the amino acid residue adjacent to the putative reactive site, and (b) to dissect hirudin inhibitory activity and
to localize binding sites of the resulting peptides Ala<sup>48</sup>-hirudin<sup>48-57</sup> and Ala<sup>48</sup>-hirudin<sup>48-65</sup> on α-thrombin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials were obtained from the following sources: Tos-Gly-Pro-Arg-AMC from Bachem; Tos-Gly-Pro-Arg-p-nitroanilide (Worthington grade), calf pancreatic elastase Lyn-C-T4 DNA polymerase, T4 gene 32 protein and restriction endonucleases from Boehringer Mannheim; DEAE-Phenex A-25 and 20 columns from Pharmacia LKB Biotechnology Inc.; Immobilon transfer membrane from Millipore; human fibrinogen from Kabl Vitrum; porcine pancreatic elastase, 7-amino-4-methylcoumarin, and reagents for electrophoresis from Serva; SDS-7 molecular weight standard from Sigma. Natural hirudin was a gift of Dr. R. Maschler, Plantorganwerk Bad Zwischenah, West Germany; United States standard human thrombin (lot J, 510 NIH units/vial) was kindly provided by Dr. G. Murano, National Center for Drugs and Biologics, Bethesda, MD.

**Amidoxylic Assay of Thrombin Activity**—Thrombin assays were performed at 25°C in 0.1 M Tris-HCl, pH 8.3, containing 0.2 M NaCl and 0.05% Triton X-100 with 1 mM enzyme and 125 μM Tos-Gly-Pro-Arg-p-nitroanilide in a total volume of 1 ml. For kinetics in slow binding inhibition experiments assays were run as previously described (Dodd et al., 1988) under buffer and temperature conditions as mentioned above. However, active site-titrated human or bovine α-thrombin (5-20 μM) and 25 μM Tos-Gly-Pro-Arg-AMC was used. Initial velocities and concentrations were obtained over 35%. Assays were performed with an Amino SDF-500 or Perkin Elmer MFP-3 spectrofluorimeter operating in the ratio mode (λ<sub>e</sub> = 383 nm; λ<sub>f</sub> = 455 nm), and fluorescence intensities were calibrated with 7-amino-4-methylcoumarin solutions of known concentrations.

**Clotting Assay**—Inhibition of clot formation was determined in a fibrometer (KClA, Amelung, West Germany) with a 37°C warming stage, excitation (383 nm), and fluorescence intensities were calibrated with 'I-amino-4-methylcoumarin solutions of known concentrations.

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**Clotting Assay**—Inhibition of clot formation was determined in a fibrometer (KClA, Amelung, West Germany) with a 37°C warming stage. Human fibrinogen was dialysed against 0.3 M NaCl and stored at -20°C. Assays were performed as follows: 100 μl of α-thrombin (final concentration of 0.25 nM), 100 μl of buffer (50 mM imidazole, pH 7.8, containing 0.5% polyethylene glycol 6000), and 100 μl of hirudin or fragment diluted in buffer were prewarmed for 3 min. 100 μl of fibrinogen solution (final concentration of 5.9 μM) was added with the fibrometer pipette which activates the timer. Initial velocities were obtained from plots of clotting time versus inhibitor concentrations. In experiments without inhibitor, but with varying enzyme concentrations (1-50 nM), the clotting time for infinite enzyme concentration was calculated from linear regression analysis of plots of clotting time versus reciprocal thrombin activity (Fenton et al., 1986) using Equation 3.

\[ P = (v_0 + (v_e - v_0) (1 - e^{-kt})) / k_{inhib} + d \]

The symbols \(v_0\), \(v_e\), and \(k_{inhib}\) represent the initial velocity, steady-state velocity, and an apparent first-order rate constant, respectively.

The inhibitor concentration which inhibits half of the enzyme activity represents the IC<sub>50</sub> value. These values were obtained from plots of clotting times (ct) versus inhibitor concentration (Fenton et al., 1986) using Equation 3.

\[ ct_{50} = 2 \times (ct_e - ct) + ct_a \]

The abbreviations \(ct_e\) and \(ct_a\) represent clotting times without inhibitor and for infinite enzyme concentrations, respectively.

**RESULTS**

**Effect of Pro<sup>48</sup>-α-Hirudin Mutation in the Hirudin-Thrombin Interaction**—Recombinant Ala<sup>48</sup>-hirudin was isolated by ion-exchange chromatography and reverse-phase HPLC. Correct cleavage of the signal peptide was confirmed by sequencing (10 steps of Edman degradation) and determination of amino acid composition. The data were found to be in agreement with those expected for Ala<sup>48</sup>-hirudin.

The results of slow binding inhibition studies comparing the interaction of natural hirudin, r-hirudin, and Ala<sup>48</sup>-hirudin with human and bovine α-thrombin are summarized in Table I. Previously, we were able to determine the association rate constant for complex formation by native hirudin, r-hirudin, and r-hirudin mutants with bovine α-thrombin in slow binding inhibition experiments (Dodd et al., 1988). Enzyme concentrations from 10 to 20 pm were applied. In the present study, we also present data of the reaction of human α-thrombin with native and r-hirudins (Table I), which are in good agreement with those obtained by Stone and Hofsteenge.
teinase Lys-C does not lead to Pro4' → alanine positively influenced the affinity of that the 3.6-fold increased affinity was due to a 1.6-fold decrease in the rate of dissociation. The 1.9-fold decreased affinity of bovine α-thrombin for Ala4'-hirudin compared to r-hirudin was caused predominantly by the 1.9-fold increase of the dissociation rate constant. These data indicate that the affinity of human and bovine α-thrombin for r-hirudin. Mutual of Pro46 to alanine positively influenced the affinity of human α-thrombin (Kd = 1.3 ± 0.6 × 10⁻¹² M) and slightly decreased the affinity of bovine α-thrombin (Kd = 28 ± 4 × 10⁻¹² M). Comparison of the kinetic data for the reaction of r-hirudin and Ala46-hirudin with human α-thrombin indicates that the 3.6-fold increased affinity was due to a 1.6-fold increase in the rate of association and a 2.2-fold decrease in the rate of dissociation. The L9-fold decreased affinity of bovine α-thrombin for Ala46-hirudin compared to r-hirudin was caused predominantly by the 1.9-fold increase of the dissociation rate constant. These data indicate that the Pro46 → Ala mutation cause only minor differences in Kd of thrombin-hirudin complexes. However, based on the present knowledge, the reason for quite the reverse effect on Kd of the reaction of Ala46-hirudin with the two thrombin species is not obvious.

Isolation of Ala4'- Hirudin-derived Fragments—It has been shown that incubation of r-hirudin with trypsin or endoproteinase Lys-C does not lead to the cleavage of any of the three lysyl peptide bonds. However, it was possible to separate two peptides by reverse-phase HPLC from incubation mixtures of Ala4'-hirudin with endoproteinase Lys-C (Fig. 2). These peptides were rechromatographed twice and analyzed for amino acid composition and were sequenced 10 steps by Edman degradation. The peptide eluting at 29.9 min corresponds to residues 1-47 containing the three disulfide linkages and the amino-terminal valine, whereas the peptide eluting at 30.9 min contains positions 48-65 starting with an alanine.

Anticoagulant Activities of Ala46-Hirudin-derived Fragments—Unsulfated Nα-acetylhirudin46-65 has been shown to inhibit fibrinogen cleavage by binding to a noncatalytic site of thrombin (Krstensson and Mau, 1987; Mau et al., 1986; Maragano et al., 1989). Therefore we analyzed the inhibitory activity of the Ala46-hirudin fragments in a cloting assay using purified bovine α-thrombin and fibrinogen. With 0.2 NIH unit α-thrombin/assay clotting times of 21 s were obtained, whereas for infinite enzyme concentrations clotting times of 8.6 s were determined. The results of dose dependence studies comparing natural hirudin, r-hirudin, Ala46-hirudin, and Ala6-hirudin-derived fragments are shown in Table II. The carboxyl-terminal fragment Ala46-hirudin46-65 inhibited clot formation with an IC50 of 4.9 μM, whereas the potency of the amino-terminal fragment, Ala6-hirudin4-41, with an IC50 of 0.6 μM is increased 8.1-fold. Both fragments are far less effective than the intact mutant, which is comparable to r-hirudin. In comparison with intact hirudin IC50 values for the inhibition of α-thrombin-catalyzed clot formation by Ala46-hirudin46-65 and Ala6-hirudin4-41 are increased 300- and 2000-fold, respectively.

Localization of Binding Sites of Ala46-Hirudin-derived Fragments on α-Thrombin—To provide direct evidence that the
Ala^48-hirudin-derived fragments bind on \( \alpha \)-thrombin and to localize the binding regions of \( \alpha \)-thrombin in these fragments we mapped \( \alpha \)-thrombin and the complexes of \( \alpha \)-thrombin with Ala^48-hirudin and \( \alpha \)-thrombin with Ala^48-hirudin derived with either trypsin or pancreatic elastase. For these experiments we incubated 1.6 \( \mu \)M \( \alpha \)-thrombin with 35 nM trypsin and 400 nM elastase, respectively. These concentrations of trypsin and elastase completely hydrolyzed \( \alpha \)-thrombin at one predominant peptide bond in 1 h at 37 °C as was predetermined by SDS gel electrophoresis from samples of a dilution series of the appropriate enzyme with 1.6 \( \mu \)M \( \alpha \)-thrombin (Fig. 3, A and B, lane 2). To identify the cleavage sites, digests of 1 nmol of \( \alpha \)-thrombin with either trypsin or elastase were separated by SDS gel electrophoresis under nonreducing conditions. The fragments obtained by hydrolysis of thrombin with elastase or trypsin are indicated by A1 and A2 or B1 and B2, respectively. These were blotted and subjected to sequence analysis.

already published by Sonder and Fenton (1986). By sequencing peptides A1 and A2 elastase was found to hydrolyze \( \alpha \)-thrombin at position 150-151 of the \( \alpha \)-thrombin B-chain as previously has been reported for the human enzyme (Kawabata et al., 1985; Fenton and Bing, 1986). In summary, the anion-binding exosite of \( \alpha \)-thrombin is defined by the trypsin cleavage site; and, based on a \( \alpha \)-thrombin B-chain model (Fenton, 1986), the elastase cleavage site defines a region close to the fibrin groove.

In forming the \( \alpha \)-thrombin-hirudin complex, the observed elastase as well as trypsin cleavage sites were inaccessible to hydrolysis by the corresponding proteinase (Fig. 3, lanes 3). Mapping the complexes of \( \alpha \)-thrombin and Ala^48-hirudin-derived fragments for elastase and trypsin-sensitive sites we were able to determine distinct binding sites of these fragments on \( \alpha \)-thrombin. In the complex with the amino-terminal fragment Ala^48-hirudin+47, \( \alpha \)-thrombin is shielded completely for cleavage by elastase (Fig. 3, lane 7) whereas in the complex with the carboxy-terminal fragment Ala^48-hirudin+47 the rate of elastase hydrolysis appeared to be diminished (Fig. 3A, lane 5). On the other hand, \( \alpha \)-thrombin is not accessible to trypsin in the complex with Ala^48-hirudin+47 and the rate of trypsin cleavage appeared to be diminished for the complex with the amino-terminal fragment Ala^48-hirudin+47. In summary, considering the IC_{50} values, the thrombin-hirudin interaction comprises at least two binding

![Fig. 2. Reversed-phase chromatography of peptides derived from lysyl endopeptidase digest of Ala^48-hirudin. 140 nmol of Ala^48-hirudin were hydrolyzed with 0.5% (w/w) lysyl endopeptidase in 0.25 M Tris-HCl, pH 8.5, containing 10 mM EDTA for 6 h at 37 °C. The incubation mixture was applied to a Shandon ODS Hypersil column (5 \( \mu \)m, 250 x 4.6 mm) and separated in a trifluoroacetic acid system as described under “Experimental Procedures.” The chromatogram shows the separation of 20 nmol of the digest. The structures of the proteins corresponding to the peaks with amino acid composition and sequence analyses. These are indicated above the peaks.](http://www.jbc.org/)

![Fig. 3. Analysis of bovine \( \alpha \)-thrombin and thrombin-inhibitor complexes for trypsin and elastase cleavage sites. The analysis was performed under nonreducing conditions in 15% SDS-polyacrylamide gels. 1.5 \( \mu \)g of \( \alpha \)-thrombin (lane 2) were mixed with 1.5 \( \mu \)g of r-hirudin (lane 3), 37.5 \( \mu \)g of Ala^48-hirudin+47 (lane 4), and 15 \( \mu \)g of Ala^48-hirudin+48-65 (lane 5) and subjected to hydrolysis with elastase (A) or trypsin (B). Controls are shown in lane 1 (thrombin), lane 6 (elastase or trypsin), lane 7 (elastase or trypsin with hirudin), and lane 8 (hirudin). The SDS-7 standard proteins (1.5 \( \mu \)g each) were also run under nonreducing conditions. The fragments obtained by hydrolysis of thrombin with elastase or trypsin are indicated by A1 and A2 or B1 and B2, respectively. These were blotted and subjected to sequence analysis.](http://www.jbc.org/)

Table II

| Inhibitor of thrombin clotting activity | IC_{50} (nM) |
|----------------------------------------|-------------|
| Hirudin                                | 2.3         |
| r-Hirudin                              | 2.4         |
| Ala^48-Hirudin                         | 2.7         |
| Ala^48-Hirudin+47                     | 0.6 \( \mu \)M |
| Ala^48-Hirudin+48-65                   | 4.6 \( \mu \)M |
sites with distinct affinities. The results indicate direct interactions of the COOH-terminal peptide Ala\(^{48}\)-hirudin\(^{48-65}\) with the proposed anion-binding exosite and interactions between the amino-terminal fragment Ala\(^{48}\)-hirudin\(^{48-65}\) and \(\alpha\)-thrombin in the region of the elastase cleavage site. The latter seems to represent the major interaction site which accounts for tight complexing.

**DISCUSSION**

The experiments presented here have separated two hirudin binding sites on \(\alpha\)-thrombin. Two fragments, Ala\(^{48}\)-hirudin\(^{47}\) and Ala\(^{48}\)-hirudin\(^{50-65}\), were derived from the Ala\(^{48}\)-hirudin mutant. These were shown to combine with \(\alpha\)-thrombin at distinct sites, inhibiting clot formation. However, the thrombin inhibition activity of either fragment is 300-2000-fold smaller than that of the intact mutant and r-hirudin.

Evidence for the existence of an anion-binding exosite which determines thrombin specificity has been summarized (Fenton, 1981, 1986; Fenton et al., 1989). Alignment of \(\alpha\)-thrombin and \(\alpha\)-chymotrypsin sequences shows that the cleavage site producing \(\beta\)-thrombin is located within a sequence in which 6 additional basic amino acid residues have been introduced into bovine \(\alpha\)-thrombin. This sequence corresponds to the surface loop 63-85 in \(\alpha\)-chymotrypsin (Birktoft and Blow, 1972) and represents the anion-binding exosite. The 100-200-fold increase of the \(K_C\) value for the complex of hirudin with \(\beta\)-thrombin suggests that the region around the \(\beta\)-cleavage site is important for binding hirudin (Stone et al., 1987). Additional evidence for the importance of this region was obtained with antibodies to the 62-73 region of the human \(\alpha\)-thrombin B-chain which acts as a competitive inhibitor of the thrombin-hirudin interaction (Noe et al., 1988).

On the inhibitor side, the carboxyl-terminal portion of r-hirudin containing 6 negatively charged residues has been implicated in binding to the anion-binding exosite of \(\alpha\)-thrombin (Fenton, 1981). Evidence derives from mutagenesis of these acidic residues of hirudin which lowers the association rate constant for complex formation concomitant with lowering the charge of this region (Braun et al., 1988). In addition, chemically synthesized peptides of the carboxyl-terminal region were shown to recognize thrombin and to inhibit thrombin-catalyzed conversion of fibrinogen to fibrin. As the synthetic peptides only exhibit anticoagulant activity and do not inhibit hydrolysis of small peptide substrates, it was concluded that the hirudin-related peptides do not block the active site of \(\alpha\)-thrombin (Kristensky and Mao, 1987; Mao et al., 1988; Maraganore et al., 1989). Clotting inhibition by these peptides then must result from binding to a noncatalytic site on \(\alpha\)-thrombin. Indeed, the present study has demonstrated that the carboxyl-terminal part Ala\(^{48}\)-hirudin\(^{48-65}\) and residues 62-74 of the thrombin B-chain combine in thrombin-hirudin complex formation. The recognition between these sites appears therefore to be caused mainly by a complementary surface of charged amino acid residues.

However, considering the high affinity of hirudin for thrombin and the inhibition of the enzyme's catalytic as well as noncatalytic functions by intact hirudin it is obvious that the thrombin-hirudin interaction comprises an extended binding region not restricted to the anion-binding exosite. The work of Degryse et al. (1989) has identified a mixed kinetic mechanism for the thrombin-hirudin interaction including at least two binding sites. One of these appears to be involved in substrate competition. The present study provides evidence that the amino-terminal fragment Ala\(^{48}\)-hirudin\(^{48-65}\) binds to a distinct site on \(\alpha\)-thrombin. This site is located around the elastase cleavage site (Thr-B150) of the bovine enzyme corresponding to surface peptides in \(\alpha\)-chymotrypsin (Fenton and Bing, 1986). The inhibitory activity of Ala\(^{48}\)-hirudin\(^{48-65}\) may then be caused either directly by blocking a fibrinogen recognition site or indirectly by induction of a conformational change in thrombin leading to a masked binding site. Since we observed an additional inhibitory effect of Ala\(^{48}\)-hirudin\(^{48-65}\) on the hydrolysis of Tna-Gly-Pro-Arg-\(\gamma\)-nitroanilide\(^{\text{a}}\), it seems possible that the fragment interacts with the active site groove of \(\alpha\)-thrombin.

An intriguing observation is the decreased rate of \(\alpha\)-thrombin cleavage in complexes with Ala\(^{48}\)-hirudin\(^{48-65}\) by trypsin and in complexes with Ala\(^{48}\) hirudin\(^{48-65}\) by elastase. The latter may reflect a conformational change of the enzyme, as has been observed in CD spectra of complexes of \(\alpha\)-thrombin with hirudin and hirudin-related carboxy-terminal peptides (Konno et al., 1988; Mao et al., 1988). Binding of the amino-terminal fragment Ala\(^{48}\)-hirudin\(^{48-65}\) may also be associated with a significant conformational change of the enzyme displayed by the reduced trypsin cleavage rate.

The kinetic data for complex formation of hirudin with human and bovine \(\alpha\)-thrombin (Table I) show a reduced affinity of hirudin for the bovine enzyme. Recent analysis of anticoagulant activities of synthetic hirudin-related carboxy-terminal fragments toward human and bovine \(\alpha\)-thrombin (Maraganore et al., 1989) has identified a 10-fold increased potency toward human \(\alpha\)-thrombin. Thus it may be speculated that the observed species differences in complexes of hirudin with human and bovine \(\alpha\)-thrombin are attributed exclusively to differences in the interaction with the carboxy-terminal hirudin region.

The results presented here are consistent with the idea that hirudin binds to thrombin via an extended binding region by hydrophobic interactions in conjunction with general electrostatic complementarity. There is striking evidence that hirudin's carboxy-terminal tail shares the anion-binding exosite with other macromolecular substrates of thrombin and that the compact amino-terminal domain interacts with the active site. All these interactions together block a wide range of thrombin functions. With the Ala\(^{48}\)-hirudin-derived fragments at hand tools for probing the interaction of thrombin with physiological substrates are available.

\(^{\text{a}}\) T. Schmitz and J. Dodt, unpublished data.

### Table III

Structure of bovine \(\alpha\)-thrombin fragments derived by proteolysis with trypsin or pancreatic elastase.

| Fragment | Amino-terminal sequences \(^{\text{a}}\) | Structure |
|----------|----------------------------------|-----------|
| A1       | TSEDHFQPPF---                   | Intact A-chain |
| A2       | IVEQQDAEVG---                   | Amino terminus of the B-chain |
| B1       | SVAEVEQPSVL---                  | B-chain starting with S-B151 |
| B2       | IVEQQDAEVG---                   | B-chain starting with K-B74 |

\(^{\text{a}}\) All protein bands were sequenced 13-15 steps.
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