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# Interaction of site specific hirudin variants with α-thrombin

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The kinetics of complex formation between recombinant hirudin or recombinant hirudin mutants with thrombin were analyzed. In order to elucidate the inhibitor's reactive site peptide bond predetermined amino acid substitutions were introduced at positions of basic amino acid residues by means of site-directed mutagenesis of a hirudin gene. In comparison to recombinant hirudin ($K_i$ = 19 pM) only those mutant inhibitors which were modified at amino acid position $Lys^{47}$ showed a higher $K_i$ value for their complexes with thrombin. The observed effects are mainly due to increased $k_{off}$ rate constants.

Hirudin; Hirudin variant; Site-directed mutagenesis; Kinetics; α-Thrombin; Putative reactive site

## 1. INTRODUCTION

Hirudin is a polypeptide of 65 amino acid residues found in salivary gland secretions of the leech *Hirudo medicinalis* (review [1]). This protein forms stoichiometric complexes with thrombin, the central serine proteinase of blood coagulation, in which the catalytic functions of the proteinase are blocked.

The major common structural feature of proteinase inhibitor proteins is the primary contact region (= reactive site loop). The conformation of this loop is complementary to the surface of enzymes and stands out from the inhibitor to become accessible to the active sites of proteolytic enzymes [2]. Thus the reactive site amino acid residue becomes bound to the specificity pocket of the proteinase. With respect to thrombin specificity one of the three lysine residues is thought to be the reactive site peptide bond. Especially the hirudin region around $Lys^{47}$ shows a sequence homology to a target sequence of α-thrombin in the pro-part (positions 148–157) of its precursor [3]. On the other hand, the kinetics of the hirudin-thrombin interaction indicate that a single hirudin molecule may occupy more than one site on α-thrombin, one being the catalytic site and the other being distinct from the catalytic site and whose binding is dependent on ionic strength [4].

This paper deals with site-directed mutagenesis of a hirudin gene and the kinetics of mutant inhibitor proteins with thrombin in comparison to natural and recombinant hirudin (r-hirudin). These studies were performed to determine whether there is a reactive site peptide bond in hirudin, which is directly involved in the thrombin-hirudin interaction, as has been shown for other proteinase inhibitor proteins and their corresponding proteinases.

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*Abbreviations:* HPLC, high-performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; RP, reversed-phase; Tos, 4-toluolsulfonyl

## 2. MATERIALS AND METHODS

Hirudin gene mutants were generated via the M13 gapped-duplex DNA approach [5] using oligonucleotides of 17–25 bases carrying the synthetic marker. *E. coli* strains BMH 71-18: (*Δ(lac,proAB), thi, supE*; F' *lacI*$^q$, Z*Δ*M15, *proA*$^+$B$^+$); BMH-71-18 mutS: (BMH 71-18, mutS215::Tn10); MK 30-3:

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(Δ(*lac,pro*AB), *rec*A, *gal*E, *str*A; F′ *lac*I$^q$, ZΔM15, *pro*A$^+$B$^+$) and phages M13mp9am and M13mp9rev were a gift from Dr H.-J. Fritz, Munich. Gene mutants were identified by dot-blot analysis of phages with $^{32}$P-labeled oligonucleotides [6] and dideoxy sequencing of recombinant ss M13 DNA template [7]. Mutant hirudin gene fragments were isolated from ds M13 DNA after gel electrophoresis on 5% polyacrylamide gels [8] and gel elution [9]. Expression and isolation of r-hirudin variants were performed from periplasmic fractions of *E. coli* [10] as described for r-hirudin [11]. The amino acid sequences of Ile$^{27}$-, Ile$^{36}$-, Ile$^{47}$-, Glu$^{36}$- and Glu$^{47}$-hirudin were verified by amino acid sequence determinations [12]. Protein concentrations of r-hirudin variants were determined by RP-HPLC using standard solutions of recombinant hirudin. Bovine α-thrombin was isolated as described [13,14] and the purity was ascertained by 10 steps of Edman degradation. α-Thrombin concentrations were determined by active site titrations [15]. r-Hirudin isolation procedures were monitored by a thrombin inhibition assay with tos-G-P-R-*p*-nitroanilide (Boehringer) as substrate for thrombin. Assays were performed in polystyrene cuvettes at 25°C in 0.1 M Tris-HCl, 0.2 M NaCl, 0.05% Triton X-100, pH 8.3. For the kinetics of hirudin and thrombin the same buffer conditions were applied. Tos-G-P-R-7-(4-methyl)coumarylamide (Bachem) was used as a substrate for thrombin at concentrations of 20–25 μM (= 4–5-fold $K_m$). Under the specified conditions the $K_m$ of the substrate was 5.2 μM. Concentrations of thrombin were 10 pM for experiments with natural hirudins and 20–25 pM for experiments with recombinant hirudins and mutants. Inhibitor concentrations were 10–80-fold over [E$_0$] (= 10–25 pM). Assays were performed with an Aminco SPF-500 spectrofluorimeter operating in the ratio mode ($\lambda_{ex}$ = 383 nm; $\lambda_{em}$ = 455 nm) and fluorescence intensities were calibrated with 7-amino-4-methylcoumarin solutions of known concentrations.

## 3. RESULTS

### 3.1. *Construction and expression of hirudin variants*

As the reactive site peptide bond of a proteinase inhibitor protein determines its specificity the alteration of this amino acid residue should lead to a decreased affinity for its target proteinase. Concerning thrombin specificity we decided to generate hirudin mutants with single amino acid substitutions: in order to obtain definite results positions of basic amino acid residues should be replaced by apolar, uncharged and polar, negatively charged amino acid residues, respectively. According to the codons created by chemical gene synthesis [16] the AAA codons for Lys$^{27}$, Lys$^{36}$ and Lys$^{47}$ were changed to ATA for Ile and AGA for Glu; the CAG codon for His$^{51}$ was altered to CTC for Leu and GAC for Asp by the method of oligonucleotide-directed site-specific mutagenesis via the gapped-duplex (gd) DNA approach. 50–80% of examined clones showed the desired point mutation as could be verified by dot-blot hybridization of phages and dideoxy sequencing of positive clones. *Hin*dIII gene fragments of hirudin variants were obtained from ds M13mp9rev and inserted downstream of the alkaline phosphatase signal sequence as described for r-hirudin [11]. Expression products were isolated in yields of 2–6 mg/l bacterial culture.

### 3.2. *Pre-steady-state kinetics of the interaction of thrombin with hirudin, recombinant hirudin and recombinant variants*

Under assay conditions of 10–25 pM thrombin and 0.1 M Tris-HCl, 0.2 M NaCl, 0.05% Triton X-100, pH 8.3, all the inhibitors can be classified as fully competitive, slow binding inhibitors [17,18] of thrombin. Therefore, in slow binding inhibition experiments with natural hirudin enzyme concentrations of 10 pM were used to obtain reliable data for the pre-steady state, whereas for r-hirudins 20–25 pM thrombin gave good results. In the presence of buffer, substrate and the appropriate slow binding inhibitor the reaction was started by the addition of enzyme. After a rapid initial phase the initial velocity decreased to a slower steady state (fig.1). For each of the inhibitors a set of progress curves for several inhibitor concentrations was obtained and data were fitted by nonlinear regression to eqn 1:

$$P = v_s t + (v_0 - v_s)(1 - e^{-k_{app}t})/k_{app} + d \qquad (1)$$

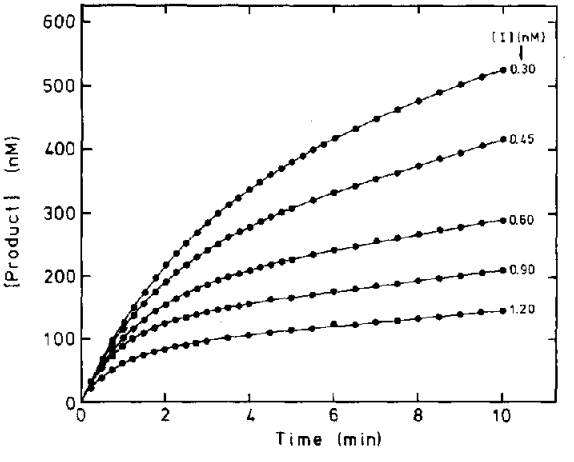



Fig.1. Slow binding inhibition of α-thrombin by recombinant hirudin. Points are experimental and lines are the best fit of the data to eqn 1. Inhibitor concentrations are given.

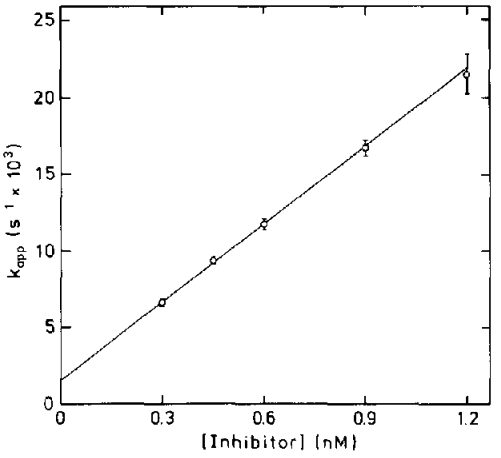


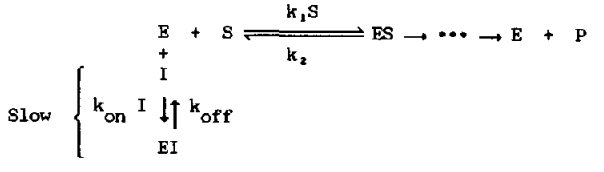

Fig.2. Plot of $k_{app}$ vs r-hirudin concentrations. The apparent rate constant ($k_{app}$) calculated for various inhibitor concentrations (fig.1) were plotted vs r-hirudin concentrations. The curve was analyzed according to eqn 2 to obtain the $k_{on}$ and $k_{off}$ rate constants. Plots for all other hirudins described in this paper were calculated accordingly.

The symbols $v_0$, $v_s$ and $k_{app}$ represent the initial velocity, steady-state velocity and an apparent first-order rate constant; $d$ is a displacement term to account for the fact that at $t = 0$ the fluorescence may not be accurately known. As the observed initial velocity of the reactions was independent of the inhibitor concentrations (cf. fig.1) and the $k_{app}$ values varied as a linear function of the inhibitor concentrations (fig.2) in our experiments hirudin, r-hirudin and variants showed the characteristics of inhibitors obeying the mechanism of scheme 1 ([17,18].

The values of $k_{app}$ were weighted according to the squared inverse of their standard errors and fitted to eqn 2 to obtain $k_{on}$ and $k_{off}$:

$$k_{app} = [k_{on}/(1 + [S]/K_m)]\,[I] + k_{off} \qquad (2)$$

From the relationship: $K_i = k_{off}/k_{on}$ the inhibition constant $K_i$ was calculated. Table 1 summarizes the data as they have been obtained for hirudin, r-hirudin and variants.




Scheme 1.

Table 1

Inhibition of thrombin by hirudin, r-hirudin and variants

| | $k_{on}$ ($\times 10^7$ $M^{-1} \cdot s^{-1}$) | $k_{off}$ ($\times 10^{-3}$ $s^{-1}$) | $K_i = k_{off}/k_{on}$ ($\times 10^{-12}$ M) |
|---|---|---|---|
| Natural hirudin | 30.8 ± 0.2 | 0.9 ± 0.4 | 3 ± 1 |
| r-Hirudin | 7.8 ± 0.2 | 1.5 ± 0.2 | 19 ± 2 |
| $Ile^{27}$-hirudin | 8.6 ± 0.2 | 1.6 ± 0.2 | 18 ± 3 |
| $Glu^{27}$-hirudin | 10.9 ± 0.02 | 0.4 ± 0.1 | 4 ± 1 |
| $Ile^{36}$-hirudin | 8.6 ± 0.8 | 1.2 ± 0.8 | 14 ± 8 |
| $Glu^{36}$-hirudin | 10.6 ± 0.6 | 1.7 ± 0.5 | 16 ± 4 |
| $Ile^{47}$-hirudin | 6.4 ± 0.4 | 4.1 ± 0.5 | 64 ± 9 |
| $Glu^{47}$-hirudin | 6.9 ± 1.1 | 13.8 ± 2.4 | 200 ± 48 |
| $Leu^{51}$-hirudin | 8.6 ± 0.5 | 3.2 ± 0.3 | 34 ± 4 |
| $Asp^{51}$-hirudin | 9.9 ± 0.8 | 1.3 ± 0.5 | 13 ± 5 |

The values of the kinetic parameters were determined by fitting primary data obtained from eqn 1 to eqn 2 and are given together with their standard errors

In this way it is shown that hirudin is the most potent thrombin inhibitor ($K_i = 2.8$ pM) as is also indicated by its individual $k_{on}$ and $k_{off}$ rate constants. These data are changed for r-hirudin to give a 6-fold increased $K_i$ value (19 pM). With respect to r-hirudin the variants which were modified at $Lys^{47}$ demonstrate a drastic effect on the inhibition constant. This is mainly caused by an increased $k_{off}$ ($1.5 \times 10^{-3}$ $s^{-1}$ for r-hirudin, $4.1 \times 10^{-3}$ $s^{-1}$ for $Ile^{47}$-hirudin and $13.8 \times 10^{-3}$ $s^{-1}$ for $Glu^{47}$-hirudin), whereas the $k_{on}$ rate constants are unaffected from the $Ile^{47}$- to $Glu^{47}$-variant and are comparable with that for r-hirudin. Remarkable is the low $K_i$ value of $Glu^{27}$-hirudin which is due to its very low $k_{off}$ ($0.5 \times 10^{-3}$ $s^{-1}$) and which is about 2-fold lower than that for natural hirudin. The $k_{on}$ rate constant, however, is similar to that of all other r-hirudins.

## 4. DISCUSSION

Studies on the inhibition of thrombin by natural hirudin, r-hirudin and r-hirudin variants were performed to obtain information about the nature of interactions involved in thrombin-hirudin complexes. With respect to natural hirudin the structure of r-hirudin is missing a sulfate group on $Tyr^{63}$: this leads to a 6-fold increase of the $K_i$ value. The data for r-hirudin are in good agreement with those for desulfato-hirudin which was produced from the natural source by arylsulfatase [19] or acid treatment [4]. In this way, tyrosine

sulfation in hirudin seems to be involved either directly in the interaction with thrombin or in stabilizing the inhibitor structure. Therefore, r-hirudin and its interaction with thrombin represents the basis for our studies.

As the reactive site determines the specificity of a proteinase inhibitor for a target proteinase [2] changing this special amino acid residue to an incompatible one gives rise to a decrease in the $k_{on}$ rate constant by some orders of magnitude. This is impressively shown for the Met → Arg exchange in $\alpha_1$-proteinase inhibitor which decreases the $k_{on}$ rate constant by 4 orders of magnitude for the interaction with elastase but increases this value 4 orders of magnitude for the interaction with thrombin [20]. As inhibitors react in a substrate-like manner with their proteinases one of the basic amino acid residues was thought to be the reactive site of hirudin. However, our results with hirudin variants showed only a weak influence of the basic amino acid residues of hirudin on the interaction with thrombin. It must be stressed that the $k_{on}$ rate constant is unchanged in all cases; the $k_{off}$ rate constant (and therewith $K_i$) however is increased for inhibitors with mutations at position $Lys^{47}$. These data imply that the recognition of hirudin and thrombin is independent of the nature of a putative reactive site, however, the stability of the complex is positively influenced by $Lys^{47}$ which may become bound to the specificity pocket of thrombin inhibiting the hydrolysis of smaller substrates.

The rapid rate of thrombin-hirudin complex formation (cf. [4] for human and this study for bovine $\alpha$-thrombin) and its dependence on ionic strength [4] suggest that this process is a diffusion-controlled reaction involving mainly ionic interactions. An extended binding region may be formed by a contiguous surface of hydrophilic and charged residues of the central core [21] in which the C-terminal part may also be involved [21]. Binding of a C-terminal peptide (residues 45–65) which recognizes thrombin but not the catalytic site [22] confirmed these observations. In this manner hirudin differs from other known proteinase inhibitor proteins as its interaction with its target proteinase $\alpha$-thrombin does not depend strongly on a putative reactive site. Investigations are in progress to elucidate more detailed regions of hirudin responsible for the specific interaction with thrombin.

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