Chemical Modifications and Amino Acid Substitutions in Recombinant Hirudin That Increase Hirudin–Thrombin Affinity†

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Received June 12, 1990; Revised Manuscript Received October 3, 1990

ABSTRACT: Recombinant hirudin (r-hirudin), unlike the naturally occurring leech protein, lacks a sulfate ester on Tyr-63 which reduces its binding affinity to thrombin by 3–10-fold. We demonstrate that nitration or iodination of Tyr-63 restores hirudin–thrombin affinity to levels similar to or exceeding that of the natural inhibitor. In contrast, nitration of Tyr-3 reduces the affinity of hirudin for thrombin. These chemical modifications result in multiple reaction products that are readily separated by reverse-phase HPLC. The mechanism of the observed changes in thrombin affinity may involve a reduction in the pK of the hydroxyl group of tyrosine due to substitution of the electrophilic iodo or nitro group on the phenyl ring, resulting in an increased negative charge at neutral pH. For Tyr-63, this effect mimics the sulfatotyrosine of natural hirudin, leading to an increased thrombin affinity at the anion-binding exosite. For Tyr-3, the increased polarity may destabilize its interaction within the apolar-binding site of thrombin. Substitution of the highly conserved Tyr-3 residue with Phe or Trp not only enables specific and quantitative chemical modification at Tyr-63 but also independently increases hirudin–thrombin affinity. Kinetic analysis of thrombin inhibition showed that enhanced binding by r-hirudin(nitro-Tyr-63) is due to an increase in the association rate between hirudin and thrombin whereas the reduced binding of r-hirudin(nitro-Tyr-3) results from a large increase in the dissociation rate. These observations indicate that specific segments within both the amino- and carboxy-terminal regions of hirudin interact with thrombin.

Hirudin is the most potent and specific known inhibitor of the blood-clotting enzyme thrombin, the serine protease that plays a key bioregulatory role in hemostasis and the pathology of thrombotic disease (Johnson et al., 1989). Hirudin is an effective anticoagulant and antithrombotic agent in animals and humans (Markwardt et al., 1984, 1988) and is uniquely suited to a wide variety of therapeutic, diagnostic, and medical device applications in cardiovascular medicine.

Hirudin inactivates thrombin by binding tightly to an extensive surface region of the enzyme upon formation of a 1:1 stoichiometric complex. The inhibition constant, K_i, has been reported to be in the range of 0.02–3 pM for leech hirudin (Stone & Hofsteenge, 1986; Dodt et al., 1988) and is significantly higher for the recombinant protein (Stone & Hofsteenge, 1986; Dodt et al., 1988; Johnson et al., 1990). The specificity of thrombin for binding macromolecular substrates is determined mainly by its insertion loops (absent in homologous trypsin-like proteases) and appears to involve interactions at three distinct regions (Bode et al., 1989; Johnson et al., 1989; Fenton & Bing, 1986): (1) the basic specificity pocket located in the active-site region that binds the side chains of arginine or lysine on the amino-terminal side of the scissile peptide bond; (2) the apolar-binding site that binds proflavin (Berliner & Shen, 1977); and (3) the anion-binding exosite, a region in the vicinity of the β-cleavage site that is rich in basic amino acids, and is important for the specific interaction of thrombin with fibrinogen (Fenton et al., 1988). The acidic carboxy-terminal region of hirudin has been shown to bind thrombin primarily at the anion-binding exosite (Chang et al., 1990; Stone et al., 1989; Mao et al., 1988). Molecular modeling studies have suggested that the high affinity and specificity of hirudin for thrombin are the result of multiple contacts within both the N- and C-terminal regions and have further implicated the amino-terminal domain of hirudin as interacting with the apolar-binding/active-site region of thrombin (Johnson et al., 1989; Lazar et al., 1991).

Hirudin variant HV-1 (65 amino acids) contains tyrosine at positions 3 and 63. The naturally occurring protein is sulfated at Tyr-63, although purified preparations may contain as much as 12% of the nonsulfated form (Chang, 1983). Recombinant hirudin produced by genetic engineering methods is not sulfated and, similarly to desulfated leech hirudin, exhibits a higher inhibition constant for thrombin (Stone & Hofsteenge, 1986; Dodt et al., 1988), suggesting that sulfation of Tyr-63 is responsible for the enhanced affinity of natural over recombinant hirudin. We demonstrate that iodination or nitration of Tyr-63 substantially reduces the inhibition constant (K_i) of hirudin for thrombin whereas nitration at Tyr-3 increases the K_i. Thrombin inhibition analysis and the effects of various amino acid substitutions at Tyr-3 suggest that the amino-terminal segment of hirudin plays an important role in the interaction with thrombin.

Experimental Procedures

Chemicals. Tetranitromethane and 3-nitro-L-tyrosine were purchased from Aldrich Chemical Co. Leech hirudin was obtained from Sigma Chemical Co. (3000 units/mg) and from BioPharm (12 000 units/mg). Enzymobeads for iodination were purchased from Bio-Rad. The chromogenic substrate H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2238) was from KabiVitrum. Active-site-titrated human α-thrombin was kindly provided by Dr. John Fenton (New York State Department of Health Research Laboratories). All solvents were reagent- or HPLC-grade.

Expression and Purification of Recombinant Hirudin. Amino acid substituted r-hirudin was produced by mutagenesis of the synthetic gene for hirudin variant 1 (HV-1) (Sze et al.,

† This work was supported by SRI Internal Research and Development Projects 870D32XJC and 391D32YSB and by funds provided by the Cigarette and Tobacco Surtax Fund of the State of California through the Tobacco-Related Disease Program of the University of California.
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0006-2960/91/0430-1271$02.50/0

submitted for publication) by using the polymerase chain reaction, an oligonucleotide primer containing the nucleotide change specifying the amino acid substitution, and a second primer. Oligodeoxyribonucleotides were synthesized by phosphoramidite chemistry (Adams et al., 1983; McBride & Caruthers, 1983) using an Applied Biosystems Model 380A DNA synthesizer. Aliquots (4 A_{260} units) of the synthesized oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis (Maniatis et al., 1982). Wild-type and mutant hirudins were produced by using an expression vector system based on the colicin E1 operon (Waleh & Johnson, 1985), in which r-hirudin was produced as a fusion protein (connected by a single methionine residue) with connective tissue activating peptide III (CTAP-III), a protein with unusually high stability in *Escherichia coli* that stabilizes hirudin to in vivo degradation (unpublished results).

Cells containing the hirudin expression plasmid were grown at 37 °C in a 16-L fermentor in media containing 50 μg/mL ampicillin. Upon reaching an optical density of 4 at 660 nm, hirudin synthesis was induced by addition of mitomycin C to the culture (1 mg/L). Cells were lysed with a Stansted cell disrupter in 25 mM Tris (pH 7.5), 10 mM EDTA, and 6 M guanidinium chloride, cellular debris was removed by centrifugation, and the extract was reacted with 0.2 M cyanogen bromide in 6 M guanidinium chloride, 0.2 M phosphoric acid, and 5 mM sodium thiosulfate to cleave the hirudin–CTAP-III fusion protein. The two proteins were then separated by reverse-phase HPLC. Pooled fractions of hirudin, typically greater than 95% pure, were then refolded (Johnson et al., 1990) and finally purified by reverse-phase HPLC. All final hirudin preparations were characterized by analytical reverse-phase HPLC, UV spectroscopy, amino acid composition, and N-terminal amino acid sequence analysis, and were judged to be greater than 98% pure.

Chemical Modification Reactions. Recombinant hirudin was reacted for 20–40 min with sodium iodide by the Enzymobead technique according to the manufacturer's instructions. A 2:1 molar ratio of NaI to r-hirudin was employed for the preparative-scale reaction of r-hirudin mutants. The iodination products were separated from unreacted material by HPLC on a reverse-phase C-4 column (Vydac 214TP54). Chromatograms were developed in 0.065% (v/v) trifluoroacetic acid with an ascending linear gradient of 15–30% acetonitrile at a rate of 0.5% acetonitrile/min. Absorbance was monitored at 215 nm.

Recombinant hirudin (2.9 nmol) was reacted with tetranitromethane in 50 mM Tris (pH 8) by addition of 23 μL of an ethanol solution of tetranitromethane (while mixing) to r-hirudin in a final volume of 50 μL (200:1 molar ratio of tetranitromethane to r-hirudin except where stated otherwise). After 1 h of constant shaking, the reaction was terminated by lowering the pH to 2 by addition of 950 μL of 0.065% trifluoroacetic acid in 15% acetonitrile. Reaction products were separated from unreacted material by reverse-phase HPLC as described for iodinated r-hirudin except that chromatograms were developed at a rate of 0.25% acetonitrile/min. UV-absorbing peaks were monitored at 215 and 360 nm (the absorbance maximum of 3-nitrotyrosine under acidic conditions). Purified products contained no detectable cross-linked species as determined by denaturing polyacrylamide gel electrophoresis (data not shown). Iodination results in 3 or 3,5 substitutions on the phenyl ring of tyrosine, whereas nitration yields a single substitution at the 3-position only.

Thrombin Inhibition Analysis. Kinetic assays were performed at room temperature, using a Vmax Kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA), by comparing the inhibition of thrombin hydrolysis of S-2238 by r-hirudin to a r-hirudin standard of known specific activity. Thrombin, r-hirudin, and substrate concentrations were 2 nM, 0.3–1 nM, and 296 μM, respectively, in 50 mM Tris buffer, 100 mM NaCl, and 0.1% poly(ethylene glycol) (PEG) 6000, pH 7.8. The 96-well plates (Nunc) were treated with 1% PEG-20 000 and dried overnight prior to assay. Release of the chromophore was monitored at 405 nm. We diluted the concentrated stock of human α-thrombin to 1 μM in 5 mM Tris buffer/0.5 M NaCl, pH 6, and stored it at −60 °C over a period of months. Aliquots were thawed and diluted to 2 nM.

The specific activity of the r-hirudin standard was repeatedly measured over a 7-month period using a recording spectrophotometer thermostated at 37 °C. Conditions were the same as described above except that reactions were performed in polystyrene cuvettes. The rate of change of the absorbance at 405 nm was converted to antithrombin units by using the relation 1 antithrombin unit/mL = 1.25 A unit/min decline (KabiVitrum technical data). Protein concentration was determined by amino acid composition analysis. The mean specific activity by this method was 10.2 antithrombin units/μg (SD 0.83).

Amino Acid Composition and N-Terminal Sequence Analysis. Amino acid analyses were determined by using a Beckman 6300 amino acid analyzer with postcolumn ninhydrin chemistry following liquid-phase hydrolysis of r-hirudin samples under vacuum in 6 N HCl at 110 °C for 24 h. For nitrated r-hirudins, quantitation of 3-nitro-L-tyrosine was achieved by comparison with an external standard. Amino acid compositions for native and mutant hirudins were in agreement with values determined from the nucleotide sequence of the synthetic genes.

The first 15 residues of the N-terminal sequence of chemically modified and unmodified r-hirudin samples were determined by using Edman chemistry on an Applied Biosystems 470A gas-phase protein sequenator with separation on a Brownlee C18 column (2.1 mm × 22 cm) at 52 °C in 110 mM sodium acetate (pH 3.9)/5% tetrahydrofuran (buffer A) to acetonitrile (buffer B), 10–37% buffer B linear gradient over 19 min. For analysis of nitrated r-hirudins, 3-nitro-L-tyrosine was employed in the standard mixture.

Tight-Binding Inhibition Analysis. Inhibition constants (K_i) were determined in steady-state velocity experiments using S-2238 as substrate. Reactions were carried out in plastic microtiter plates at room temperature (26–30 °C) in 0.1-mL final volume of 50 mM Tris, 100 mM NaCl, 0.1% PEG-6000, and 200 μM substrate at pH 7.8; reactions were initiated by addition of 200 pM thrombin. The concentration of r-hirudin was varied over a range of approximately 0.25:1 to 8:1 hirudin:thrombin ratio. Color development was followed at 405 nm by using the Vmax Microtiter plate reader and software by Molecular Devices Corp. Reactions were monitored for 1 h; steady-state velocities were generally attained within 30 min; steady-state rates were determined by linear regression analysis.

Steady-state velocities were fitted to the rate equation for tight-binding inhibition kinetics (Morrison & Stone, 1985). When steady-state velocities are plotted as a function of hirudin concentration, the resulting curve is described by the equation:

$$2TV_s/V_0 = [(K_i' + xH - T)^2 + 4K_i'T]^{0.5} - K_i' - xH + T \quad (1)$$

where H = [hirudin], T = [thrombin], x is a factor that

corrects hirudin concentration units to molarity, V_s and V_0 are steady-state rates of the inhibited and uninhibited reactions, respectively, and $K_i = K_i'/(1 + [\text{substrate}]/K_m)$ for competitive inhibition. The K_m was taken to be 3.63 μM as determined by Stone and Hofsteenge (1986) using identical conditions except for temperature, which they controlled at 37 °C. The data were fitted to the equation by nonlinear regression analysis, yielding estimates of x, V_0, and K_i'.

Slow Tight-Binding Inhibition Analysis. By increasing the ionic strength, the rate of interaction between hirudin and thrombin can be inhibited sufficiently that the steady-state velocity is attained slowly. Under this condition, the progress curve of formation of the enzyme–inhibitor complex is described by the set of equations (Morrison & Stone, 1985):

$$P = V_f t + [(1 - g)(V_i - V_f)/kg] \ln [(1 - ge^{-kt})/(1 - g)] \quad (2)$$

$$g = (K_i' + H + T - Q)/(K_i' + H + T + Q) \quad (3)$$

$$Q = [(K_i' + T + H)^2 - 4TH]^{0.5} \quad (4)$$

$$V_f = V_i(T - H - K_i' + Q)/2T \quad (5)$$

where t = time, V_i is the initial reaction rate, V_f is the final steady-state rate, $k = k_{on}'Q$, and k_{on}' is the apparent association rate constant. Nonlinear regression analysis yields estimates of K_i' and k_{on}'; the apparent dissociation rate constant, k_{off}', is the product of K_i' and k_{on}'. The inhibition constant K_i was calculated from K_i' as given above, taking the K_m at 0.52 ionic strength to be 3.1 μM (Stone et al., 1989).

Slow tight-binding experiments were performed identically with tight-binding studies except that the NaCl concentration was raised to 0.5 M and the hirudin:thrombin ratio was varied from approximately 0.5:1 to 6:1 or, in the case of r-hirudin nitrated at Tyr-3 (which had relatively weak-binding characteristics), from 1:1 to 12:1. Computer software provided by Dr. Craig Jackson (American Red Cross, Southeastern Michigan Chapter) was used to acquire progress curves and smooth them using a cubic spline technique. Software for nonlinear regression analysis of both tight-binding and slow tight-binding data was kindly provided by Dr. Stuart Stone (Friedrich Miescher Institute, Basel, Switzerland).

Results

Iodination of r-Hirudin. The reaction products formed by iodination of r-hirudin were separated by reverse-phase HPLC as shown in Figure 1. The reaction conditions were designed to produce approximately equal quantities of the various reaction products. There are three sets of peaks; the left-most set (pool I) contains unreacted r-hirudin (at the far left) and two additional peaks. The middle set of peaks (pool II) contains three peaks, which are not completely resolved. The resolution of material at the right (pool III) is not as high as the earlier two sets of peaks; however, a higher level of iodine substitution resulted in three peaks eluting at this position (data not shown).

Each of the three sets of peaks in Figure 1 was individually pooled, and the thrombin inhibition properties were compared with unreacted hirudin. No attempt was made to separate the three sets of peaks into their individual components. Preliminary data (not shown) indicated that iodine substitution caused a significant reduction in the binding constant of r-hirudin for thrombin (described below). Due to the absence of free cysteines in native hirudin, the reaction product distribution probably represents various combinations of monoiodo- and diiodotyrosine and unsubstituted tyrosine at positions

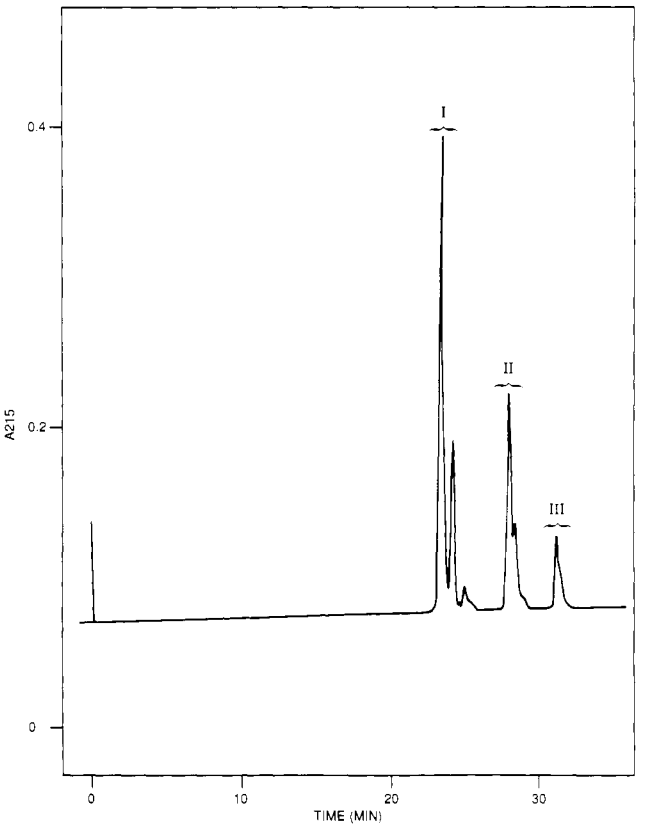

FIGURE 1: Separation of reaction products formed by iodination of r-hirudin. Products resulting from iodination of 5.7 nmol of r-hirudin with 4.6 nmol of NaI by the Enzymobead technique were separated by reverse-phase HPLC on a 4.6 by 250 mm Vydac C-4 300-Å column. The eluent was 0.065% (v/v) trifluoroacetic acid in a linear gradient of acetonitrile from 15 to 30% at a rate of 0.5% acetonitrile/min at a flow rate of 2.0 mL/min. Pools I–III, which contain incompletely resolved peaks, were combined as shown and further characterized by thrombin inhibition properties, as described in the text.

3 and 63, and possibly monoiodo- and diiodohistidine-51 and unsubstituted His-51.

Nitration of r-Hirudin. We attempted to simplify the complexity of the reaction products by evaluating the effect of another tyrosine-specific chemical modification reaction. Tetranitromethane was used to selectively introduce a single nitro group onto accessible tyrosines. Figure 2 shows a typical reverse-phase HPLC separation of tetranitromethane-reacted r-hirudin. Control reactions lacking protein indicated that the two early peaks (at the far left) are from tetranitromethane. Peak I is unreacted r-hirudin based on its retention time and the absence of absorbance at 360 nm (specific for nitrotyrosine); peaks II, III, and IV are nitrated r-hirudin because they have altered retention times and absorbance properties at 360 nm characteristic of nitrotyrosine. Peaks II and III have approximately equal 360:215 absorbance ratios, suggesting similar levels of nitro substitution. Peak IV has a higher absorbance ratio (360:215 nm), suggesting that it is nitrated at both Tyr-3 and Tyr-63.

The kinetics of product formation were assessed by plotting peak area as a function of tetranitromethane concentration at a fixed level of r-hirudin (Figure 3). Production of peaks II and III is approximately parallel throughout the range of tetranitromethane concentrations tested and exceeds peak IV production up to the highest level tested (200:1 tetranitromethane:r-hirudin ratio), at which point peak IV becomes the predominant product. These results suggest that production of peak IV is dependent on prior formation of either or both of peaks II and III. Therefore, evidence from both reaction

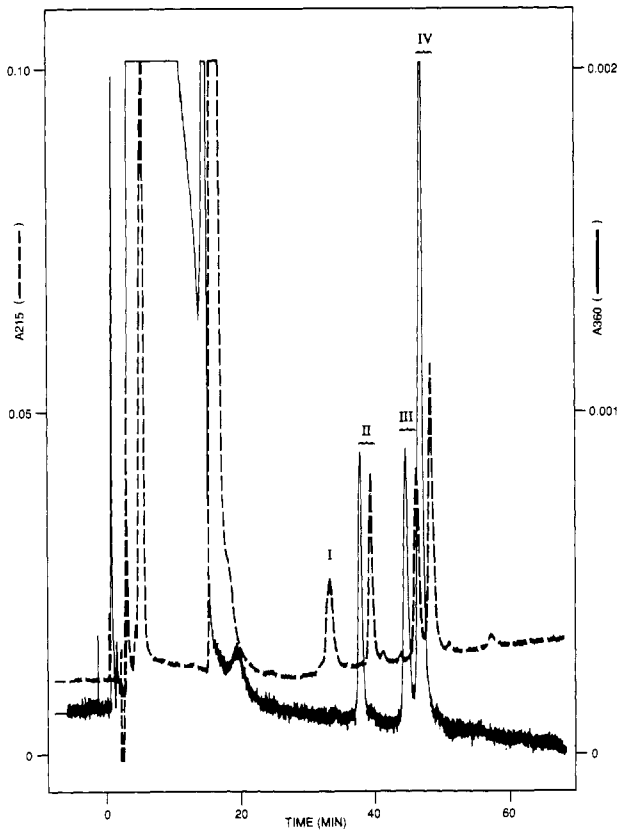

FIGURE 2: Separation of reaction products formed by nitration of r-hirudin. The products resulting from nitration at pH 8 of 2.9 nmol of r-hirudin with a 200-fold molar excess of tetranitromethane were separated by reverse-phase HPLC on a Vydac C-4 column. The eluent was 0.065% trifluoroacetic acid in a linear gradient of acetonitrile from 15 to 30% at a rate of 0.25% acetonitrile/min at a flow rate of 2.0 mL/min. Absorbance was monitored at 215 and 360 nm. The time scale corresponds to the A_{215} trace; the A_{360} trace is offset by 1.6 min. The identities of peaks I–IV were established by amino acid composition and N-terminal sequence analysis, and were found to correspond to (I) unmodified r-hirudin, (II) nitro-Tyr-3 r-hirudin, (III) nitro-Tyr-63 r-hirudin, and (IV) dinitro-Tyr-3,Tyr-63 r-hirudin. The absorbance at 360 nm is characteristic of 3-nitrotyrosine under acidic conditions; consequently, no absorbance at 360 nm is observed for unmodified r-hirudin (peak I).

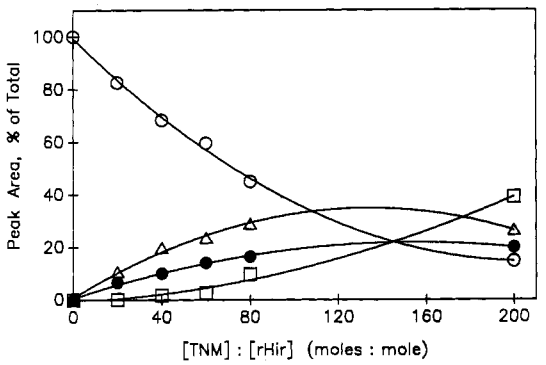

FIGURE 3: Nitration kinetics of r-hirudin as a function of tetranitromethane concentration. Peak I [unmodified r-hirudin] (○); peak II [r-hirudin(nitro-Tyr-3)] (●); peak III [r-hirudin(nitro-Tyr-63)] (△); peak IV [r-hirudin(dinitro-Tyr-3,Tyr-63)] (□).

kinetics and the ratios of absorbance at 360 to 215 nm indicates that peaks II and III are mononitrated forms of r-hirudin and that peak IV is nitrated on both tyrosine residues. Furthermore, these results indicate the difficulty of selective modification of either tyrosine since the two residues exhibit similar reaction kinetics.

N-Terminal sequence analysis of nitrated r-hirudin demonstrated that peaks II and IV contain only 3-nitro-L-tyrosine at position 3 whereas Tyr-3 of peak III is unmodified; amino acid composition analysis confirmed that 3-nitro-L-tyrosine in peaks II and III is 48% and 46%, respectively, of total tyrosine, close to the maximum theoretical value of 50% for a protein nitrated on only one of the two available tyrosine residues. Peak IV contains 100% 3-nitro-L-tyrosine. In summary, our findings indicate that peak II is nitrated at Tyr-3 but not Tyr-63, peak III is nitrated at Tyr-63 but not Tyr-3, and peak IV is nitrated on both tyrosines.

Table I: Influence of r-Hirudin Nitration on the Inhibition Constant (K_i) for Human α-Thrombin at Low and High Ionic Strength

r-hirudin nitration	K_i (fM)[a]	
	I = 0.12	I = 0.52
none	240 (100)	1100 (100)
nitro-Tyr-3	3000 (1200)	6800 (600)
nitro-Tyr-63	76 (32)	550 (48)
nitro-Tyr-3,Tyr-63	360 (150)	2600 (230)

[a] Assays were performed at 200 μM S-2238 in 50 mM Tris, 0.1% PEG 6000, 100 mM NaCl at ionic strength (I) = 0.12, or 500 mM NaCl (I = 0.52), pH 7.8, and were initiated with 200 pM thrombin. Data were analyzed as described under Experimental Procedures under Tight-Binding Inhibition Analysis (I = 0.12) and Slow Tight-Binding Inhibition Analysis (I = 0.52). Values in parentheses are percentages relative to unmodified r-hirudin tested at the same ionic strength. Standard deviations for binding constants ranged from 2% to 21% of the mean values.

Table II: Influence of Nitration on Apparent Inhibition Parameters of r-Hirudin for Human α-Thrombin Determined at 0.52 Ionic Strength

r-Hir nitration	apparent binding and rate constants[a]		
	K_i' (M × 10^{12})	k_{on}' [1/(M·s) × 10^{-6}]	k_{off}' (1/s × 10^6)
none	74 (100)	6.1 (100)	450 (100)
nitro-Tyr-3	440 (600)	4.9 (81)	2180 (480)
nitro-Tyr-63	36 (48)	14.0 (230)	500 (110)
nitro-Tyr-3,Tyr-63	170 (230)	11.0 (180)	1900 (410)

[a] Reaction conditions were as described in Table I for ionic strength = 0.52. Results were analyzed as described under Experimental Procedures under Slow Tight-Binding Inhibition Analysis. Standard deviations for binding and rate constants ranged from 2% to 17% of the mean values.

Thrombin Inhibition Analysis of Nitrated r-Hirudin. Table I compares inhibition constants for the different nitrated forms of r-hirudin at two ionic strengths, 0.12 and 0.52. As expected, high ionic strength reduces the binding of r-hirudin and all modified forms of r-hirudin to thrombin. At either ionic strength, r-hirudin(nitro-Tyr-63) binds thrombin more tightly than does nonnitrated r-hirudin, whereas binding of r-hirudin(nitro-Tyr-3) to thrombin is reduced.

Figure 4 shows the reaction progress curves for the slow-binding inhibition analysis of nitrated r-hirudin. Table II summarizes apparent association and dissociation rate constants (k_{on}', k_{off}') calculated from the progress curves determined at high ionic strength. The reduction in K_i for r-hirudin(nitro-Tyr-63) is almost entirely attributable to the 2.3-fold increase in k_{on}'; the apparent dissociation rate constant (k_{off}') was largely unaffected by nitration at this position. For r-hirudin(nitro-Tyr-3), the major cause of the large increase in K_i compared to unmodified r-hirudin is the 4.8-fold increase in k_{off}'.

r-Hirudin(dinitro-Tyr-3,Tyr-63) behaves as a hybrid between the two singly substituted r-hirudin analogues with respect to changes in K_i', k_{on}', and k_{off}'. The increase in its association rate due to nitration at Tyr-63 is more than offset by the increase in the dissociation rate caused by nitration at

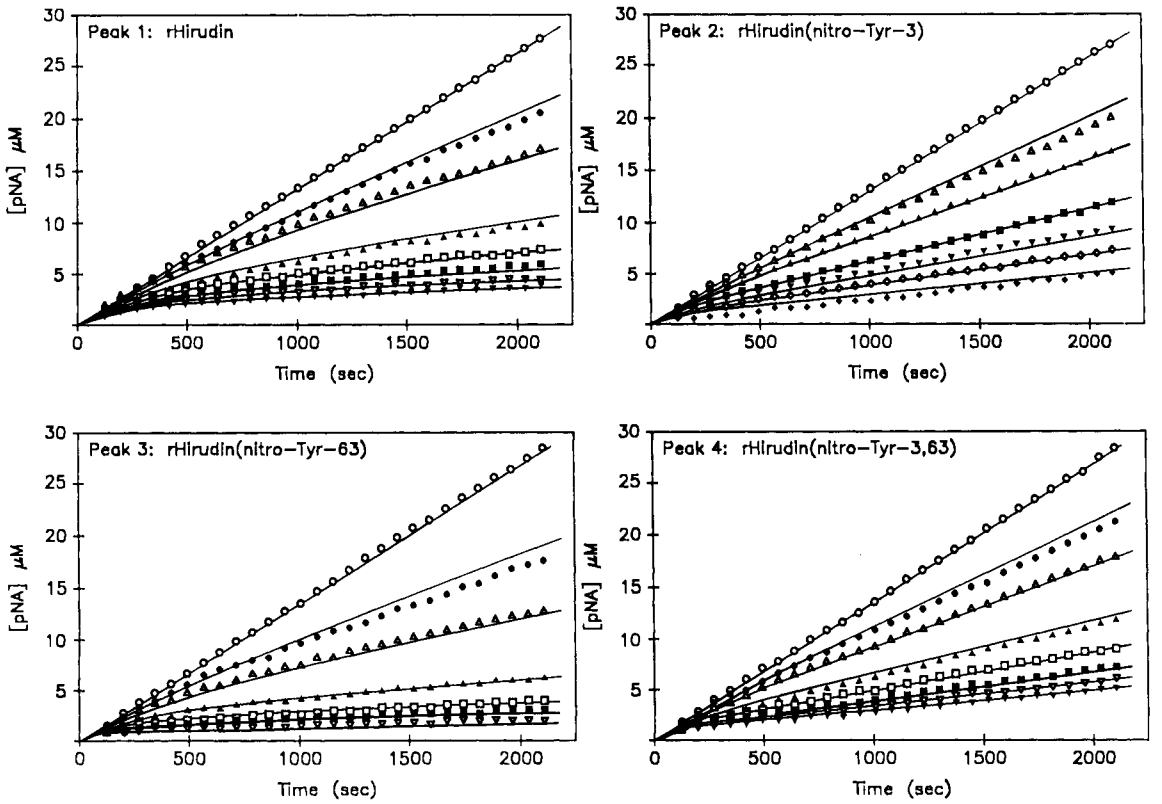

FIGURE 4: Slow-binding inhibition of thrombin by nitrated recombinant hirudin. Reactions were initiated by addition of 200 pM thrombin to microtiter wells containing hirudin and 200 μM S-2238 substrate, 50 mM Tris, 0.5 M NaCl, and 0.1% PEG, pH 7.8, in a total volume of 0.1 mL. Hirudin concentrations are as follows: 0 (○); 50 pM (●); 100 pM (△); 200 pM (▲); 300 pM (□); 400 pM (■); 500 pM (▽); 600 pM (▼); 800 pM (◇); 1000 pM (◆).

Tyr-3, resulting in a 2.3-fold increase in K_i'.

Chemical Modification of r-Hirudin Mutants. Mutants of r-hirudin with Phe or Trp substituted for Tyr-3 were constructed in an attempt to increase the specificity of the chemical modification for Tyr-63. Because each mutant has only a single reactive tyrosine (Tyr-63), nitration of both r-hirudin(Phe-3) and r-hirudin(Trp-3) produced only single homogeneous products that were readily purified by reverse-phase HPLC (data not shown). Amino acid composition analysis confirmed that tyrosine of both r-hirudin mutants is 100% nitrated.

In the case of the iodination reaction, which normally produces several iodinated products, we were able to identify the modified r-hirudin because higher levels of chemical substitution cause increased retention times on reverse-phase HPLC (data not shown). Upon iodination of r-hirudin(Phe-3), we recovered three reaction products designated I-1-, I-2- and I-3-r-hirudin(Phe-3), in order of increasing retention time. Because tyrosine is more readily iodinated than histidine, the I-1 and I-2 derivatives are most likely to be mono- and di-iodinated, respectively, at the 3- or 3,5-positions on the phenyl ring of tyrosine-63; I-3-r-hirudin(Phe-3), in addition to two iodo groups on tyrosine-63, may be further iodinated on His-51. For r-hirudin(Trp-3), only two products were generally observed, designated I-1- and I-2-r-hirudin(Trp-3). However, after longer reaction times, we also observed a third derivative designated I-3-r-hirudin(Trp-3), but we did not further analyze this form.

Table III presents the thrombin inhibition constants for wild-type r-hirudin and chemically modified and unmodified mutant proteins. Comparisons of mutant and wild-type r-hirudin demonstrate that both mutations cause approximately 2-fold decreases in K_i relative to wild-type r-hirudin (t test, $p < 0.01$). The binding constant for r-hirudin(Phe-3) is significantly lower ($p < 0.05$) than that of r-hirudin(Trp-3). Both iodination and nitration cause further significant decreases in the binding constants for both mutants. In the case of modified r-hirudin(Phe-3), the I-2 derivative binds thrombin more tightly than either the I-1 form ($p < 0.01$) or the I-3 form ($p < 0.05$).

Table III: Influence of Mutations at Position 3 and Chemical Modification of Mutants on the Inhibition Constant (K_i) of Recombinant Hirudin for Human α-Thrombin

hirudin	chemical modification	K_i (fM)[a] (SD)	N[b]
r-hirudin(Tyr-3)	none	319 (44.1)	16
r-hirudin(Phe-3)	none	126 (12.8)	5
r-hirudin(Phe-3)	I-1	81 (20.8)	9
r-hirudin(Phe-3)	I-2	54 (11.0)	7
r-hirudin(Phe-3)	I-3	70 (13.3)	6
r-hirudin(Phe-3)	nitration	68 (9.1)	5
r-hirudin(Trp-3)	none	165 (28.4)	5
r-hirudin(Trp-3)	I-1	93 (7.3)	5
r-hirudin(Trp-3)	I-2	66 (21.6)	10
r-hirudin(Trp-3)	nitration	66 (15.5)	10
leech[c]	none	98 (17.5)	5

[a] Reaction conditions were as described in Table I with ionic strength = 0.12. Results were analyzed as decribed under Experimental Procedures under Tight-Binding Inhibition Analysis. [b] N is the number of independent determinations. [c] Data shown are for Biopharm leech hirudin; an identical K_i was obtained for Sigma leech hirudin.

Compared to the singly and multiply iodinated forms of r-hirudin(Phe-3), the nitrated derivative has an intermediate binding constant. These apparent differences are not statistically significant ($p > 0.05$) in comparing the nitrated form with I-1 or I-3 ($p > 0.05$), but the K_i of I-2-r-hirudin(Phe-3) is significantly lower than that of nitro-r-hirudin(Phe-3) ($p < 0.05$). For r-hirudin(Trp-3), the binding constants for both the I-2 and nitro derivatives are lower than for the I-1 form ($p < 0.05$), but they do not differ from one another. No

differences were detected between the two mutations in r-hirudin following chemical modification of a specific type [e.g., comparing I-1-r-hirudin(Phe-3) to I-1-r-hirudin(Trp-3)].

The data presented in Table III demonstrate a 3.3-fold lower binding constant for leech hirudin than for wild-type r-hirudin, which is in reasonable agreement with previous reports (Stone & Hofsteenge, 1986; Dodt et al., 1988). However, the binding constant for the I-2-r-hirudin(Phe-3) mutant is reduced approximately 2-fold ($p < 0.01$) below that of leech hirudin.

Discussion

The combination of a single amino acid substitution for Tyr-3 and chemical modification of Tyr-63 produces r-hirudin derivatives with up to 6-fold lower inhibition constants for human α-thrombin in comparison to unmodified r-hirudin. Substitution of Tyr-3 with Phe or Trp independently lowers the K_i of r-hirudin and prevents the formation of undesirable reaction products arising from nitration that reduce the affinity of the modified r-hirudin for thrombin. This makes it possible to perform tyrosine-specific chemical modification reactions quantitatively using inexpensive reagents and simple reaction conditions that result in high yields of the chemically modified r-hirudin mutants.

Since tyrosine is conserved at position 3 in all 20 hirudin variants described to date (Scharf et al., 1989), it is surprising that Trp and Phe substitutions actually enhance activity. The importance of an aromatic residue at position 3 is supported by the fact that Thr substitution of Tyr-3 results in a 450-fold increase in K_i while multiple modifications of residues 1–5 can result in nearly complete loss of thrombin inhibition activity (Lazar et al., 1991). These results suggest that the amino-terminal segment of hirudin plays a very important role in the interaction of hirudin with thrombin, a conclusion that was previously inferred from molecular modeling studies of the thrombin–hirudin complex suggesting the possible interaction of the amino-terminal region of hirudin with the apolar-binding/active-site region of thrombin (Johnson et al., 1989). The recent crystal structure determination of human thrombin complexed with H-D-Phe-Pro-Arg chloromethyl ketone (Bode et al., 1989) indicates that the Phe side chain of this inhibitor occupies the S3 specificity site within the apolar-binding site formed by Ile-174, Trp-215, Leu-99, His-57, Tyr-60A, and Trp-60D. This result, together with our molecular modeling data, indicates that Tyr-3 of hirudin also may occupy this position in the hirudin–thrombin complex and, therefore, corresponds to the P3 residue of thrombin peptide substrates. Thus, the decrease in affinity of r-hirudin(nitro-Tyr-3) for thrombin (Table I) may result from disruption of the hydrophobic interaction of Tyr-3 with the apolar-binding site of thrombin due to the increased polar character of nitrotyrosine. Furthermore, the greater affinity of r-hirudin(Phe-3) for thrombin compared to r-hirudin(Tyr-3) is consistent with the fact that Phe-8 is the P3 residue of fibrinogen (Ni et al., 1989), a natural substrate of thrombin.

The discovery that either iodination or nitration of r-hirudin(Tyr-63) enhances binding to thrombin (Table III) is interesting in light of the findings that the absence of the sulfate moiety in r-hirudin or its removal from Tyr-63 in leech hirudin results in a 3–10-fold increase in K_i (Table III; Stone & Hofsteenge, 1986; Dodt et al., 1988). Thus, sulfatotyrosine-63 is implicated in forming an ionic interaction within the region of the anion-binding exosite of thrombin along with several other acidic groups in the carboxy-terminal region of hirudin (Stone et al., 1987; Owen et al., 1988). We suggest that the introduction of an electrophilic ortho substituent on the tyrosine ring, which lowers the pK of the hydroxyl group and results

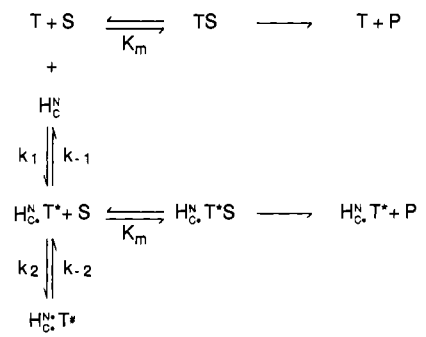

FIGURE 5: Mechanism of thrombin inhibition by hirudin. The following designations represent reaction components: T, thrombin; T*, altered conformational form of thrombin induced by the binding of C-terminal region of hirudin; T#, altered conformational form of thrombin in the tight-binding complex; S, chromogenic substrate; P, hydrolysis products of S; $H_C^N T$ and $H_C^N T$, complexes of thrombin with the C- and N-terminal regions of hirudin, respectively.

in an increased negative charge on the Tyr-63 at neutral pH, effectively mimics the acidic sulfatotyrosine residue in leech hirudin. The pK's of monoiodo- and diiodotyrosine are 8.2 and 6.4, respectively (Edelhoch, 1962), whereas 3-nitrotyrosine has a pK between 6.8 and 7.0 (Means & Feeney, 1971), compared to 10.1 for tyrosine. Thus, the ordering of the pK's of modified tyrosines (I-2-Tyr < nitro-Tyr < I-1-Tyr < Tyr) is the same as the order we observed in binding constants for the corresponding substituted r-hirudin mutants.

Iodination experiments of r-hirudin(Phe-3) indicated that, in addition to the formation of mono- and diiodo-Tyr-63, a third product (designated I-3) was formed that may represent iodination of His-51. Interestingly, I-3 was less readily formed in r-hirudin(Trp-3) (data not shown). This is consistent with the observation of others (Wolf & Covelli, 1969) that histidine is less effectively iodinated in proteins, such as chymotrypsin, containing tryptophan. Although the explanation for this is not clear, we have examined the chymotrypsin crystal structure and observed that two of the eight tryptophans are each within 10 Å of each of the two histidines (the remainder are between 17 and 28 Å distant). On the basis of our previous molecular modeling studies (Johnson et al., 1989), the distance between Trp-3 and His-51 may also be approximately 10 Å, suggesting that the proximity of tryptophan may affect the chemical reactivity of the histidine imidazole ring.

Hirudin inhibition of thrombin consists of two major steps (Figure 5). Step 1, described by k_1, involves both ionic (Stone et al., 1989) and hydrophobic (Owen et al., 1988) interactions between the thrombin anion-binding exosite and the C-terminal segment (C) of hirudin. Although it has been observed that the association rate constant, k_{on}, is in the range of diffusion-controlled reaction rates, the association rate does not appear to be, strictly speaking, diffusion-controlled since various small changes in the hirudin molecule such as Tyr-63 modification (which are unlikely to change the diffusion coefficient) clearly affect k_{on}. Since the association rate for hirudin inhibition of chromogenic substrate hydrolysis by thrombin is independent of substrate concentration (Stone & Hofsteenge, 1986), the proposed rate-limiting step of hirudin–thrombin association (k_1) does not involve binding of hirudin to the active site of thrombin. Formation of the initial thrombin–hirudin complex (k_1) appears to induce a conformational change in thrombin (T*) since binding of a C-terminal synthetic peptide fragment of hirudin alters thrombin's circular dichroism (Mao et al., 1988). The thrombin crystal structure (Bode et al., 1989) indicates the importance of two insertion loops, comprising the decapeptide containing residues 146–150 and the pentapeptide about residue 50, that help to

form the active-site/substrate-binding cleft and whose structure appears to restrict access to most small-protein inhibitors. The conformational change induced by binding the C-terminal region of hirudin may involve movement of the less rigid loop structure (146–150) to provide access to the amino-terminal region of hirudin. Step 2 (Figure 5), described by k_2, is a molecular rearrangement to form a tight-binding complex which may involve predominantly hydrophobic interactions between the active-site/apolar-binding site region of thrombin (with altered conformation, T#) and the N-terminal region (N) of hirudin.

For the dissociation of hirudin from thrombin, we propose that k_{-1}, not k_{-2}, is the rate-limiting step (Figure 5). When the active site of thrombin is available, substrate binding inhibits formation of a hirudin–thrombin tight-binding complex (since k_2 is not the rate-limiting step, there is no effect of substrate concentration on the forward rate). Increasing substrate concentration increases the concentration of the noninhibited intermediate (i.e., H^N_CTS, the substrate-binding form of the hirudin–thrombin complex), thus increasing the rate of dissociation of the hirudin–thrombin complex.

Nitration of Tyr-63 increases the apparent association rate constant (k_{on}') by 2.3-fold (Table II). This increase may be due to the increased negative charge on Tyr-63, which permits formation of an ion-pair interaction with thrombin, raising its affinity for the anion-binding exosite of thrombin and enhancing the rate-limiting step, k_1. The apparent dissociation rate constant (k_{off}') is not significantly changed.

Nitration of Tyr-3 results in a 5-fold increase in the apparent dissociation rate constant (k_{off}') compared to unmodified hirudin. The rate constant k_{-1} (Figure 5) may not be changed, but the rate is increased at least in part because of an increase in the concentration of the noninhibited hirudin–thrombin complex (H^N_CT*) complex. This is consistent with the proposal that placing a negative charge on Tyr-3 directly affects the stability of the tight-binding complex by destabilizing the hydrophobic interaction at the apolar-binding site. The result is a lower forward rate constant, k_2, which raises the concentration of the noninhibited hirudin–thrombin intermediate, thus increasing the rate of dissociation of free hirudin and thrombin.[1]

We have demonstrated that it is possible to construct r-hirudin analogues whose affinity for thrombin is higher than that of natural hirudin (Table III). Hirudin analogues with enhanced thrombin affinity may be particularly useful for therapeutic applications. It was recently shown for hirudin variant HV-2 that a 10-fold reduction in K_i was accompanied by a 100-fold reduction in the effective dose (ED_{50}) of hirudin necessary to inhibit clot formation in the rabbit Wessler venous thrombosis model (Degryse et al., 1989). An understanding of how hirudin–thrombin affinity can be increased will be important to maximize activity of other types of useful analogues that may have diminished activity compared to the native form. Hirudin analogues will have significant applications in cardiovascular medicine and as unique reagents for studying the function and mechanism of action of thrombin and its important bioregulatory role in hemostasis.

[1] In describing the association between two macromolecules such as hirudin and thrombin which involve multiple interacting regions, the distinction between experimentally determined rates and rate constants may be ambiguous. Measured rates may change due to a change in a rate constant at a rate-determining step, a change in the concentration of intermediates, or both. A change in rate constant implies an altered structure of the complex involved in the rate-determining step, while a change in the structure of a complex not involved in the rate-determining step could change the concentration of an intermediate and thus indirectly change the observed rate.

ACKNOWLEDGMENTS

We thank Debra Hudson and Ping Sze for their excellent technical assistance.

Registry No. Hirudin, 8001-27-2; thrombin, 9002-04-4.

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