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A new class of potent thrombin inhibitors that incorporates a scissile pseudopeptide bond

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A synthetic hirudin peptide analog corresponding to N^{α}-acetyl [D-Phe^{45}, ArgΨ(COCH$_2$)^{47}, Gly^{48}]desulfo hirudin$^{45-65}$ (P79) was synthesized. Comparative kinetic studies showed that while recombinant hirudin (HV2) is a slow-tight binding inhibitor, P79 behaves as a classical competitive inhibitor of human α-thrombin ($K_i = 3.7 \pm 0.3 \times 10^{-10}$ M) and bovine α-thrombin ($1.8 \pm 0.7 \times 10^{-9}$ M). P79 showed saturable inhibition of plasma APTT. The P_1'subsite of P79 is isosteric with the glycine residue of the natural thrombin substrate fibrinogen, but is proteolytically stable due to the incorporation of a ketomethylene pseudopeptide bond. The model active site-directed tripeptide [D-Phe-Pro-ArgΨ(COCH$_2$)CH$_3$COOCH$_3$, P79L] corresponding to the amino terminal of P79 also binds competitively to the active site of α-thrombin and inhibited the proteolysis of a tripeptidyl substrate with a $K_i = 17.9 \pm 2.1$ μM (human) and 10.3 ± 3.6 μM (bovine) α-thrombin. NMR experiments indicated that P79L and the corresponding amino terminal residues of P79 occupy a mutually exclusive binding site on bovine α-thrombin while the carboxyl terminal tail of the latter adopts a similar bound conformation as the fragment hirudin$^{55-65}$ which is known to interact with the 'anion' exosite. Taken together these results provide conclusive evidence that the high antithrombin activity of N^{α}-acetyl[D-Phe^{45}, ArgΨ(COCH$_2$)^{47}, Gly^{48}]desulfo hirudin$^{45-65}$ stems from the concurrent interaction with the catalytic site and the putative 'anion' exosite through its respective NH_2- and COOH-terminal recognition sites.

Hirudin analog; Thrombin inhibitor; Pseudopeptide; Active site; Exosite

1. INTRODUCTION

Thrombin is the principal enzyme that acts in concert with other activated factors of the hemocoagulative system to regulate clot formation in response to vascular tissue damage. Although α-thrombin is highly specific toward fibrinogen as its natural substrate, its structural homology to other serine proteases may preclude the development of specific and effective active-site directed inhibitors which could be useful to treat thrombotic disorders. Indeed a large number of synthetic or substrate-related inhibitors have been reported [1], but most of them suffer from various drawbacks such as low potency, poor selectivity or potential toxicity. The most potent inhibitor of α-thrombin known is hirudin [2,3], a 7 kDa family of isoproteins isolated from the glandular extracts of the leech *Hirudo medicinalis*. Hirudin forms a high affinity non-covalent stoichiometric complex with α-thrombin ($K_i = 10^{-11}$ to 10^{-14} M), but does not inhibit trypsin, kallikrein or other hemocoagulant serine proteases [4]. It is now known that the high affinity is due to the multiple interactions of hirudin with α-thrombin while the specificity stems from the interaction with at least one complementary binding site that is unique to α-thrombin [5,6]. This putative 'anion' exosite may also be required for fibrinogen binding.

We have previously demonstrated that desulfo-hirudin$^{45-65}$ corresponding to the carboxyl terminal fragment of recombinant hirudin variant 1 (r-HV1) can inhibit the amidolytic activity of human α-thrombin towards synthetic substrates [7]. Although the inhibition by this native fragment was weak and the mechanism partially competitive compared to r-hirudin, a modification of the NH_2-terminal residues by substituting D-Phe and Arg in positions 45 and 47 respectively, afforded an inhibitor with a $K_i = 2.8 \pm 0.9$ nM against human α-thrombin. The enhancement of enzyme affinity compared to the corresponding native fragment was attributed to favored interaction with the S_1 and S_3 subsites of the catalytic center while the C-terminal region comprising residues 55–65 could serve as an anchor that binds to the putative 'anion' exosite.

In this communication, we report that this model was used to obtain a new class of reversible inhibitors prototyped by P79 (Fig. 1) with high enzyme affinity and whose in vitro plasma antithrombin activity approaches the level of r-hirudin despite the drastically reduced

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Abbreviations: NOE, nuclear Overhauser enhancement; Boc-AOGO, 5*N*-*tert*-butyloxycarbonyl-4-oxo-8-tosyl guanidinooctanoic acid; TFA, trifluoroacetic acid; AMC, 7-amino-4-methyl coumarin; APTT, activated partial thromboplastin time

size. Further, on the basis of NMR measurements, we show that this inhibitor mimics the mechanism of hirudin by interacting concurrently with both the active site and the putative 'anion' exosite of α-thrombin.

2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant hirudin variant 2 (r-HV2), bovine and human α-thrombins, the fluorogenic and chromogenic substrates Tos-Gly-Pro-Arg-amc/Benzoyl-Phe-Val-Arg-p-nitroanilide were purchased from Sigma. APTT reagents were also obtained from Sigma. Protected amino acids and solvents for peptide synthesis were obtained from Applied Biosystems. Reagents for chemical synthesis were purchased from Aldrich.

2.2. *Peptide synthesis, purification and analytical methods*

N^{α}-acetyl[D-Phe^{45},Arg Ψ(COCH$_2$)^{47},Gly^{48}]desulfo hirudin$^{45-65}$ was synthesized on an Applied Biosystems 430A peptide synthesizer as described previously [7]. The synthetic amino acid N^{α}-Boc-L-Arg Ψ[COCH$_2$]CH$_2$COOH (Boc-AOGO) was prepared by a multistep procedure that will be reported in detail elsewhere. This novel N^{α}-protected ketomethylene amino acid was obtained enantiomerically pure, $[\alpha]^{25}$ = +18 (c = 2,MeOH), and was incorporated into the polymer-bound peptide using a standard coupling procedure mediated by dicyclohexylcarbodiimide/N-hydroxybenzotriazole. The crude peptide obtained following hydrogen fluoride treatment was purified to homogeneity in one step using reverse phase chromatography on a Vydac octadecyl silica glass column as described previously [7]. The peptide was more than 98% pure as judged by analytical HPLC on a Vydac 5 μm, C_{18} column using UV detection at 215 nm. Alternatively, Boc-AOGO could first be methylated using diazomethane, deblocked with trifluoroacetic acid and subsequently incorporated as the tripeptide N^{α}-Cbz-(D)-Phe-Pro-Arg(Tos)Ψ[COCH$_2$]CH$_2$COOCH$_3$ using standard solution methods and purification on silica gel. The blocked tripeptide was treated with HF as described above and lyophilized. For acetylation, the crude peptide was redissolved in 1 ml of cold sodium phosphate buffer, pH 7.5, and treated with 100 μl of acetic anhydride and the mixture maintained at 0°C for one hour [8]. The solution was diluted with water, frozen in a dry ice/acetone bath and lyophilized. The product was purified as described above. The resulting tripeptide is designated as P79L (Fig. 1). Amino acid analysis was performed on a Beckman model 6300 amino acid analyzer following hydrolysis on a Waters Pico-Tag work station. Mass spectral analyses were conducted on a SCIEX API III spectrometer equipped with an ionspray inlet and were in agreement with calculated molecular weights.

2.3. *Assay of thrombin inhibition and data analysis*

Amidolytic assays for the synthetic inhibitors were carried out at 25°C in 0.1 M Tris-HCl buffer, pH 7.8 containing 0.1 M NaCl and 0.1% polyethyleneglycol (PEG 8000) using 35 pM enzyme and substrate concentrations ranging between 2.5 and 20 μM. Experiments were conducted on a Cary 2000 spectrophotometer in the fluorescence mode in the ratio (λ_{ex} = 383 nm, λ_{em} = 455 nm). Initial velocities were recorded following the addition of thrombin to a solution of substrate dissolved in 0.1 M Tris-HCl buffer, pH 7.8 and variable concentrations of inhibitor in the same buffer adjusted to final volume of 3 ml. Fluorescence intensities were calibrated with known concentrations of 7-amino-4-methyl coumarin. The data were analyzed by using the non-linear regression program RNLIN in the IMSL library [9] on a micro VAX 3500 computer. Data points were fitted to equation 1 describing competitive inhibition which was determined on the basis of combined Dixon and Lineweaver-Burk plots [10].

$$v = V_{max}S/K_m(1 + I/K_i) + S \quad (1)$$

The slow-tight binding inhibition of human α-thrombin by r-HV2 was evaluated using a fixed enzyme and substrate concentration of 20 pM and 25 μM, respectively. A set of progress curves were obtained for several hirudin concentrations ranging from 20 to 70 pM. The resulting data points were fitted by non-linear regression analysis to equation 2 [11–13] where P is the concentration of product formed at time t. These analyses yielded values for K_i, association rate constant k_1 and dissociation rate constant k_2.

$$P = V_s t + \frac{(v_0 - v_s)(1-\gamma)}{k\gamma} \cdot \ln\left(\frac{1-\gamma e^{-kt}}{1-\gamma}\right) \quad (2)$$

2.4. *Activated partial thromboplastin time*

The anticoagulant activity of r-HV2 and synthetic peptides was evaluated spectrophotometrically on a Varian DMS 90 spectrophotometer at 405 nm. 50 μl of reconstituted citrated plasma control was mixed with 50 μl of thromboplastin solution at 37°C. This solution was treated with either 200 μl of Tris-HCl buffer, pH 7.8 containing 0.1 M NaCl, 0.1% PEG 6000 or variable concentrations of inhibitor in the same buffer. The clotting time was recorded following recalcification with 100 μl of 25 mM $CaCl_2$ solution. The average clotting time without inhibitors was 35 s.

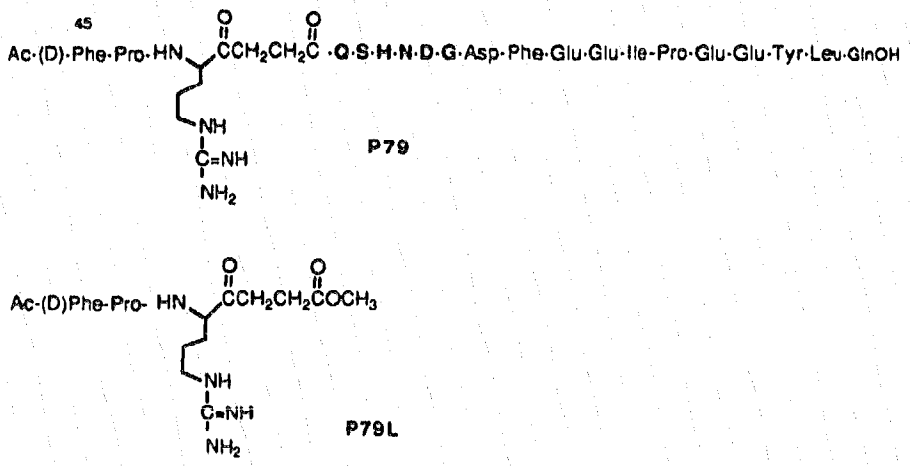


Fig. 1. Chemical structure of N^{α}-acetyl[(D)-Phe^{45},ArgΨ(COCH$_2$)^{47},Gly^{48}]desulfo hirudin$^{45-65}$ (P79) and P79L. The amino acids represented by the single letter code in P79 comprise the spacer that links the components of the catalytic site (left) and the auxiliary 'anion' exosite (right).

2.5. *NMR experiments*

The interaction of P79 and P79L (Fig. 1) with bovine α-thrombin was studied using both one- and two-dimensional NMR spectroscopy following the procedures described elsewhere [14]. For two-dimensional transferred NOE experiments, P79 was dissolved at 3 mM concentration in aqueous buffer (150 mM NaCl, 50 mM phosphate and 0.2 mM EDTA at pH 5.3) in the presence of 0.2 mM α-thrombin. For experiments involving P79L, the peptide concentration was 5 mM in the same aqueous buffer either in the absence or in the presence of 0.3 mM α-thrombin. For displacement experiments of P79L by P79, the peptide P79 was added to a preincubated mixture of α-thrombin and P79L to a final concentration of 0.6 mM (P79). NMR spectra were recorded on a Brüker AM-500 spectrophotometer and spectral analyses and plotting were carried out on a Brüker Aspect 3000 workstation.

3. RESULTS AND DISCUSSION

In the previous paper in this series we reported that the acetylated carboxyl terminal hirudin fragment comprising residues 45–65 inhibits both the fibrinogen clotting activity of α-thrombin and the amidolytic activity of the enzyme toward tripeptidyl substrates (K_i = 110 ± 30 nM, human α-thrombin). This result is at variance with other reports [15] and with recent X-ray structure determinations of the complex between human α-thrombin and either r-HV2-Lys^{47} [5] or r-HV1 [6] which clearly demonstrate that hirudin binds in the region of the catalytic cleft through its NH_2-terminal three residues. By inference, deletion of residues 1–44 could expose new recognitive elements within the thrombin substrate-like sequence consisting of residues Thr-Pro-Lys-Pro whose spatial disposition permits interaction with the catalytic site and substitute for the native NH_2-terminal residues of intact hirudin, albeit not as effectively. The probability of this mechanism is corroborated by our previous observation showing that substituting Thr^{45} and Lys^{47} by (D)Phe and Arg, respectively, confers potent and competitive inhibitory activity (P53, K_i = 2.8 ± 0.9 × 10^{-9}, human α-thrombin). The present results demonstrate that a significant improvement in potency and stability is achieved by appropriate modification of the scissile bond and structure of the P_1' subsite of P53.

Reasoning by analogy with fibrinogen, which incorporates a glycine residue at P_1', we have successfully incorporated a ketomethylene surrogate [16] into P53 in order to mimic the scissile Arg-Gly peptide bond in the natural substrate fibrinogen. This new structural prototype (P79) N^{α}-acetyl[D-Phe^{45},ArgΨ(COCH$_2$)^{47}, Gly^{48}]desulfo hirudin$^{45-65}$ (Fig. 1) competitively inhibits the proteolysis of the tripeptidyl substrate Tos-Gly-Pro-Arg-amc with K_i = 3.7 ± 0.3 × 10^{-10} M (Fig. 2A) and 1.8 ± 0.7 × 10^{-9} M for human and bovine α-thrombins, respectively. These values represent up to 450-fold increased affinity for the enzyme compared to the original native fragment N^{α}-acetyl desulfo hirudin$^{45-65}$ described previously [7], but still approximately 1000-fold lower than intact recombinant hirudin (r-HV2). Under the present experimental conditions r-HV2 behaved as a slow tight-binding inhibitor (K_i = 0.28 ± 0.07 pM) (Fig. 2A) in agreement with previous studies [8,13] that have implicated a probable two-step mechanism for hirudin binding. In contrast to hirudin, P79 did not display tight binding inhibition and the progress curves for product formation were linear over time. Despite the drastically reduced size compared to hirudin, amidolytic experiments using trypsin showed no inhibition up to concentrations of 50 μM of P79, thus retaining the specificity conferred by the fibrinogen recognition site as is the case with intact hirudin. Expectedly, incubation of P79 with human α-thrombin over 24 h at 37°C resulted in no

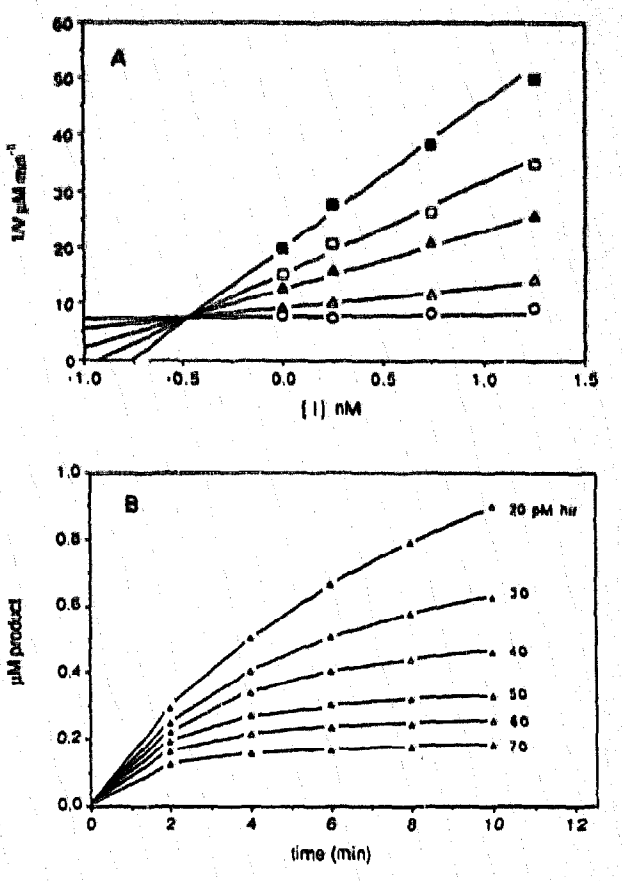


Fig. 2. (A) Dixon plot of the inhibition of human α-thrombin-mediated hydrolysis of Tos-Gly-Pro-Arg-AMC by N^{α}-acetyl[(D)-Phe^{45},ArgΨ(COCH$_2$)^{47},Gly^{48}]desulfo hirudin$^{45-65}$ (P79). The K_m for the substrate was determined to be 3.8 and 5.5 μM for human and bovine α-thrombin, respectively. Assays were performed as described in the experimental section in the presence of 2.5 (■), 3.5 (□), 5 (▲), 10 (△) and 20 μM (○) substrate. The calculated K_i values for human and bovine α-thrombins were: P79, 3.7 ± 0.3 × 10^{-10} M and 1.8 ± 0.7 × 10^{-9} M; P79L, 17.9 ± 2.1 × 10^{-6} and 10.3 ± 3.6 × 10^{-6} M. (B) Slow-binding inhibition of human α-thrombin by r-hirudin (r-HV2). The conditions were as described under the experimental section in 100 mM Tris buffer, pH 7.8 containing 100 mM NaCl. Enzyme and substrate concentrations were fixed at 20 pM and 25 μM, respectively. The determined association and dissociation rate constants were 1.9 ± 0.2 × 10^{8} $M^{-1}\cdot s^{-1}$ and 5.3 ± 1.4 × 10^{-4} s^{-1} respectively with a corresponding K_i = 2.8 ± 0.7 × 10^{-13} M.

observable proteolysis in contrast to its progenitor N^{α}-acetyl[D-Phe^{45},Arg^{47}]desulfo hirudin$^{45-65}$ (P53) which was slowly hydrolyzed on the carboxyl side of Arg^{47} [7]. Since thrombin-mediated proteolysis of P53 was not a factor in the time frame of kinetic measurements, we conclude that the enhanced enzyme affinity of P79 compared to P53 could be a consequence of a preferred conformational effect at P_1' or greater polarization of the ketone versus the amide function in the catalytic site. Fig. 3 shows that P79 prolongs the activated partial thromboplastin time of normal human plasma in a concentration dependent manner. Compared to hirudin, both P79 and P53 cause a saturable effect on APTT. However, the clotting time at the saturating level of P79 was more than double that of P53 over the same concentration ranging from 0.2 to 0.6 μg/400 μl.

The interaction of thrombin with the active site directed component of P79 was investigated by evaluating the inhibitory activity and identifying the enzyme-induced broadening of the NMR proton resonances of the model tripeptide N^{α}-acetyl-(D)-Phe-Pro-ArgΨ(COCH$_2$)CH$_2$COOCH$_3$ which is designated P79L and is structurally related to the active site alkylating agent PPACK [17]. P79L inhibited the hydrolysis of the fluorogenic substrate Tos-Gly-Pro-Arg-amc mediated by human α-thrombin competitively with a K_i = 17.9 ± 2.1 μM but was slightly more effective against the bovine isozyme (K_i = 10.3 ± 3.6 μM) suggesting subtle structural differences also in the respective active site of these two enzymes. The sequence denoted by amino acids using the single letter code in Fig. 1 represents the region that we have designated as the 'spacer' required to bridge two remote binding sites [7]. Accordingly, the N-terminal residues to the left of the spacer can interact with the catalytic site while the C-terminal residues to the right of the spacer have recognitive elements characteristic of the fibrinogen groove or exosite [17,18]. The simultaneous occupancy of these two sites manifests synergistic inhibitory activity. We have previously found that the fragment corresponding to N^{α}-acetyl hirudin$^{55-65}$ doubles the fibrinogen clotting time of human α-thrombin at 0.7 μM, while the inhibitory dissociation constant for the fragment corresponding to hirudin$^{1-45}$ has been reported to be 0.3 μM [19]. Using these values, the expected K_D of hirudin would be in the order of 0.2 pM which is in agreement with the experimentally determined value of 0.28 ± 0.07 pM found in this study. On the other hand, the determined K_i value of P79 (0.37 ± 0.03 nM) deviates slightly from the expected value of 13 pM if the inhibitory dissociation constant were the simple product of the individual components as is the case with hirudin. This divergence could reflect the different binding mode of P79 with thrombin compared to intact hirudin whose hydrophobic NH$_2$-terminal residues interact with the putative apolar binding site in a direction opposite to what would be expected of a substrate and without occupying the specificity pocket [5,6]. Moreover, the 6-residue sequence that separates the catalytic site and 'anion' exosite in P79 may not be optimum compared to the large but compact conformationally-defined core of hirudin [20,21]. However the short spacer of P79 could be modified to further enhance enzyme affinity.

Fig. 4A shows the NMR proton spectrum of the aromatic region of P79L. The spectrum shows that the

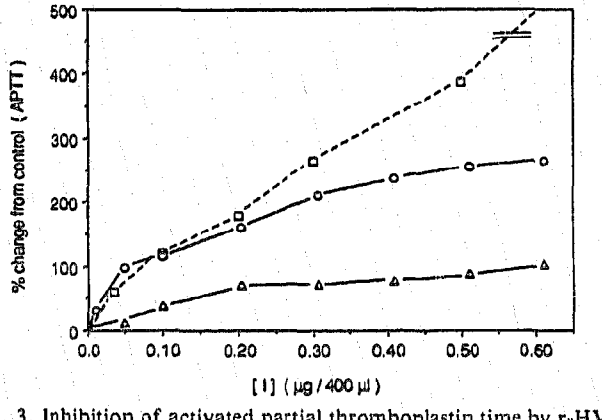


Fig. 3. Inhibition of activated partial thromboplastin time by r-HV2 (□), P79 (○) and P53 (△). The anticoagulant activity was measured as described in the methods using normal human plasma. Each point is the mean of three determinations.

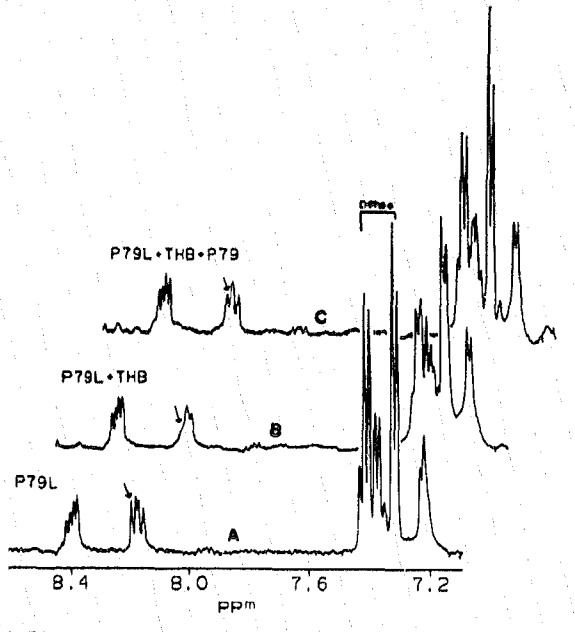


Fig. 4. NMR spectra of the NH and the aromatic region of P79L 5 mM in the absence (A) and in the presence (B) of 0.3 mM of bovine α-thrombin. Spectrum C shows the same region in the presence of 0.3 mM thrombin and 0.6 mM P79. The NH proton resonances of D-Phe and Arg are at 8.4 and 8.17 ppm, respectively, while the peaks between 7.2–7.5 ppm are the ring proton resonances of D-Phe. Note that only one of the NH doublets (indicated by the arrows) of Arg^{47} is broadened by thrombin binding (middle panel). This effect, together with the line broadening of the aromatic ring protons, was reversed upon addition of the bifunctional inhibitor, P79 (C).

Pro-Arg amide bond of the tripeptide exists in an equal population of *cis* and *trans* isomers as reflected by the splitting of the NH resonances (doublets of doublets) of D-Phe (8.4 ppm) and Arg (8.17 ppm). In the presence of thrombin (Fig. 4B), only one of the conformeric α NH protons of Arg was broadened by α-thrombin, while the αNH proton of the D-Phe residue was unaffected. Therefore while only the amide proton of the arginine residue is implicated in binding to thrombin, only one conformational isomer is compatible with the binding site. The most pronounced line broadening was observed for the resonance signals corresponding to the aromatic ring protons of (D)-Phe as illustrated in Fig. 4B. However, addition of P79 (0.6 mM) to a preformed complex of thrombin (0.3 mM) with P79L (5 mM) was found to reverse the thrombin-induced line broadening (Fig. 4C). This suggests that the NH_2-terminal moiety of P79 interacts with the same binding site as P79L, which would be expected to be the apolar region of the catalytic cleft due to its homology with the active site inhibitor PPACK.

Using NMR transferred NOE measurements, we have found that residues Glu^{61} to Gln^{65} in a peptide fragment corresponding to desulfo hirudin$^{55-65}$ form a helical structure when bound to bovine α-thrombin [13]. The resulting conformation orients the non-polar side chains of residues Phe^{56}, Ile^{59}, Pro^{60}, Tyr^{63} and Leu^{64} in a characteristic hydrophobic cluster in contact with thrombin. The NOEs for this structure of hirudin$^{55-65}$ were those between the αCH proton of Glu^{61} and the NH and side chain protons of Leu^{64} and between the side chain protons of residues Phe^{56}, Ile^{59}, Pro^{60}, Tyr^{63} and Leu^{64}. Similar transferred NOEs were also observed within residues Phe^{56} to Gln^{65} of the inhibitor P79 (Fig. 5), suggesting that the C-terminal portion of P79 binds to thrombin in a similar conformation as that of the peptide fragment hirudin$^{55-65}$. Furthermore, all of the resolved resonances of residues Asp^{55} to Gln^{65} in P79 are affected by thrombin binding to a similar extent as hirudin$^{55-65}$ (data not shown). These results indicate that residues Asp^{55} through Gln^{65} in P79 interact with the thrombin exosite similar to the peptide fragment derived from the C-terminal region. Taken together, the above results provide conclusive evidence that P79 behaves as a bifunctional inhibitor that interacts concurrently with the catalytic site and 'anion' exosite of thrombin analogous to hirudin.

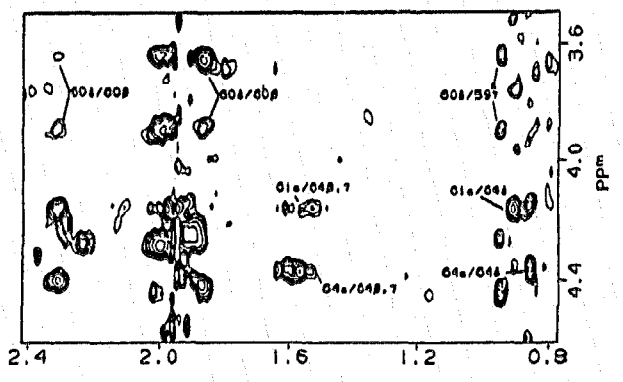


Fig. 5. A portion of the two-dimensional NOE spectrum of P79 (3 mM) in the presence of bovine α-thrombin (0.2 mM). The labels indicate the proton pairs that give rise to the particular NOE crosspeak(s). The NOEs between residues Glu^{61} and Leu^{64} ($61\alpha/64\beta,\gamma$ and $61\alpha/64\delta$), together with the NOE between the αCH proton of Glu^{61} and the NH proton of Leu^{64} (not shown), indicate that residues Glu^{61} through Leu^{64} form a helical conformation when P79 binds to thrombin. Other observed NOEs not displayed in the spectrum include those between the δCH protons of Phe^{56} and the δCH protons of Ile^{59}, between the γCH and the δCH protons of Ile^{59} and the δCH protons of Tyr^{63}, between δCH protons of Pro^{60} and the δCH protons of Tyr^{63} and between the δCH protons of Tyr^{63} and δCH protons of Leu^{64}.

N^{α}-acetyl[D-Phe^{45},ArgΨ($COCH_2$)^{47},Gly^{48}]desulfo hirudin$^{45-65}$ remains the most potent inhibitor of α-thrombin reported to date other than hirudin itself. The enhanced affinity for α-thrombin compared to the native congener N^{α}-acetyl desulfo hirudin$^{45-65}$, the analog N^{α}-acetyl[D-Phe^{45},Arg^{47}]desulfo hirudin$^{45-65}$ [7] or analogous hirulogs [22] can be attributed directly to (1) greater primary specificity and secondary recognition at S_1 and S_3 conferred by Arg^{47} and D-Phe^{45}, respectively and (2) the incorporation of a ketomethylene pseudopeptide bond which is isosteric with glycine in the scissile bond of the natural substrate. Inclusion of the ketomethylene function confers complete enzymatic resistance toward α-thrombin and would also be expected to increase the electrophilic properties of the carbon atom bearing the ketone group. Previously described hirulogs have been designated as non-competitive thrombin inhibitors [22]. It is interesting to note that the inhibition of α-thrombin by P79 is purely competitive with respect to the tripeptidyl substrate Tos-Gly-Pro-Arg-amc, but that the presence of a proline residue at P_1' has a modulating effect on the mechanism of inhibition [7]. Furthermore, the polyglycine spacer characteristic of hirulogs may contribute a subtle qualitatively different mode of interaction with thrombin that could affect the active site. A detailed structural analysis is in progress for a better understanding of the mode of binding of this new class of thrombin inhibitors.

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REFERENCES

[1] Stürzebecher, J. (1984) in: The Thrombin, Vol. 1 (Machovich, R. ed.), CRC Press, Chap. 7, pp. 131–160.
[2] Markwardt, F. (1970) Methods Enzymol. 19, 924–932.
[3] Bagdy, D., Barabas, E., Graf, L., Petersen, T.E. and Magnusson, S. (1976) Methods Enzymol. 25, 669–678.

[4] Seemüller, U., Dodt, J., Fink, E. and Fritz, H. (1986) In: Proteinase Inhibitors (Barrett, A.J., Salvesen, G. eds) Chap. 8, Elsevier.
[5] Rydel, T.J., Ravichandran, K.G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C. and Fenton, J.W. II (1990) Science 249, 277–280.
[6] Grutter, M.G., Priestle, J.P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J. and Stone, S. (1990) EMBO J. 9, 2361–2365.
[7] DiMaio, J., Gibbs, B., Munn, D., Lefebvre, J., Ni, F. and Konishi, Y. (1990) J. Biol. Chem. 265, 21698–21703.
[8] Wallace, A., Dennis, S., Hofsteenge, J. and Stone, S.R. (1989) Biochemistry 28, 10079–10084.
[9] IMSL (1987) IMSL Library Reference Manual, 9th edition version 1.0, IMSL, Houston.
[10] Segel, I.H. (1975) In: Enzyme Kinetics, Chap. 3, John Wiley, New York.
[11] Cha, S. (1976) Biochem. Pharmacol. 25, 2695–2702.
[12] Williams, J.W. and Morrison, J.F. (1979) Methods Enzymol. 63, 437–467.
[13] Stone, S.R. and Hofsteenge, J. (1986) Biochemistry 25, 4622–4628.
[14] Ni, F., Konishi, Y. and Scheraga, H.A. (1990) Biochemistry 29, 4479–4489.
[15] Krstenansky, J.L. and Mao, S.J.T. (1987) FEBS Lett. 211, 10–16.
[16] Almquist, R.G., Chao, W.R., Ellis, M.E. and Johnson, H.L. (1980) J. Med. Chem. 23, 1392.
[17] Bode, W., Mayr, I., Maumann, U., Hüber, R., Stone, S.R. and Hofsteenge, J. (1989) EMBO J. 8, 3467–3475.
[18] Chang, J.Y.C., Ngai, P.K., Rink, H., Dennis, S. and Schlaeppi, J.M. (1990) FEBS Lett. 261, 287–290.
[19] Chang, J.Y.C., Schlaeppi, J.M. and Stone, S.R. (1990) FEBS Lett. 260, 209–212.
[20] Clore, G.M., Sukumaran, D.K., Nilges, M., Zarbock, J. and Gronenborn, A.M. (1987) EMBO J. 6, 529–537.
[21] Folkers, P.J.M., Clore, G.M., Driscoll, P.C., Dodt, J., Köhler, S. and Gronenborn, A.M. (1989) Biochemistry 28, 2601–2617.
[22] Maraganore, J.M., Bourdon, P., Jablonsky, J., Ramachandran, K.L. and Fenton, J.W. II (1990) Biochemistry 29, 7095–7101.