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Proton Bridging in the Interactions of Thrombin with Hirudin and Its Mimics

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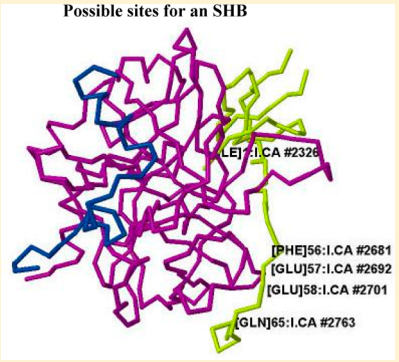
ABSTRACT: Thrombin is the pivotal serine protease enzyme in the blood cascade system and thus a target of drug design for control of its activity. The most efficient nonphysiologic inhibitor of thrombin is hirudin, a naturally occurring small protein. Hirudin and its synthetic mimics employ a range of hydrogen bonding, salt bridging, and hydrophobic interactions with thrombin to achieve tight binding with K_i values in the nano- to femtomolar range. The one-dimensional ^{1}H nuclear magnetic resonance spectrum recorded at 600 MHz reveals a resonance 15.33 ppm downfield from silanes in complexes between human α-thrombin and r-hirudin in pH 5.6−8.8 buffers and between 5 and 35 °C. There is also a resonance between 15.17 and 15.54 ppm seen in complexes of human α-thrombin with hirunorm IV, hirunorm V, an Nα(Me)Arg peptide, RGD-hirudin, and Nα-2-naphthylsulfonyl-glycyl-DL-4-amidinophenylalanyl-piperidide acetate salt (NAPAP), while there is no such low-field resonance observed in a complex of porcine trypsin and NAPAP. The chemical shifts suggest that these resonances represent H-bonded environments. H-Donor−acceptor distances in the corresponding H-bonds are estimated to be <2.7 Å. Addition of Phe-Pro-Arg-chloromethylketone (PPACK) to a complex of human α-thrombin with r-hirudin results in an additional signal at 18.03 ppm, which is 0.10 ppm upfield from the observed signal [Kovach, I. M., et al. (2009) *Biochemistry 48*, 7296−7304] for thrombin covalently modified with PPACK. In contrast, the peak at 15.33 ppm remains unchanged. The fractionation factors for the thrombin−hirudin complexes are near 1.0 within 20% error. The most likely site of the short H-bond in complexes of thrombin with the hirudin family of inhibitors is in the hydrophobic patch of the C-terminus of hirudin where Glu$^{57'}$ and Glu$^{58'}$ are embedded and interact with Arg^{75} and Arg^{77} and their solvate water (on thrombin). Glu$^{57'}$ and Glu$^{58'}$ present in the hirudin family of inhibitors make up a key binding epitope of fibrinogen, thrombin's prime substrate, which lends substantial interest to the short hydrogen bond as a binding element at the fibrinogen recognition site.



A key serine protease enzyme in blood clotting is α-thrombin.[1−8] Thrombin catalyzes the hydrolysis of one to four peptide bonds in more than a dozen large protein precursors operating in the blood cascade system.[6,7,9−14] Thrombin fulfills two strictly coordinated roles: procoagulant and anticoagulant. Five cofactors participate in substrate binding and the interconversion between the two catalytic states termed "fast" and "slow".[7,8,15−17] Cofactors bind to external sites distant from the active site of thrombin to exert a subtle allosteric effect implemented by a slight conformational change. As maintenance of the hemostatic balance has broad implications in human health, the regulation of human α-thrombin with a broad range of inhibitors has been a main target of investigations and drug design.[18−21]

Our interest in the regulation of thrombin function has been in the physicochemical interactions underlying the inhibitory power of certain thrombin effectors. Previously, we examined H-bonding interactions in the covalent adduct between human α-thrombin and PPACK mimicking the oxyanionic tetrahedral intermediate in the acylation step and phosphate and phosphonate ester adducts of thrombin resembling the anionic tetrahedral intermediate in the deacylation step in substrate hydrolysis, using kinetics and high-resolution, low-field ^{1}H nuclear magnetic resonance (NMR) signals.[22] Both types of tetracovalent adducts of thrombin, as many transition-state analogue adducts of enzymes with inhibitors, yield a unique resonance in high-resolution ^{1}H NMR spectra between 14 and 21 ppm downfield from silanes.[24−38] These low-field resonances have also been observed below pH 6 with some native enzymes that catalyze proton transfer.[23,24,34,36−38] The deshielding phenomenon had been attributed to the presence of a short, strong H-bond (SSHB) at the active site of the enzyme upon protonation of a key base catalyst (His), which occurs even at above pH 6 when the enzyme interacts with a covalent modifier. It has since been shown that the H-bond is most likely one formed between His^{57} δNH and Asp^{102} γO in serine proteases.[22,25−33,35,36,39] The stabilization of SSHBs has been attributed to electrostatic effects, charge, polarization, and

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resonance. Whereas H-bond donor and acceptor distances associated with the low-field resonances are typically between 2.4 and 2.7 Å and donor−H-acceptor angles are >150°, the unusual strength of the H-bonds described previously[40] has rarely been substantiated.[41−47] In recognition of this circumstance, the short H-bond (SHB) notation is used in this paper.

The proposal that the H-bond donor−acceptor distances across the catalytic triad contract during catalysis originates from the interpretation of solvent deuterium isotope effects on protease-catalyzed reactions.[48−56] The contraction of distances most likely lowers the activation barrier for nucleophilic attack on carbonyl at the reaction center in serine protease catalysis, as a quantum chemical calculation for RNase A catalysis shows.[40] As thrombin is a very efficient catalyst of the breakdown of its natural and analytical substrates, it also undergoes similar structural changes to stabilize the transition states for hydrolysis of its substrates. This notion is supported by solvent deuterium isotope effects observed between 2.5 and 3.5 in the hydrolysis of many thrombin substrates.[51,52]

We have recently posed a new question of whether SHBs exist in tight-binding interactions between enzymes and their cognate (large) substrates. If they do, tight-binding substrate analogue inhibitors may exhibit a signal in low-field ^{1}H NMR at high resolution. Members of the hirudin family of inhibitors mimic the propensity of binding of fibrinogen to thrombin. Among inhibitors, hirudin presents the greatest affinity for thrombin with a K_i of 20 fM.[7] Hirudin binds to thrombin in a parallel and nonsubstrate mode, which sets it apart from many peptide inhibitors. There are at least 13 intermolecular H-bridges in the X-ray structure of the α-thrombin−r-hirudin (variant 2, Lys^{47}) complex (2.3 Å resolution) interspersed with salt bridges and hydrophobic interactions.[57−59] Most of these interactions occur at the fibrinogen recognition site (FRS or exosite I) of α-thrombin. The FRS is a critical site for the allosteric regulation of α-thrombin catalysis and inhibition. The C-terminal section of hirudin is more ordered in the complex[57−59] than in the solution (two-dimensional) ^{1}H NMR structure of r-hirudin alone,[60−63] when bound to the FRS of α-thrombin. In addition to the intermolecular H-bonds formed in the complex, intramolecular H-bonds near the N-terminus of r-hirudin become shorter and numerous short H-bonds form with water.[58] Hirudin mimics,[64−66] which have most key binding elements of hirudin incorporated, became available in recent years. Comparative studies of thrombin complexes with hirudin mimics can guide the elucidation of the location of SHBs and enhance our understanding of the specific environment of the SHBs.

Herein, we report the observation and characterization of SHBs that human α-thrombin forms with the noncanonical r-hirudin, its mimics, and canonical (binding as antiparallel β-sheets) peptide inhibitors. The deuterium isotope effect on the SHBs and their robustness tested in pH dependence, Na^+ ion dependence, and temperature studies of the line width of the resonance are also discussed.

MATERIALS AND METHODS

Materials. All buffer salts were reagent grade and were purchased from either Aldrich, Fisher, or Sigma Chemical Co. Anhydrous DMSO, heavy water with 99.9% deuterium content, and anhydrous methanol were purchased from Aldrich Chemical Co. The proton sponge 1,8-bis(dimethylamino)-naphthalene, MUGB, and NAPAP at >93% purity (Fluka), porcine trypsin (Type IX, 24000 Da, >15000 BTA units/mg, 98% protein), and r-hirudin type 2 (lyophilized product, >95% pure) were from Sigma Chemical Co. r-Hirudin type 1 (lyophilized product, >95% pure) was from Centerchem Inc. H-D-Phe-Pip-Arg-4-nitroanilide-HCl (S-2238, 99%) (TLC) was purchased from Diapharma Group Inc. The inhibitor PPACK was purchased from BioMol Co. Human α-thrombin (36500 Da) was purchased either from Enzyme Research Laboratories Inc. with 3010 NIH units/mg activity in 0.05 M sodium citrate buffer (pH 6.5), 0.2 M NaCl, and 0.1% PEG-8000 or from Haematologic Technologies Inc. at 3664 NIH units/mg activity in 0.01 M sodium phosphate buffer (pH 6.5), 0.2 M NaCl, and 0.1% PEG-8000. r-RGD-hirudin (constructed by introducing RGD in place of ^{32}SDG^{34} in wild-type hirudin type 2) was a gift from W. Mo (Molecular Medicine, Ministry of Education, Fudan University, Shanghai, China),[60] and hirunorms IV and V were gifts from V. Pavone (University of Padova, Napoli, Italy).[21,64] D-Cha-Pro-Nα(Me)Arg-Thr-$(Gly)_5$-^{10}Asp-Tyr-Glu-Pro-Ile-Pro-$(Glu)_2$-Ala-Cha-^{20}D-Glu [Nα(Me)Arg peptide] was a gift from T. Steinmetzer (Institute of Pharmaceutical Chemistry, Philipps Universität, Marburg, Germany).[65,66]

Solutions. Buffer solutions were prepared from the appropriate analytical grade salts using doubly distilled deionized water. All buffers were further filtered using a 0.2 μm Nylon Membrane Filter. All measurements of pH were taken with a Delta electronic pH meter.

Instruments. ^{1}H NMR spectra of inhibited human α-thrombin were recorded with a Varian INOVA 600 MHz NMR instrument (Agilent Technologies, Santa Clara, CA) at the Department of Chemistry of Rutgers University and a 600 MHz BRUKER instrument at the Center for Biomolecular Structure and Organization of the Department of Chemistry and Biochemistry of the University of Maryland. Spectrophotometric measurements were performed with a Perkin-Elmer Lambda 6 or Lambda 35 UV−vis spectrophotometer connected to a personal computer. The temperature was monitored using a temperature probe connected to a digital readout device. Either a Neslab RTE-4 or a Lauda 20 circulating water bath was used for temperature control.

Thrombin Activity Assay. Assays were conducted in the absence or presence of inhibitors in a total volume of 1.0 mL with a 10 μL injection into each appropriate solution of the substrate and thrombin. Initial rates of hydrolysis of 3−5 × 10^{-5} M (>10K_m) S-2238 were measured by monitoring the release of 4-nitroaniline at 400 nm. Thrombin concentrations were then calculated from V_{max} values using a k_{cat} of 95 ± 20 s^{-1} in 0.02 M Tris buffer (pH 8.2) at 25.0 ± 0.1 °C.[51] The decline in thrombin activity was monitored for several hours after inhibition.

Trypsin Activity Assay. The inhibition of trypsin was monitored fluorometrically by the excitation of 4-methyl-umbelliferon at 365 nm and emission at 445 nm, as released from the active-site titrant MUGB in a stoichiometric amount with respect to trypsin upon reaction in 0.02 M Tris buffer (pH 8.2) at 25.0 ± 0.1 °C.

Low-Field ^{1}H NMR Measurements. The samples were prepared by mixing 0.2−0.5 mM human α-thrombin with a slight excess of r-hirudin or its analogues, the Nα(Me)Arg peptide and PPACK, and a > 5-fold excess of D-NAPAP in 0.05 M sodium citrate buffer (pH 6.5) or in 0.01 M phosphate buffer (pH 6.7), 0.2 M NaCl, and 0.1% PEG-8000. Identical concentrations and conditions were used for samples of human α-thrombin, r-hirudin, and hirunorm V alone. The D_2O content was ∼5−7% in general and ∼45−59% for the

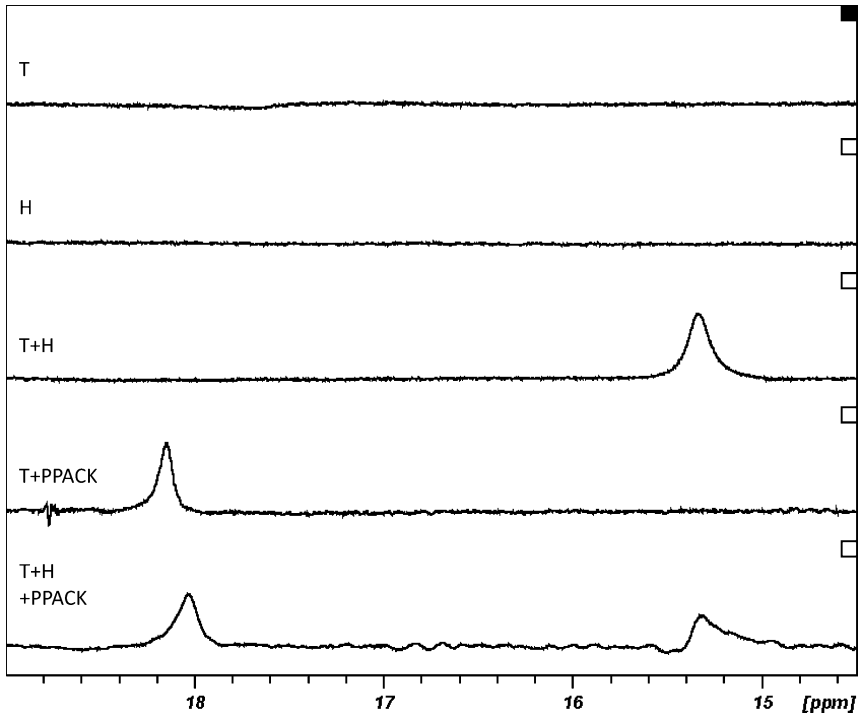

Figure 1. Low-field sections of 3000 transients of 600 MHz ^{1}H NMR spectra of human α-thrombin and its inhibited forms at pH 6.5 in 0.2 M NaCl and 0.01% PEG-800 at 30.0 ± 0.1 °C and 7% D_2O: T, human α-thrombin in 0.05 M citrate buffer; H, r-hirudin in 0.01 M phosphate buffer; T+H, r-hirudin-inhibited thrombin in 0.05 M citrate buffer; T+PPACK, PPACK-inhibited thrombin in 0.05 M citrate buffer; and T+H+PPACK, PPACK added to the thrombin−r-hirudin complex. The line width at half-height is 60 Hz for the thrombin−PPACK adduct and 98 Hz for the thrombin−hirudin complex.

isotope fractionation studies. The human α-thrombin−r-hirudin and RGD-hirudin complexes were also probed in 0.020−0.040 M citrate (pH 6.5) or 0.04−0.10 M phosphate buffer (pH 6.5) that contained between 0.09 and 0.31 M NaCl. Thrombin activity was measured before and after inhibition; 97 and 98% activity losses were observed with the Nα(Me)Arg peptide and NAPAP inhibition, respectively, and a >99% loss of activity was achieved with r-hirudin and its mimics.

Trypsin samples (1.8 mM) were made with and without an ∼3-fold excess of D-NAPAP in 0.10 M phosphate buffer (pH 7.00), 0.1% PEG-4000, and 7% D_2O. The level of inhibition was >70%.

D/H fractionation factors were measured from deshielded resonances at maximal sensitivity by dividing samples identically into two, one in buffered H_2O and the other in identically buffered D_2O. Initially, the integrated peaks were referenced to a CH_2 signal on thrombin, and NMR data analysis was performed in the Mestrelab Research software or using SpinWorks. This method was not always dependable; thus, all quantitative measurements were replicated with an external standard.

For the D/H fractionation experiment, 270 μL of a sample was transferred in a NMR micro tube (New Era Enterprises, Vineland, NJ). A concentric capillary that contained ∼60 μL of the external standard was inserted, the standard being a 1 mM CD_3CN solution of proton sponge 1,8-bis(dimethylamino)-naphthalene, which had been titrated with H_2SO_4. When using the BRUCKER spectrometer, water excitation was avoided by using a 1331-pulse sequence and a 90° pulse width of 30 μs was applied for a 512 ms acquisition time, including a 2.5 s relaxation delay. One-dimensional ^{1}H NMR spectra were acquired at controlled temperatures using the INOVA NMR spectrometer operating at a proton frequency of 599.7 MHz and equipped with a 5 mm triple-resonance HCN probe. Typically, 2K to 16K scans of 128K complex data points over a 25 kHz spectral width were collected, preceded by a relaxation delay of 2 s. All spectra were acquired with the carrier offset placed on the water resonance that was reduced by tailored excitation, using WATERGATE[67,68] with a typical 90° pulse width of 7 μs. The interpulse delay of the 3−9−19 WATERGATE block was set to 66 μs so that the spectral region of interest would be at the center of the excitation profile.

Data sets were processed on a Sun Blade 150 workstation (Sun Microsystems Inc., Palo Alto, CA) using the vnmrj software package (Agilent Technologies). To improve the signal-to-noise ratio and the definition of the SHB NMR peak, we applied a line broadening of 50 Hz followed by baseline correction. Spectra were referenced to the sponge standard that was referenced versus DSS at 0.00 ppm.

Fractionation factors (ϕ) were calculated from the integrated signals in ∼5−7 and ∼45−59% D_2O buffers as follows: $I = [I_{max}(X)]/[\phi(1 - X) + X]$, where X is the mole fraction of H_2O, I is the observed intensity, and I_{max} is the maximal intensity when $X = 1.0$.

The pH dependence of the resonance observed with the thrombin−r-hirudin sample was studied between pH 7.5 and 8.8 by elevating the pH of a sample, as described above, in five increments by adding aliquots of a 0.1 or 1.0 M Na_2CO_3 solution and recording the spectrum at 25 °C with the proton sponge present. Another sample was studied by decreasing the pH between 6.7 and 5.6 by adding aliquots of a 0.2 M HCl solution and recording the spectrum under identical conditions.

RESULTS

Low-Field ^{1}H NMR Spectra. Low-field segments of high-resolution ^{1}H NMR spectra are shown in Figure 1: one obtained for a pure human α-thrombin solution, one for a pure r-hirudin solution, one for a solution of the human α-thrombin−r-hirudin complex, one for a solution of the active-site-modified human α-thrombin with PPACK, and one for a solution containing PPACK-modified thrombin and r-hirudin. No signals could be observed with chemical shifts greater than 12 ppm from DSS in buffered solutions of human α-thrombin and r-hirudin or hirunorm V (not shown). In our earlier work, buffered solutions of human α-thrombin between pH 5.3 and 8.5 did not display any resonances with chemical shifts greater than 12 ppm in ^{1}H NMR spectra recorded at 600 MHz.[22] A signal at 18.13 ppm is present for the covalently bound and cross-linked adduct with PPACK, and one at 15.33 ppm is exhibited for the complex with r-hirudin. Addition of 2 equiv of r-hirudin to the PPACK-inhibited enzyme does not cause a perceptible change in the spectrum, but addition of PPACK to the thrombin−r-hirudin complex gives a new resonance at 18.03 ppm, shifted 0.1 ppm upfield from the resonance obtained in the absence of r-hirudin. The resonances in Figure 1 are for SHBs at the active site in the case of PPACK and at a binding site with r-hirudin. Spectra identical to those shown were obtained in three repeats with PPACK-inhibited and with r-hirudin-inhibited human α-thrombin from two different sources. Both r-hirudin type 2 and r-hirudin type 1 give the resonance. The proton exchange rate constant could be estimated from the peak width at half-peak height at ∼60 Hz for the PPACK adduct and ∼98 Hz for the thrombin−r-hirudin complex. If there is no dipolar contribution to the line width,[28,69] the proton exchange rate constants with solvent can be calculated to be 188 and 307 s^{-1} at 30 °C for the thrombin adduct with PPACK and the r-hirudin complex, respectively. Polarization effects seemed to cause peak broadening at 5 °C. This observation is consistent with an earlier report of Frey and co-workers.[28]

Significantly, the 15.33 ppm resonance observed with the thrombin−r-hirudin complex remains unchanged in chemical shift, line width, and intensity between pH 5.6 and 8.8. This precludes the participation in a H-bond of groups that change protonation state in this pH range.

In Figure 2, low-field spectra for complexes of thrombin with hirudin mimics and D-NAPAP are shown. The resonances for the thrombin complexes are at 15.54 ppm with RGD-hirudin, 15.23 and 15.17 ppm with hirunorm IV and V, respectively, and 15.40 ppm with the Nα(Me)Arg peptide. The thrombin complex with D-NAPAP yields a weak signal at 15.35 ppm from DSS. Although we used the racemic form of NAPAP, thrombin presents an ∼1000-fold preference for the D-enantiomer. D-NAPAP was in >5-fold excess of thrombin. An analogous experiment with 1.3 mM porcine trypsin−D-NAPAP complex at pH 7.0 did not display a resonance above 15 ppm. We have examined the resonance for the thrombin−hirunorm IV complex in the temperature range of 5−35 °C, as shown in Figure 3. The resonance shifts to a lower field with decreasing temperature, consistent with a stronger H-bond at a lower temperature. The peak broadening is probably due to polarization or to decreasing solubility and aggregation of the complex.

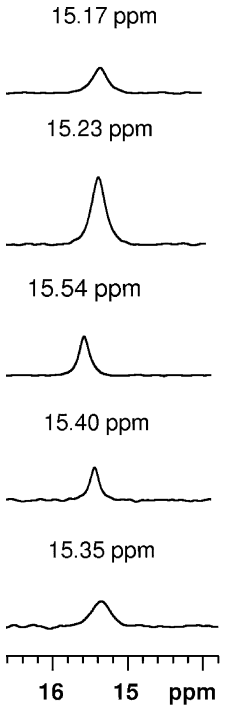

Figure 2. Low-field region of the spectra (3−4000 transients) obtained for complexes of human α-thrombin with hirunorm V, hirunorm IV, RGD-hirudin, Nα(Me)Arg peptide, and NAPAP from top to bottom, respectively, at pH 6.5 in 0.025−0.050 M citrate buffer, 0.10−0.20 M NaCl, and 0.005−0.010% PEG-8000 at 30 °C. The corresponding peak widths at half-height are 91, 82, 43, 38, and 125 Hz, respectively.

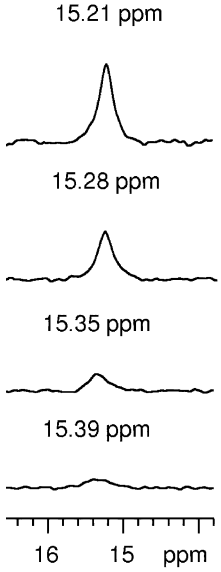

Figure 3. Temperature dependence of the low-field resonance for the human α-thrombin−hirunorm IV complex at pH 6.5 in 0.020 M citrate buffer, 0.10 M NaCl, and 0.005% PEG-8000. The spectra were recorded at 35, 25, 15, and 5 °C (from top to bottom, respectively).

The resonances for the human α-thrombin−r-hirudin and RGD-hirudin complexes are insensitive to the concentration of Na^+ ions in the concentration range of 0.09−0.31 M.

For studies of equilibrium deuterium isotope effects, the r-hirudin-inhibited human α-thrombin solution was divided into two equal parts of 125 μL, and equal volumes of water in one

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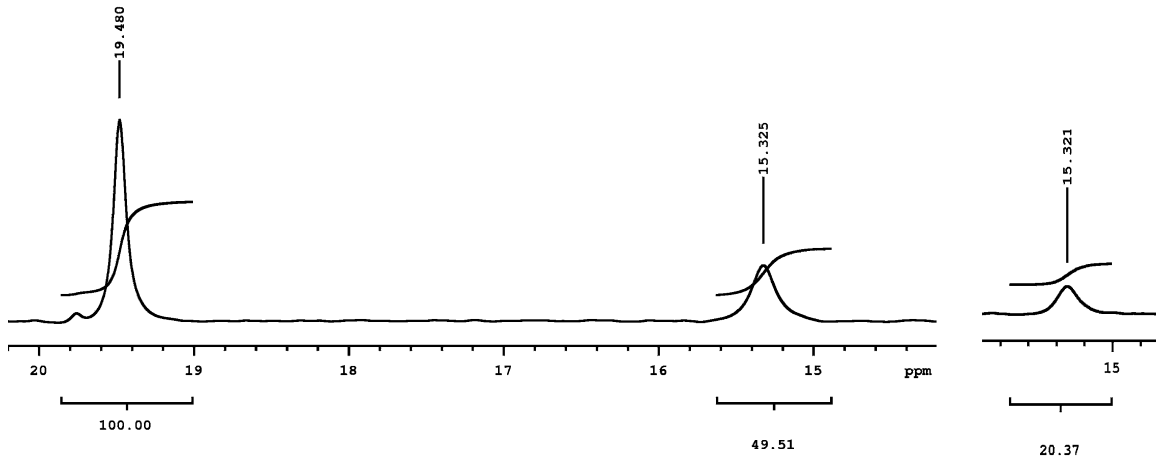

Figure 4. Display of the integration of the resonances obtained for the proton sponge (left) and the thrombin hirudin complexes in 6.6% D_2O (middle) and 58% D_2O (right), both buffered at pH 7.5 in 0.30 M HEPES buffer, 0.30 M glycine, 0.10 M NaCl, and 0.005% PEG-8000 at 25.0 ± 0.1 °C.

case and heavy water in the other were added to the solutions to yield ~5−7 and 45−59% D content, respectively. This method was used consistently for solutions of thrombin inhibited with r-hirudin, hirunorms IV and V, and RGD-hirudin. The resonances obtained for complexes with r-hirudin have chemical shifts of 15.33 ± 0.05 ppm in 6.6% D_2O and 15.32 ppm in 58% D_2O, independent of the concentration within the 0.1−0.5 mM range (assuring that monomeric complexes are responsible for the observation). The pair of spectra for the thrombin−r-hirudin complexes is shown in Figure 4. The resonance positioned at 19.40 ± 0.05 ppm is for the proton sponge integration standard dissolved in CD_3CN.

Fractionation factors between 0.67 and 1.0 were calculated for r-hirudin-inhibited human α-thrombin from the resonances at 15.33 ppm at pH 6.5 in 0.020 M citrate buffer and 0.10 M NaCl and 25.0 and 30.0 ± 0.1 °C. The fractionation factor of 1.0 for the hirunorm V-inhibited thrombin was calculated from the 15.17 ppm resonances in 0.025 M sodium citrate buffer and 0.10 M NaCl at pH 6.5 and 30 ± 0.1 °C. The fractionation factor calculated at total salt concentrations of >0.30 M was 1.15 for the complexes of thrombin with r-hirudin and RGD-hirudin. The estimated precision in the integration is 20%.

DISCUSSION

SHBs Formed at the Active Site of Thrombin. The complex of thrombin with D-NAPAP, the noncovalent active-site modifier, yielded a weak and broad signal at 15.35 pm from 16000 scans, with a line width of 125 Hz at half-peak height in contrast to the narrow peaks obtained from 3 to 8000 scans with the longer peptides. H-Bonds between thrombin and D-NAPAP in the predominant orientations are located in the X-ray structure at the binding site, near the active site of thrombin, as is the case with PPACK.[70,71] These inhibitors bind in the canonical mode; i.e., they form an antiparallel β-sheet with the Ser^{214}−Gly^{216} segment of thrombin. Twin H-bonds are prevalent between the backbone N and O atoms of Gly^{216} and the O and N atoms of Gly in the P1 position of D-NAPAP (these also occur in PPACK-modified thrombin). The X-ray crystal structures suggest an opportunity for a good H-bond. The peak at 15.35 ppm probably indicates the presence of an SHB in solution. The binding of D-NAPAP to trypsin is very similar to that of thrombin,[72] which prompted us to investigate the presence of a ^1H NMR signal in the region of interest, but we found none.

Previously, resonances with chemical shifts larger than 14 ppm have been reported for many enzymes using acid−base catalysis when the catalytic base (mostly His) is protonated (at low pH) or the enzyme is bonded covalently to an appropriate active-site modifier: these observations have been interpreted to indicate the shortening of H-bridges at the active site of the enzymes.[36] These H-bridges have been termed SSHBs on the basis of different correlations of H-bond length and chemical shift in model compounds,[36] proton exchange rates, and fractionation factors below unity, rather than energetic considerations.[41−46,73,74] By these criteria, we have established the presence of a SHB in the PPACK adduct of human α-thrombin (Figure.1), an analogue of the oxyanionic tetrahedral intermediate for acylation in substrate hydrolysis. As already mentioned, this SHB is almost certainly one formed between His^{57} δNH and Asp^{102} γO. The H-donor−acceptor distance for this pair in thrombin has been reported to be between 2.50 and 2.65 Å in various crystal structures of inhibited thrombin at 1.8−2.5 Å resolution.[7,13,57−59,75] Previously, we also described a signal at 17.34 ppm occurring in anionic phosphorylated/phosphonylated adducts of thrombin, which are analogues of the tetrahedral intermediate in deacylation in substrate hydrolysis, and again form an SHB between His^{57} δNH and Asp^{102} γO.[22] The proton exchange rates in these adducts at 30 °C are low by comparison to those of SHBs occurring at other enzyme active sites. This observation is consistent with the character and the location of the thrombin active site in a deep canyon.[7] Deshielded ^1H NMR signals observed at the active site of enzymes with or without effectors result from H-bonds occurring in low-dielectric environments,[44−46] often in a cluster of other H-bonds,[47] and/or due to shortening of the distance between H-donor and acceptor pairs.[40] SHBs are often ionic and have been termed short ionic (SI) HBs.[47]

Notably, the resonances for the two covalently modified anionic thrombin adducts are nearly identical to those observed in the corresponding analogues of tetrahedral intermediates in the double-displacement mechanism of ester hydrolysis catalyzed by cholinesterases.[36−38] The greatest chemical shifts have been observed for ionic H-bonds between the protonated catalytic His and the carboxylate ion of Asp or Glu at the active site. The ^1H NMR resonances for SHBs occurring at the

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catalytic site of serine proteases, which have been modified to form oxyanionic tetrahedral adducts, are constant between pH 5 and 9 because they have elevated pK_a values.[28,29,33−36,39] Neutral adducts exemplified by the hemiacetal that chymotrypsin forms with *N*-acetyl-L-leucyl-L-phenylalanal have chemical shifts near 15 ppm.[35] His^{57} in the complex of thrombin with NAPAP is likely to be protonated at pH 6.5, and no low-field ^{1}H NMR resonance is present in native thrombin at this pH. If NAPAP binding results in the sequestering or compressing of the active site of thrombin, an SHB may form in the catalytic triad, but the occurrence of an intermolecular SHB at a binding site is just as possible.

The SHBs had appeared to be unique to the attainment of catalytic perfection, but this idea was refuted later.[41−47] Furthermore, a surprising discovery has been a ^{1}H NMR resonance at 18.0 ppm originating not from the catalytic triad in the native rhamnogalacturonan acetylesterase but from an adjacent SHB between two Asp residues near the oxyanion hole.[76] In fact, SHBs are frequent stabilizing elements of tertiary protein structure in compressed regions or in low-dielectric fields.[77] These are the features that characterize thrombin inhibition with canonical peptide inhibitors, such as NAPAP, at the P1 and P2 binding site and even more the thrombin−hirudin interface at the FRS.

SHBs in Binding Interactions at Remote Sites. The small molecular modifiers of the thrombin active site lack critical remote interactions of natural substrates at exosites so vital to the precise function of thrombin. The exosites, especially the FRS, determine the substrate selection, which is in turn regulated by Na^+ binding at an adjacent location.[78] The regulation is mediated by water channels, which involve some short H-bridges. No ^{1}H NMR resonances with chemical shifts greater than 12 ppm have previously been reported in protein−modifier interactions occurring outside the active site of an enzyme. The binding of the hirudin family of protein/peptide inhibitors (Table 1) to α-thrombin is unique in that it is extended and includes remote binding sites, which makes the complexes erstwhile candidates for studies of the presence, origin, and location of SHBs.

Hirudin is an allosteric effector of the fast conformation of α-thrombin. It is a 65-residue protein produced in the salivary glands of *Hirudo medicinalis*, the common leech, in 20 varieties with similar sequence homologies.[79,80] Three of the most common variants contain three disulfide bonds and sulfated Tyr$^{63\prime}$, Tys$^{63\prime}$. Hirudin interacts noncovalently but tightly with α-thrombin near the active-site cleft as well as with the FRS, which contains an abundance of basic residues.[79]

The first X-ray structure (2.3 Å) of the α-thrombin−r-hirudin complex (variant 2, Lys$^{47\prime}$) affords a complex picture of the key interactions:[57−59,75] It displays a bound r-hirudin structure that becomes more compact and forms numerous intermolecular H-bridges when it interacts with thrombin in comparison to the NMR solution structure of r-hirudin, which reveals many intramolecular H-bridges within r-hirudin,[59] yet r-hirudin and hirunorm V alone do not yield a resonance with a chemical shift greater than 12 ppm in high-resolution ^{1}H NMR spectra (Figure.1), precluding the presence of intramolecular SHBs; however, that changes when they bind to α-thrombin.

Do the resonances so consistently appearing between 15.17 and 15.54 ppm derive from SHBs at different locations with canonical and noncanonical inhibitors?

While hirudin and its mimics bind near the active-site region, the X-ray structure of the complex of thrombin with this family of inhibitors presents a variety of H-bonding patterns around the active site. In fact, the S1 binding site on thrombin is not used as a result of the parallel binding mode of these inhibitors, which is in stark contrast to the canonical binding of NAPAP and PPACK to thrombin.

Again, the possibility of compression of the active site and the consequent recruitment of the traditional SHB between the catalytic His^{57} δH and Asp^{102} γO, as a result of the tight binding of inhibitors to thrombin, comes to mind. However, crystal structures of thrombin with hirudin show the same geometry of the catalytic residues, a short distance (<2.7 Å) between N and O and an angle of >150°, as in native thrombin.[7,79] More importantly, the pH profile for the binding of hirudin to thrombin yields pK_a values of 7.1 for His^{57}, 8.4 for the N-terminal amino group of hirudin, and 9.2 for the α-amino group of Ile^{16} on thrombin engaged in a salt bridge with Asp^{194}.[81] The pK_a values of 7.1 and 9.2 are identical to those reported for the native enzyme, yet the 15.33 ppm resonance is independent of the ionization of the His^{57}-Asp^{102} pair and the ionization of the N-terminal amino group of hirudin between pH 5.6 and 8.8 for the r-hirudin complexes of thrombin. This seems to preclude the location of the SHB at the active site of thrombin in the complexes with r-hirudin. The N-terminal head of r-hirudin forms a parallel β-strand with thrombin (residues 214−219), making a non-substrate-like interaction. The first three residues at the N-terminus (Ile$^{1\prime}$-Thr$^{2\prime}$-Tyr$^{3\prime}$, variant 2) of hirudin penetrate the active site and aryl binding site where they

Table 1. Comparison of the Amino Acid Sequences[a] of r-Hirudin,[57−59,75] Hirunorms,[64] R-Peptide,[65] and NAPAP[72]

amino acid	hirudin, variant 2 (variant 1)	hirunorm IV	hirunorm V	R-peptide	NAPAP
1	Ile$^{1\prime}$ (V)	Chg	Chg	D-Cha	2Ngl
2	Thr$^{2\prime}$ (V)	Arg	Val	Pro	Phe
3	Tyr$^{3\prime}$	2-Nal	2-Nal	N-Me-Arg	Pip
4	Thr$^{4\prime}$	Thr	Thr	Thr	
5	Asp$^{5\prime}$	Asp	Asp	|	
6	|	D-Ala	D-Ala	|	
7	|	Gly	Gly	$(Gly)_5$	
8	|	B-Ala	B-Ala	|	
9	Pro$^{48\prime}$	Pro	Pro	|	
10	Glu$^{49\prime}$	Glu	Glu	|	
11	Ser$^{50\prime}$	Ser	Ser	|	
12	His$^{51\prime}$	His	His	|	
13	Asn$^{52\prime}$	h-Phe	h-Phe	|	
14	Asx$^{53\prime}$	Gly	Gly	|	
15	Gly$^{54\prime}$	Gly	Gly	|	
16	Asp$^{55\prime}$	Asp	Asp	Asp	
17	Phe$^{56\prime}$	Tyr	Tyr	Tyr	
18	Glu$^{57\prime}$	Glu	Glu	Glu	
19	Glu$^{58\prime}$	Glu	Glu	Pro	
20	Ile$^{59\prime}$	Ile	Ile	Ile	
21	Pro$^{60\prime}$	Pro	Pro	Pro	
22	Glu$^{61\prime}$	Aib	Aib	Glu	
23	Glu$^{62\prime}$	Aib	Aib	Glu	
24	Tyr$^{63\prime}$	Tyr	Tyr	Ala	
25	Leu$^{64\prime}$	Cha	Cha	Cha	
26	Gln$^{65\prime}$	D-Glu	D-Glu	D-Glu	

[a]Abbreviations: Aib, α-aminoisobutyric acid; Cha, β-cyclohexyl-Ala; Chg, cyclohexyl-Gly; h-Phe, (+)-α-amino-4-phenylbutyric acid; 2-Nal, β-(2-naphthyl)alanine; 2-Ngl, β-(2-naphthyl)glycine.

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interact with the S2 and S3 specificity site and form H-bonds to His[57] and Ser[214].

The chemical shift at 18.03 ppm appears after addition of PPACK to the human α-thrombin−r-hirudin complex (Figure 1). PPACK probably dissociates the thrombin−hirudin complex, and the spectrum may simply display two binary complexes of thrombin. The resonance for the PPACK-inhibited enzyme is 0.1 ppm upfield in the presence of r-hirudin, which may indicate an increased level of shielding as a result of a less tightly bound PPACK in a ternary complex[82] or a changed electrostatic environment in the active-site cavity. The chemical shift at 15.33 ppm for the thrombin−r-hirudin complex remains unchanged as if the conformation of hirudin in the vicinity of the SHB remained undisturbed in the complex, but the peaks lean toward each other and become broader, which may indicate proton exchange between sites.

The central portion of hirudin is globular and is more loosely attached to α-thrombin. A C-terminal fragment of hirudin spanning residues 53−65 binds the tightest to residues 62−73 of the B-chain on α-thrombin. Strong electrostatic interactions, including at least 13 H-bonds, hold this segment together, but the last five residues form a 3_{10}-helical turn, which engages in hydrophobic interactions. Native hirudin with the sulfate group on Tys[63′] enhances the binding constant by ∼15-fold versus that of the desulfo form.[7,79,83] The intrinsic fluorescence of α-thrombin has been exploited for measurements of binding parameters, because r-hirudin binding causes key Trp residues of thrombin be buried more deeply in the interior and thus enhance its fluorescence.[84] From two studies,[15,84] it emerged that first the C-terminal segment of residues 53−65 is preoriented and binds rapidly to the FRS because of the complementary electrostatic forces between the two. This is followed by the fitting of the three-residue N-terminal segment, which is ∼300 times slower than the first step.

When hirunorms are complexed to α-thrombin, the ^{1}H NMR resonance becomes a little more shielded at 15.17−15.23 ppm than in the thrombin−r-hirudin complexes. Hirunorms[21,64,85,86] were designed to be "true hirudin mimics" by containing the functionalities that interact with the α-thrombin active-site region, specifically the Ser[214]−Gly[216] segment, and with the FRS.[79] A three-residue segment [D-Ala[6″]-βAla[7″](or Gly)-βAla[8″]] is the spacer in place of the larger Cys[6′]−Lys[47′] core in hirudin. Among five hirunorms, hirunorms IV and V are the most potent.[64] X-ray structures of α-thrombin−hirunorm IV[85] and α-thrombin−hirunorm V complexes[86] show that the hirunorms bind along the B-chain, partly blocking the active-site cleft by interacting with key residues in a parallel manner. The chains stretch out of the cleft and arch over to the FRS where the C-termini interact tightly. Binding probably commences with the C-terminal end as shown for r-hirudin.[15,84] The primary sequences of hirunorms IV and V differ only at the second residue, and only slightly along the C-terminal sequence. The H-bonding potential between α-thrombin and the C-terminal region of these inhibitors is similar to that of hirudin, but not so at the active site. The hirunorms bind with ∼4 kcal/mol less energy than hirudin does.[21,85]

r-RGD-hirudin is r-hirudin embellished with an ^{32}RGD^{34} (instead of SDG) sequence known to be critical for binding to the FRS of thrombin.[60] The interaction with thrombin is also enhanced at the C-terminus, where more Glu, Asp, and Pro residues are present to enhance hydrophobicity. Certainly, the deshielding of the ^{1}H NMR signal by 0.21 ppm relative to r-hirudin is consistent with changes in the milieu of the key binding region at the FRS.

The ^{1}H NMR signal obtained with the complex of the Nα(Me)Arg peptide with thrombin is essentially the same as that for the thrombin−r-hirudin complex, implying that the same or similar binding elements are involved in the two cases. The Nα(Me)Arg peptide was designed for enhanced binding affinity and storage stability by introducing methylation of the Arg at position P1.[65] A close analogue of the C-terminal peptide of hirudin is tethered to the N-terminal segment with a poly-Gly linker in the Nα(Me)Arg peptide, which interacts with human α-thrombin with a K_i of 37 pM.

The resonances measured between 15.17 and 15.54 ppm are consistent with donor−acceptor distances of <2.70 Å, on the basis of correlations between chemical shift and H-donor−acceptor distances in small molecular crystals measured by X-ray.[36−38,40,87−89] This compares very well with the crystallographic data for the donor−acceptor distance in certain H-bonds between α-thrombin and r-hirudin, RGD-hirudin, and hirunorms. The exchange rates with solvent are smaller than those observed for SHBs in other enzymes,[36,88] which suggests a hydrophobic or dry environment of the SHB. In fact, contraction of the H-donor−acceptor distance would lead to water exclusion.[90]

Sequence Specificity of the ^{1}H NMR Resonances. On the basis of the X-ray structure,[3,57,58] three regions can be identified at the α-thrombin−r-hirudin interface, where the SHB associated with the 15.33 ppm signal may originate. The candidates from r-hirudin participating are Ile[1′], Tyr[3′], Val[21′], and the Glu[57′]-Glu[58′] pair, the binding epitope in fibrinogen. (1) The pH and Na^+ ion independence of the 15.33 ppm resonance demonstrates the H-bond at the N-terminal amino group is not the origin of the chemical shift in the thrombin−r-hirudin complex. (2) Hirunorms contain a β-(2-naphthyl)-alanine at the P3 site, which is an unlikely H-bonding partner to H-donors and acceptors at the active site of thrombin, yet the resonance at 15.17 ppm persists in the ^{1}H NMR spectrum. (3) Participation of Val[21′] is abrogated in the hirudin mimics, yet they display the 15.17 or 15.33 ppm ^{1}H NMR resonance. (4) This leaves the most plausible site of the SHB to be, imbedded among hydrophobic residues at the C-terminus of hirudin, between Glu[57′] or Glu[58′], as H-acceptors, and Arg[75] and Arg[77] of thrombin as H-donors. In fact, Arg[75] and Arg[77] are solvated with water, which are reported to involve H-donor−acceptor distances of 2.6−2.8 Å.[57,58] The most likely site of SHBs is the region shown in Figure 5. The pH independence between pH 5.6 and 8.8 of the 15.33 pm peak is consistent with an SHB between a carboxylate ion and guanidinium ion (Arg).

Character of Proton Bridges in Binding. The fractionation factors for the signal at 15.17−15.54 ppm are near 1.0 or somewhat lower with an estimated error of 20%. Whereas these values are not as precise as desired, they certainly are in line with what has been found with H-bridges that resonate near 15 ppm. A D/H fractionation factor (ϕ) for a H-bridge is essentially an equilibrium constant for the exchange of H/D between a site on a protein and the protic solvent, i.e., L_2O (L = H or D). In this case, proton bridging occurs at a tight-binding site. The equilibrium constant for the process can be defined as ϕ = [binding site D][H solvent]/[binding site H][D solvent], indicating the preference of the binding site for D over H in reference to the solvent, i.e., an inverse deuterium solvent isotope effect. In general, the shorter and stronger the H-bond, the smaller the value of ϕ.[36,73,91] Fractionation factors

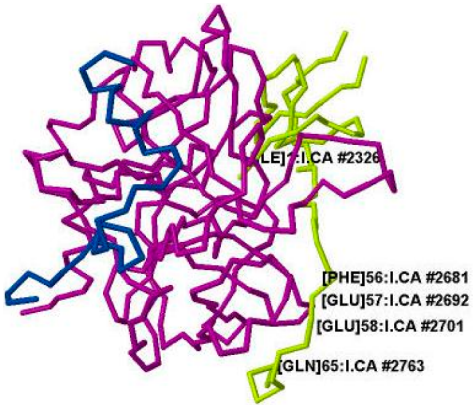

Figure 5. Strand structures of the A chain (blue) and B chain (cyan) of thrombin complexed with hirudin (yellow). $Ile^{1'}$ and the most likely site of an SHB occurring in the thrombin−r-hirudin complex, residues 55−65 of r-hirudin, are labeled. The image is from Protein Data Bank entry 4HTC[58] modeled with Jmol version 12.0.41.

measured for H-bonds that resonate at low field are generally smaller than what we observed with these systems; for example, the 18.13 ppm signal for thrombin covalently modified by PPACK has a fractionation factor of 0.45 ± 0.04.[22]

The temperature dependence of the chemical shift for the thrombin−hirunorm IV complex demonstrates that the SHB remains in slow exchange with solvate and other proton donors or acceptors up to at least 35 °C.

In conclusion, using 600 MHz ^{1}H NMR on aqueous solutions of thrombin allowed us to identify SHBs in the interaction of human α-thrombin with several tight-binding inhibitors. On the basis of our earlier findings the transition state for binding of the small inhibitors does not show changes in the status of the H-bridges, but the stable adducts of the inhibitors with thrombin show one unique SHB at the active site. In this work, we show that strong proton bridges also enforce tight binding at external binding sites, most probably at $Glu^{57'}$-$Glu^{58'}$ in hirudin and its mimics or in the water channels. Members of the hirudin family of inhibitors mimic the binding propensity of fibrinogen.[79] It is therefore of substantial interest that an SHB be present when r-hirudin is bound to α-thrombin.

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ABBREVIATIONS

Cha, cyclohexyl-β-alanine; Chg, cyclohexylglycine; DMSO, dimethyl sulfoxide; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; MUGB, 4-methylumbelliferyl 4-guanidinobenzoate hydrochloride; NAPAP, Nα-2-naphthylsulfonyl-glycyl-DL-4-amidinophenylalanyl-piperidide acetate salt; Nα(Me)-Arg peptide, D-Cha-Pro-Nα(Me)Arg-Thr-$(Gly)_5$-^{10}Asp-Tyr-Glu-Pro-Ile-Pro-$(Glu)_2$-Ala-Cha-^{20}D-Glu; Pip, piperidyl; PPACK, Phe-Pro-Arg-chloromethylketone; r-hirudin, recombinant hirudin type 1 or 2; r-RGD-hirudin, recombinant ^{32}SGD^{34} type 2 hirudin; S-2238, H-D-Phe-Pip-Arg-pNA; SHB, short hydrogen bond; TLC, thin layer chromatography.

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