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Thrombin Exosite for Fibrinogen Recognition Is Partially Accessible in Prothrombin*

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From the ‡Biotechnology Research Institute, National Research Council of Canada, Montréal, Québec H4P 2R2, Canada, the ¶American Red Cross Blood Services, South Eastern Michigan Region, Detroit, Michigan 48232, and the ‖New York State Department of Health, Wadsworth Center for Laboratories and Research, Albany, New York 12201

The procoagulant α-thrombin is produced by the proteolytic cleavages of a minimum of two peptide bonds Arg^{274}-Thr^{275} and Arg^{323}-Ile^{324} in prothrombin. The Arg^{323}-Ile^{324} cleavage is required for the expression of the active site of thrombin (Morita, T., Iwanaga, S. Suzuki, T. (1976) *J. Biochem. (Tokyo)* 79, 1089–1108; Hibbard, L. S., Nesheim, M. E., and Mann, K. G. (1982) *Biochemistry* 21, 2285–2292). It is not yet clear to what extent the proteolytic events are responsible for exposing protein recognition exosites on thrombin. We employed high resolution NMR spectroscopy to examine interactions of prothrombin and thrombin with synthetic hirudin peptides targeted toward the fibrinogen recognition exosite of thrombin. The hirudin tail synthetic analogues (acetyl-Asp^{55}-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln^{65}/Gly^{65}-OH) exhibited similar NMR relaxation enhancements (line broadening patterns and transferred nuclear Overhauser effects) with human prothrombin as with human α-thrombin, indicating that both proteins bind the peptide in a similar manner. The protein-induced relaxation enhancements are specific to the interaction of the hirudin peptides with the fibrinogen recognition exosite of thrombin since no significant effects were observed with either human serum albumin or with human γ-thrombin, which has an impaired recognition exosite. The binding affinities were determined from NMR relaxation time measurements, which gave approximate K_d values of 500 μM and <100 μM for prothrombin and α-thrombin, respectively. Since the hirudin tail fragment binds specifically to the fibrinogen recognition exosite in α-thrombin, this exosite appears to be partially accessible in prothrombin in a proenzyme form.

Thrombin is the central regulatory enzyme in hemostasis (1, 2). During normal circulation, it exists almost entirely as its inactive precursor prothrombin. Upon stimulation, procoagulant thrombin is generated from prothrombin by a delicately controlled cascade of zymogen activation reactions (3). The conversion of prothrombin to active thrombin has been the subject of intense study (4–9). It has been shown that the minimum requirement for the expression of the proteolytic active site is the cleavage of the Arg^{323}-Ile^{324} peptide bond producing the activation intermediate meizothrombin (4). The full expression of the clotting activity, however, requires a further cleavage at peptide bond Arg^{274}-Thr^{275} connecting the activation fragment 1.2 with the protease domain prethrombin 2 or α-thrombin (4, 9). The protease domains as a result also bind specifically to fibrinogen, presumably via the macromolecular substrate (fibrinogen) recognition exosite (1, 2, 7, 10). Thus, it appears that activation fragments 1 and 2 might obstruct the fibrinogen recognition exosite, resulting in reduced clotting activity for meizothrombin.

The properties of the fibrinogen recognition exosite in thrombin have been investigated with hirudin (10–12) and with hirudin tail peptides (13–15), also referred to as hirugen (16), targeted specifically for this thrombin exosite (17–19). Interestingly, it has been shown that hirugen binds to meizothrombin, as well as prethrombin 2 and α-thrombin, but not to prothrombin (20). This finding led to the conclusion that the hirugen or the fibrinogen binding exosite is not expressed before the cleavages of either the Arg^{274}-Thr^{275} or the Arg^{323}-Ile^{324} peptide bond in prothrombin. This conclusion, on one hand, is consistent with the results from previous studies employing the protein substrate fibrinogen (7). On the other hand, all these studies utilized methods that are sensitive only to tight binding interactions in the micromolar (μM) range. There is a possibility of weaker but specific binding between prothrombin and hirudin peptides that escaped previous detection. We have undertaken high resolution NMR studies on the interaction of hirudin tail peptides with both prothrombin and thrombin. Our results suggest that the fibrinogen recognition exosite in prothrombin is at least partially accessible to peptide binding with a bound conformation indistinguishable from that recognized by thrombin.

EXPERIMENTAL PROCEDURES

Peptides and Proteins—The hirudin peptides acetyl-Asp^{55}-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln^{65}-OH (peptide P24) and a substituted analog, acetyl-Asp^{55}-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gly^{65}-OH (peptide P35), were synthesized as described previously (14, 19). Human serum albumin was obtained from Sigma as a dried powder and used without further purification. Human α-thrombin was prepared as described previously (21). Stock solutions of thrombin (0.64–1.25 mg/ml) were stored in a deep freeze (<−70 °C) before use. The material used in the current study had a specific activity of 3000–3300 NIH units/mg. Human γ-thrombin was prepared by controlled trypsin digestion of α-thrombin (22, 23). This preparation of γ-thrombin (1.8 mg/ml) had an intact active site (93% by active-site titration), but had a very low clotting activity of <10 NIH units/mg, indicating that the fibrinogen recognition exosite is destroyed by trypsin cleavages (22). For transferred NOE[1] experiments, stock thrombin solutions were exchanged to the desired NMR solvent (see below) and concentrated to 15 mg/ml using CentriPrep-10 concentration cells. Human prothrombin was purified from human plasma (8). Prothrombin (40 mg/ml) was stored at −70 °C in an aqueous solution containing 2 mM Tris at pH 7 and was used without further concentration.

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[1] The abbreviation used is: NOE, nuclear Overhauser effect.

NMR Spectroscopy—NMR samples of peptides were prepared in an aqueous solution that was 150 mM in NaCl, 50 mM in sodium phosphate, and 0.2 mM in EDTA at pH 5.3. One- and two-dimensional NMR experiments were carried out on a Brüker AM-500 MHz superconducting spectrometer as described previously (14, 15, 24). Selective T_1 experiments were carried out by inverting a peptide proton resonance of interest, followed a variable delay before the detection of the peptide spectrum. The intensity of the irradiated resonance was fitted to a mono-exponential decay to obtain the relaxation time. Control experiments showed that the proton signals of the hirudin peptides remain equally sharp (spectra not shown) within the entire range of peptide concentrations (0.005–10 mM), indicating the peptides remain in a monomeric state as established previously (15). No enzyme degradation was observed after the NMR experiments as judged by use of SDS-polyacrylamide gel electrophoresis under reducing conditions.

Measurement of Binding Affinity—Selective T_1 relaxation times of the peptides were measured in the presence of a certain concentration of prothrombin or thrombin at pH 7.0 and a temperature of 25 °C. The initial peptide concentration was 1.36 mM and the initial prothrombin concentration was 0.064 mM. For the thrombin-peptide sample, the initial peptide concentration was 0.70 mM and the initial thrombin concentration was 0.053 mM. Volumes of 10 or 15 μl of the P35 stock solution was added in each subsequent titration step for both prothrombin and thrombin binding. The observed relaxation enhancement, $1/T = 1/T_{1,obs} - 1/T_{1,fp}$, is related to the equilibrium binding constant K_d and the total concentrations of the peptide $[L]$ and the protein $[E]$ by $[E]T = q\,(K_d + [L])$ where q is a scaling constant (25, 26). The binding constant K_d can thus be obtained from the horizontal intercept of a linear titration plot.

RESULTS AND DISCUSSION

Protein-induced Differential Line Broadening—In aqueous solution, small peptides usually exhibit well resolved proton NMR signals. Fig. 1*A* shows the amide NH and the aromatic region of a proton NMR spectrum of peptide P24 in the presence of human prothrombin. The aromatic signals from Tyr^{63} of the peptide remain relatively sharp compared to those of the free peptide (Fig. 1*C*). But the δCH_2 ring protons of Phe^{56} is selectively broadened by prothrombin binding (Fig. 1*A*). For the amide protons, those of residues Asp^{55}, Phe^{56}, and Glu^{57} are obviously sharper and have higher signal intensity than those from the rest of the peptide. Particularly, there is an almost uniform line broadening (and decrease in signal intensities) for residues Glu^{58} and Ile^{59} and for residues Glu^{62} to Gln^{65} (Fig. 1*A*). The NH signal of Glu^{61}, on other hand, is about as sharp as the NH of Glu^{57}, thus forming an apparent break within the stretch of broadened residues Glu^{58} to Gln^{65}. Fig. 1*B* displays the broadening pattern of the peptide resonances in the presence of human α-thrombin. Except for some resonance shifts due to the slight difference in the pH values of the samples, there is thrombin-induced line broadening for the NH resonances of residues Glu^{58}, Ile^{59}, Glu^{62}, Leu^{64}, and Gln^{65}. The NH signal of Tyr^{63} is also broadened if one compare the reduced intensity of the overlapped resonances for Phe^{56} and Tyr^{63} with that of residue Asp^{55} (Fig. 1*B*). Therefore, there is again a uniform line broadening from residue Glu^{58} to Gln^{65} with a break at residue Glu^{61} if peptide P24 interacts with human α-thrombin. These same patterns of NH line broadening were also observed for the interaction of hirudin peptides with bovine α-thrombin (14). These observations indicate that human prothrombin indeed binds to the hirudin peptide, since line broadening patterns are indicative of the binding environments of peptides on the thrombin surface (14, 27, 28).

Not only the peptide NH protons are affected, the aliphatic side-chain protons of the peptide are also selectively broadened by prothrombin or thrombin binding. Fig. 2 compares the methyl proton resonances of Ile^{59} and Leu^{64} in the presence of prothrombin (*panel A*) and thrombin (*panel B*) and from the free peptide (*panel C*). It is obvious that the γCH_3 proton resonances are more broadened compared to other resonances in the same spectrum (*panel A* or *B*). The δCH_3 proton resonance of Ile^{59} was also broadened as judged by spectra of peptide P35 where the $Gln^{65} \rightarrow Gly$ substitution shifted the overlapping δCH_3 proton resonance of Leu^{64} away from the δCH_3 protons of Ile^{59} (not shown).

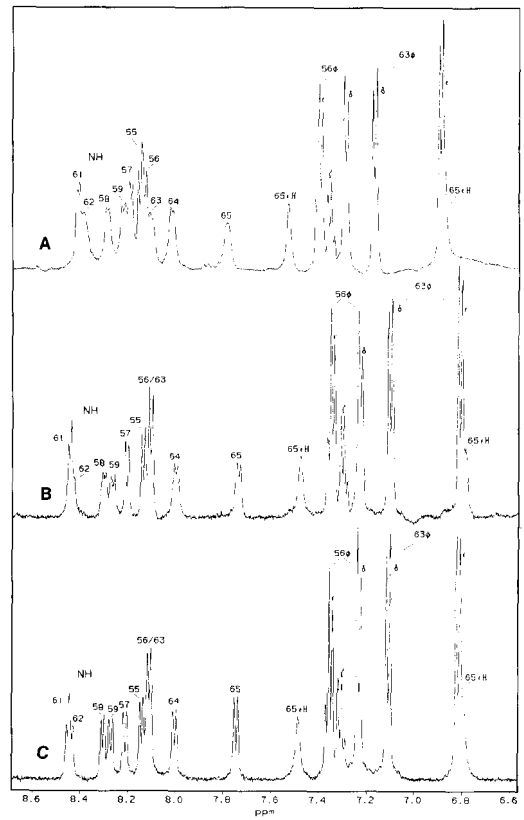

FIG. 1. **Proton NMR spectra of the hirudin tail peptide (acetyl-Asp^{55}-Phe^{56}-Glu^{57}-Glu^{58}-Ile^{59}-Pro^{60}-Glu^{61}-Glu^{62}-Tyr^{63}-Leu^{64}-Gln^{65}-OH or peptide P24) in the presence of human prothrombin (*A*) and human α-thrombin (*B*) compared to the free peptide (*C*).** The spectra were acquired at 25 °C with a peptide concentration of 6.3 mM and a prothrombin concentration of 0.222 mM at pH 5.0 (*A*), a peptide concentration of 3.2 mM and a thrombin concentration of 0.135 mM at pH 5.2 (*B*), or a peptide concentration of 4.6 mM at pH 5.2 (*C*). Resonance assignments were established in a previous publication (14). The NH proton resonances are indicated by the corresponding residue numbers with the side-chain proton resonances of residues Phe^{55}, Tyr^{63}, and Gln^{65} labeled for internal reference. The same patterns of line broadening were also observed with a peptide concentration of 0.05 mM (50 μM) and a prothrombin concentration of 0.005 mM (5 μM) that is close to the plasma concentration (2 μM) of prothrombin (29).

In general, there is more peptide line broadening in the peptide-prothrombin spectra (Fig. 1*A*) compared to the spectra in the presence of thrombin (Fig. 1*B*). This phenomenon may be explained by the increased molecular weight of the prothrombin molecule, hence increased effects of relaxation (26). Differential peptide line broadening could still be observed with a prothrombin concentration of 5 μM, close to the normal plasma level of this protein (29), and the observed line broadening persists at a higher pH of 7.0 (spectra not shown) for both prothrombin and thrombin binding. These results establish that pH variation within the range of 5–7 does not affect the interaction of the hirudin peptide with these proteins. Furthermore, after NMR experiments with prothrombin, the protein was found to be intact without degradation or activation cleavages, as judged by gel electrophoresis under reducing conditions (data not shown). The differential peptide line broadening should be a result of specific interactions with prothrombin (or α-thrombin) since human serum albumin did not produce any significant peptide line broadening under similar experimental

conditions (spectra not shown). Human γ-thrombin, a form of thrombin with an impaired fibrinogen recognition exosite (10, 12, 23), also failed to induce differential line broadening for any of the peptide resonances at a γ-thrombin concentration of 20 μM. This result indicates that the damaged fibrinogen recognition exosite in γ-thrombin shows a significant loss of binding capacity even compared to prothrombin. On the other hand, blocking of the active site of α-thrombin by (d)-Phe-Pro-Arg-CH_2Cl (PPACK) did not interfere with the binding of the hirudin tail peptides (14). All these observations indicate that peptide line broadening must be a result of specific interactions with the fibrinogen recognition exosite in prothrombin or α-thrombin.

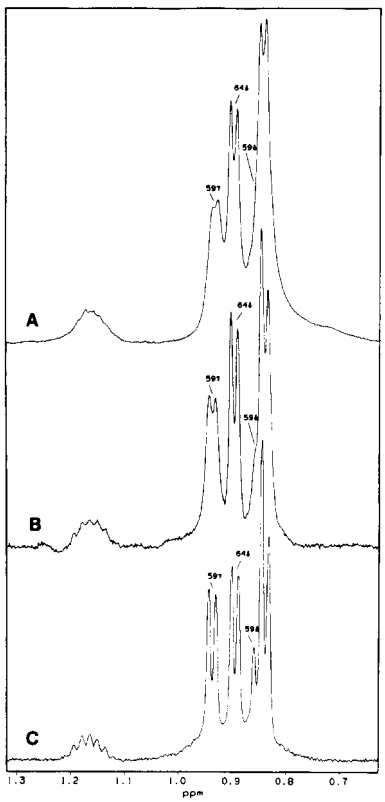

FIG. 2. **Line broadening on the side-chain proton resonances of residues Ile^{59} and Leu^{64} of the hirudin peptide.** Experimental conditions are the same as in Fig. 1. Indicated are the methyl proton resonances of the two residues. The second δCH_3 methyl group of Leu^{64} overlaps with the δCH_3 proton resonance of Ile^{59}. But the spectral overlaps were resolved in another hirudin peptide where Gln^{65} was replaced by Gly^{65}. The spectra were acquired in the presence of prothrombin (*A*), human α-thrombin (*B*), or peptide alone (*C*). Differential line broadening of the γCH_3 and δCH_3 methyl groups of Ile^{59} was also observed with physiological concentrations of prothrombin (see Fig. 1).

Transferred Nuclear Overhauser Effects—Apart from resonance line broadening, an alternative way to examine ligand binding is through the observation of transferred NOEs (27). Fig. 3 displays the fingerprint regions of the two-dimensional NOE spectra of peptide P24 in the presence of human prothrombin (*panel A*) or α-thrombin (*panel B*). In the absence of binding, this spectral region should only contain cross-peaks arising from through-bond J interactions between the NH and the αCH protons of each residue in the peptide (14). However, the cross-peaks in both spectra contain similar sequential (transferred or binding-induced) NOE contacts between adjacent amino acid residues. There are also similar medium range NOEs between the αCH proton of Glu^{61} and the NH protons of Tyr^{63} and Leu^{64} and between the αCH proton of Glu^{62} and the NH proton of Leu^{64} (Fig. 3) characteristic of a 3_{10}-helical turn from residue Pro^{60} to Gln^{65} (14, 15). Furthermore, there are side-chain NOE interactions between the δCH_2 protons of Pro^{60} and the δCH_2 protons of Tyr^{63} and other NOEs (not shown) that were previously established for the interaction of peptide P24 with α-thrombin (14, 15). The existence of these characteristic NOEs indicates that the hirudin tail peptide not only binds, it binds to both prothrombin and thrombin in a very similar conformation.

The thrombin-bound conformation of the hirudin peptide was determined previously by distance geometry calculations incorporating transferred NOE distance constraints (14) and by x-ray crystallography (18). The critical feature of the bound structure is the burial of the side chain of Ile^{59} surrounded by the hydrophobic side chains of Phe^{56}, Pro^{60}, Tyr^{63}, and Leu^{64} in the hirudin peptide and by the hydrophobic residues Phe^{34T}, Leu^{65T}, Tyr^{76T}, and Ile^{82T} in thrombin (15, 18). In such a hydrophobic cluster, the side-chain methyl groups of Ile^{59} are embedded between the aromatic rings of the thrombin residues Phe^{34T}, Tyr^{76T}, and the peptide residue Tyr^{63}. Such a spatial arrangement would explain the significant line broadening of the γCH_3 and the δCH_3 protons of Ile^{59} (Fig. 2) in that there must be significant ring-current shifts for these methyl protons in the protein-peptide complex (26, 28). The similarity of the bound environment around the methyl groups of Ile^{59} again indicates that the fibrinogen recognition exosite exists in both prothrombin and thrombin.

Binding Affinities from Selective Relaxation Time Measurements—For quantitative measurements, peptide P35 was used, which differs from P24 by a single Gly substitution at Gln^{65} with a slight decrease in inhibition potency toward thrombin (19). Fig. 4*A* shows a titration plot of the observed selective T_1

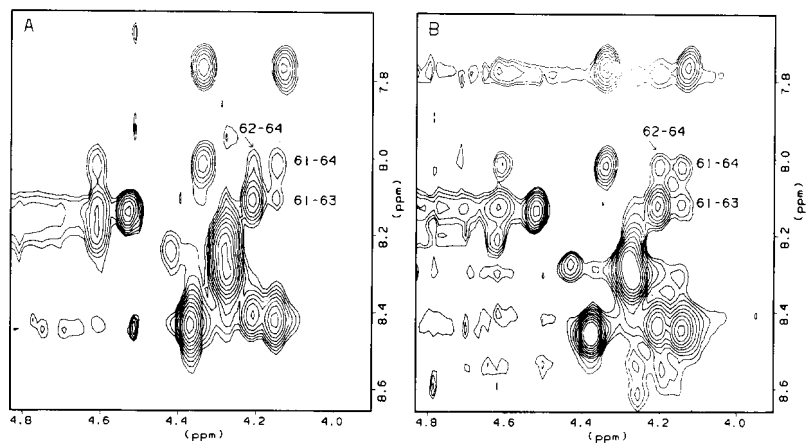

FIG. 3. **Transferred NOE cross-peaks between the NH and the αCH protons of the hirudin peptide.** The spectra were acquired with an NOE mixing time of 200 ms at a temperature of 25 °C. *A*, the concentration of the peptide was 6.3 mM with a peptide-prothrombin molar ratio of 28:1 and a pH value of 5.0. *B*, the peptide concentration was 7.7 mM with a peptide-thrombin molar ratio of 21:1 and a pH value of 5.3. Indicated are the medium range transferred NOEs between residues Glu^{61}, Glu^{62}, Tyr^{63}, and Leu^{64}. These NOEs are characteristic of a helical structure for the involved residues of the peptide in the thrombin-bound state (14, 15).

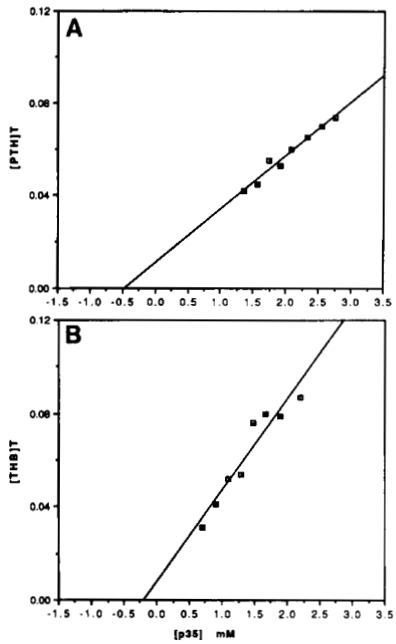

FIG. 4. **NMR titration plots for the interaction of prothrombin (*A*) or thrombin (*B*) with the hirudin peptide P35 (acetyl-Asp^{55}-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gly^{65}-OH).** The starting concentration of prothrombin was 0.064 mM and for thrombin 0.053 mM. The horizontal intercept gave an approximate K_d value of 500 μM for prothrombin binding and <100 μM for thrombin binding.

of the δCH_2 protons of Phe^{56} in peptide P35 with the peptide-prothrombin ratios between 21:1 and 40:1. In agreement with line broadening experiments, the peptide protons relax faster (shorter T_1 or enhanced relaxation) with increases in the fraction of bound peptides. An approximate K_d of 500 μM was estimated for prothrombin binding from the horizontal intercept of the titration plot. Similar experiments were also carried out with human thrombin with peptide-thrombin ratios between 13:1 and 35:1 (Fig. 4*B*). However, NMR relaxation experiments can determine binding constants accurately only in the millimolar range (25). The large scatter in the data suggests a K_d < 100 μM for the thrombin-peptide interaction. The binding of both hirudin peptides P24 and P35 to thrombin has been studied by the inhibition of thrombin-induced fibrinogen clot formation (19, 30). Based on an inhibition constant (K_i) of 1–2 μM for peptide P24, we can estimate a binding constant K_d of 2–100 μM for the interaction between human thrombin and peptide P35.

Results obtained from the present NMR measurements clearly demonstrate that the fibrinogen recognition exosite in prothrombin is partially open for peptide binding without the cleavage of any of the activation peptide bonds. Interactions of prothrombin with hirudin peptides are specific, as shown by identical patterns of differential line broadening and transferred NOEs observed for prothrombin and thrombin binding. However, prothrombin shows a weaker affinity (K_d ~ 500 μM) toward hirudin peptides than thrombin (K_d ~ 2 μM). This enhanced affinity with thrombin could be due to some conformational changes in the thrombin domain brought about by the cleavages of the Arg^{274}-Thr^{275} and the Arg^{323}-Ile^{324} peptide bonds during prothrombin activation. The binding energetics of the fibrinogen recognition exosite in thrombin have been analyzed in detail by a study of the interaction of thrombin with designed hirudin analogs (12, 30). It appears that the stability (as reflected by k_{off}) of the thrombin-inhibitor complex is determined primarily by hydrophobic contacts between the hirudin tail and the fibrinogen exosite on thrombin. The hydrophobic framework may already exist in the prothrombin exosite, as established by specific peptide line broadening effects observed in this study. Thus, reduced affinities may be a result of decreased electrostatic interactions between the prothrombin exosite and the hirudin tail peptides, leading to a decrease in the ligand on-rate (k_{on}). It is conceivable that there are transient interactions between the thrombin exosite and the flexible and negatively charged regions of fragment 1 or 2 in prothrombin (31), thereby partially neutralizing the positive electrostatic field required for the recognition of the negatively charged hirudin tail peptides. Such questions may be addressed in more detail by a direct NMR study of the structure and dynamics of prothrombin in solution.

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