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High-Resolution NMR Studies of Fibrinogen-like Peptides in Solution: Interaction of Thrombin with Residues 1–23 of the Aα Chain of Human Fibrinogen[†]

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ABSTRACT: The interaction of the following human fibrinogen-like peptides with bovine thrombin was studied by use of one- and two-dimensional NMR techniques in aqueous solution: Ala(1)-Asp-Ser-Gly-Glu-Gly-Asp-Phe(8)-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg(16)-Gly(17)-Pro-Arg(19)-Val(20)-Val-Glu-Arg (F10), residues 1–16 of F10 (fibrinopeptide A), residues 17–23 of F10 (F12), residues 1–20 of F10 (F13), residues 6–20 of F10 with Arg(16) replaced by a Gly residue (F14), and residues 6–19 of F10 with Arg(16) replaced by a Leu residue (F15). At pH 5.3 and 25 °C, the Arg(16)–Gly(17) peptide bonds of both peptides F10 and F13 were cleaved instantaneously in the presence of 0.6 mM thrombin, whereas the cleavage of the Arg(19)–Val(20) peptide bonds in peptides F12, F13, and F14 took over 1 h for completion. On the basis of observations of line broadening, fibrinopeptide A was found to bind to thrombin. While resonances from residues Ala(1)–Glu(5) were little affected, binding of fibrinopeptide A to thrombin caused significant line broadening of NH and side-chain proton resonances within residues Asp(7)–Arg(16). There is a chain reversal within residues Asp(7)–Arg(16) such that Phe(8) is brought close to the Arg(16)–Gly(17) peptide bond in the thrombin–peptide complex, as indicated by transferred NOEs between the aromatic ring protons of Phe(8) and the $C^{\alpha}H$ protons of Gly(14) and the $C^{\gamma}H$ protons of Val(15). A similar chain reversal was obtained in the *isolated* peptide F10 at a subzero temperature of −8 °C. The titration behavior of Asp(7) in peptide F13 does not deviate from that of the reference peptide, *N*-acetyl-Asp-NHMe at both 25 and −8 °C, indicating that no strong interaction exists between Asp(7) and Arg(16) or Arg(19). Peptides with Arg(16) replaced by Gly and Leu, respectively, i.e., F14 and F15, were also found to bind to thrombin but with a different conformation, as indicated by the absence of the long-range NOEs observed with fibrinopeptide A. Residues Asp(7)–Arg(16) constitute an essential structural element in the interaction of thrombin with fibrinogen.

The specific removal of fibrinopeptides A and B by thrombin exposes complementary polymerization sites near the N-termini of the Aα and Bβ chains (Laudano & Doolittle, 1980) located in the central domain of the soluble plasma protein fibrinogen (Telford et al., 1980), an event that initiates the spontaneous polymerization of the resulting fibrin monomer into the insoluble fibrin clot (Scheraga, 1983, 1986, and references cited therein). Much has been learned in recent years about the mechanism of the interaction of thrombin with fibrinogen, especially about the cleavage of the Arg–Gly peptide bond in the Aα chain of human fibrinogen (Scheraga, 1983, 1986, and references cited therein). Among other things, by use of an active-site mapping approach, it has been shown that residues Asp(7) and Phe(8), which are located 10 and 9 residues away, respectively, from the thrombin cleavage site, influence the effectiveness of the binding of synthetic peptide substrates to thrombin (Meinwald et al., 1980; Marsh et al., 1982, 1983). These kinetic data help explain the observations

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that Asp(7) and Phe(8) are strongly conserved in many species (Blombäck, 1967; Henschen et al., 1983) and that mutations of Asp(7) to Asn(7) and of Arg(19) to Asn(19) result in delayed release of fibrinopeptide A (FpA,[1] or F11; see Figure 1) and produce human bleeding disorders (Henschen et al., 1983; Mênachê, 1983, and references cited therein).

Through physicochemical techniques such as NMR spectroscopy, attempts have been made to identify conformational features of synthetic peptide substrates of thrombin in aqueous and in nonaqueous solutions such as DMSO (von Dreele et al., 1978; Rae & Scheraga, 1979; Marsh et al., 1985). As a continued effort, we have recently completed the proton resonance assignments of a synthetic peptide corresponding to residues Ala(1)–Arg(23) (F10) of the Aα chain of human fibrinogen (Ni et al., 1988). An NMR conformational analysis suggested that a β-bend might exist within residues Gly(12)–Val(15) of this larger peptide in aqueous environment. We have subsequently carried out an NMR study of various synthetic fibrinogen-like peptides complexed to thrombin. In this paper, we report evidence for the interaction of thrombin with specific residues within 1–23 of the Aα chain of human fibrinogen. Transferred NOE measurements of thrombin–peptide complexes also provided further information about the structural aspects of the interaction process. In the paper that follows (Ni et al., 1989), we describe the structure of a thrombin-bound peptide corresponding to residues Asp(7)–Arg(16) of the Aα chain of human fibrinogen.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification. The tripeptide Gly-Pro-Arg was a commercial product (Bachem, Switzerland) and was used without further purification. Synthesis and purification of peptides F10, F11, and F12 (Figure 1) were carried out as described in our previous work (Ni et al., 1988).

Peptides F13, F14, and F15 were prepared by solid-phase peptide synthesis using Fmoc-amino acids (Konishi & Rivetna, 1989). Except for serine and arginine, the amino acids used in the synthesis were Fmoc-L-amino acid pentafluorophenyl esters (Fmoc-L-amino acid *O*-Pfp) purchased from either Advanced ChemTech or Milligen. The *tert*-butyl group was used to protect the side chains of Asp and Glu. Fmoc-L-serine oxobenzotriazine ester was used for serine and was purchased from Milligen. Fmoc-L-argine (4-methoxy-2,3,6-trimethylphenylsulfonyl)[Fmoc-Arg-(Mtr)-OH] was used for arginine and was purchased from Advanced ChemTech and Milligen. *p*-Methylbenzhydrylamine resin hydrochloride used for peptide synthesis was purchased from Fluka Chemical Corp. After the last amino acid was attached, the coupling procedure was terminated at a point at which the peptide product retained the Fmoc group. The peptides on the resin were then washed twice with methylene chloride and dried under vacuum. The peptides were cleaved from the resin by using the conventional HF cleavage procedure at −15 to −5 °C for 1 h. A mixture of 10% anisole and 5% thioglycolic acid was used as a scavenger. The cleaved peptides were dissolved in TFA and then filtered through a polypropylene filter (Econo-column, Bio-Rad) and precipitated by adding this solution to ethyl ether.

Before removal of the Fmoc group, the peptides were dissolved in 50% acetic acid/water (TFA was added if necessary) and purified on an RPLC column (C_4, 10 × 250 mm, SynChrom, Inc.) by a 5–70% acetonitrile gradient in water in the presence of 0.3% TFA. The Fmoc groups on the purified peptides were then removed by using a procedure described elsewhere (Konishi & Rivetna, 1989). The purity of the deprotected peptides was assessed by use of an analytical RPLC column (C_4, 4.6 × 250 mm, Vydac) with a 0–45% acetonitrile gradient in water in the presence of 0.3% TFA. The purified peptides were further characterized by amino acid analyses that were performed with a Waters Pico Tag amino acid analysis system at the Cornell University Biotechnology Program Facility. The samples were hydrolyzed and derivatized according to the method of Bidlingmeyer et al. (1984). The amino acid composition of peptide F13 was Asp (1.99, 2), Glu (2.04, 2), Ser (0.97, 1), Gly (6.03, 6), Arg (2.10, 2), Ala (1.99, 2), Pro (1.03, 1), Val (1.81, 2), Leu (1.03, 1), and Phe (1.01, 1). That of F14 was Asp (0.95, 1), Glu (1.03, 1), Gly (6.01, 6), Arg (1.14, 1), Ala (0.91, 1), Pro (1.09, 1), Val (1.96, 2), Leu (0.97, 1), and Phe (0.94, 1). That of F15 was Asp (0.96, 1), Glu (0.98, 1), Gly (5.26, 5), Arg (1.06, 1), Ala (0.91, 1), Pro (1.10, 1), Val (0.95, 1), Leu (1.84, 2), and Phe (0.95, 1).

Preparation of Bovine Thrombin. Bovine thrombin was obtained from bovine plasma barium citrate eluate (Sigma) by using an activation procedure similar to that described previously (Ghosh & Seegers, 1980). Briefly, the lyophilized powders of the plasma citrate eluate were dissolved up to 110 mg/mL in a buffer that was 50 mM in Tris, 150 mM in NaCl, and 0.1% in PEG 6000 at pH 7.4. The dissolved plasma eluate was gel filtered (Sephadex G-25) into the same buffer by using a PD10 minicolumn (Pharmacia). The gel-filtered solution was then diluted to 10 mg/mL in plasma eluate, and 100 mM $CaCl_2$ was added to a final Ca^{2+} concentration of 10 mM. Activation commenced upon addition of crude *Echis carinatus* venom (Sigma, 1.0 mg/mL in Tris buffer, pH 7.4) to a final concentration of 0.1 mg/mL. The activation was completed after incubation at 37 °C for 1 h.

The activation mixture was gel filtered (Sephadex G-25) into a buffer that was 25 mM in sodium phosphate at pH 6.5. Purification of thrombin was carried out by FPLC (Spectra Physics Model 8700) on a Mono S HR 10/10 (Pharmacia) cation-exchange column equilibrated with the sodium phosphate buffer. Thrombin was eluted by a gradient of 0–500 mM NaCl in 60 min at a flow rate of 2 mL/min. The pool containing bovine thrombin was shown to be homogeneous by SDS–PAGE. Active-site titration using p-NPGB (Chase & Shaw, 1969) showed that the thrombin preparation was more than 95% intact. Pooled thrombin was concentrated by an ultrafiltration apparatus (Amicon) and gel filtered (Sephadex G-25) into a buffer that was 50 mM in sodium phosphate, 150 mM in NaCl, and 0.5 mM in EDTA at pH 5.3. The final thrombin concentration was determined to be 25–35 mg/mL by its absorbance, using the value of $E_{1\%}^{280} = 19.5$ (Winzor & Scheraga, 1964). The concentrated thrombin solution was divided into 0.1-mL aliquots and stored at −70 °C before use.

NMR Sample Preparation and Measurements. A stock solution of peptide F10 was prepared by dissolving 26.6 mg of the peptide in 0.1 mL of D_2O (Cambridge Isotopes) and 0.3 mL of an aqueous solution (H_2O) that was 150 mM in NaCl, 50 mM in sodium phosphate, and 0.1 mM in EDTA, with the pH adjusted to 5.3. Stock solutions of F11 and F12

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser and exchange spectroscopy; FID, free induction decay; DMSO, dimethyl sulfoxide; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; EDTA, ethylenediaminetetraacetic acid; Fmoc, N^{α}-9-fluorenylmethyloxycarbonyl; FpA, fibrinopeptide A; PEG 6000, polyethylene glycol with an average molecular weight of 6000; FPLC, fast protein liquid chromatography; RPLC, reverse-phase high-performance liquid chromatography; p-NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

were prepared at 12 and 4 mg/mL, respectively, in a 50 mM sodium phosphate buffer that was 150 mM in NaCl and 0.1 mM in EDTA, with the pH adjusted to 5.3. The solutions of F11 and F12 were then diluted to 0.5 mL with the same buffer to make up three samples of mixtures of F11 and F12 at volume ratios of 0:1, 0.75:1, and 3.2:1 (all at a constant concentration of ~1.3 mM in F12) of the stock solutions. The pH values of the resulting peptide solutions were then adjusted to 5.13 (F10) and 5.26 (F11/F12 mixtures) by using trace amounts of 1 M HCl or 1 M NaOH. Samples of the F11 and F12 mixtures were then freeze-dried; the dried powders (containing peptides and salts) were taken up in 0.04 mL of D_2O and 0.29 mL of H_2O, producing 0.33 mL of the final samples with a pH of 5.37. The pH values of the aqueous samples were all checked by use of an Ingold electrode without correction for the presence of D_2O. NMR samples of the peptide–thrombin solutions were prepared immediately before the experiment by mixing appropriate volumes of the thrombin stock solution and the peptide solutions prepared above.

For peptides F13, F14, and F15, sample preparation was more complicated because of their lower solubility. In general, dilute solutions of the peptides were first made by use of a buffer with a lower salt concentration, and the pH of the resulting solutions was adjusted to 5.3. The pH-adjusted solutions were then freeze-dried; the dried powders (including salt and peptides) were taken up in appropriate amounts of H_2O (90%) and D_2O (10%) so that the concentrations of the peptides were all about 25 mM. The peptide solutions were then centrifuged to remove possible precipitates that may have formed during the adjustments of pH.

For the pH titration of the protons resonances, eight samples of approximately 1 mM each in F13 were prepared in a buffer (90% H_2O/10% D_2O) that was 150 mM in NaCl, 50 mM in sodium phosphate, and 0.1 mM in EDTA, with DSS added as internal references (at 0 ppm). The pHs of these samples were then adjusted to 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.5, respectively, without the correction for the effect of D_2O.

NMR experiments were carried out on a GN-500 500-MHz spectrometer described previously (Ni et al., 1988). Spectral data were analyzed on a Sun 3/60 workstation using the NMR1/NMR2 software system (Levy et al., 1986) provided by New Methods Research Inc. (Syracuse, NY). All one-dimensional data were processed with a 0.5-Hz Lorentzian apodization to increase the signal-to-noise ratio in the spectra.

Monitoring the Cleavages of the Arg(16)–Gly(17) and Arg(19)–Val(20) Peptide Bonds by Thrombin. The cleavages of the Arg(16)–Gly(17) and Arg(19)–Gly(20) peptide bonds were followed at various times by recording one-dimensional proton spectra of the peptide–thrombin mixtures in 90% solutions.

For peptides F10, F13, F14, and F15, 0.1 mL of the pH-adjusted peptide solutions was mixed with 0.3 mL of the thrombin stock solution (giving a peptide concentration of ~6 mM, or less if there was precipitate formed in the preparation of the peptide stock solutions, and a thrombin concentration of ~6 mM) immediately before (1–3 min) the acquisition of spectra data was begun. A total of 32 accumulations was collected at each time point with a total acquisition time of 1.2 min. For F11/F12 mixtures, 0.07 mL of the thrombin stock solutions was introduced into the NMR sample tubes containing the 0.33-mL peptide mixtures prepared as described in the previous section. This gave a final thrombin concentration of about 0.14 mM and a F12 concentration of about 1.3 mM for all three F11/F12–thrombin samples with different concentrations of peptide F11. The recording of one proton spectrum for the F11/F12 mixtures required 128 accumulations with a total acquisition time of 5.8 min at each time point.

Two-Dimensional Transferred NOE Measurements. The pulse sequence for measuring a two-dimensional transferred NOE spectrum (Clore et al., 1986; Andersen et al., 1987; Meyer et al., 1988) was the same as for the regular NOESY procedure used previously (Ni et al., 1988). Residual transverse magnetization and multiple-quantum coherences (except for zero-quantum) were eliminated by a short z_1-gradient pulse at the beginning of the mixing period (Jeener et al., 1979; Macura et al., 1981). This homospoil pulse also served to eliminate any residual water signal in the transverse plane due to imperfections of the 90° pulses (Basus, 1984). To avoid excitation of the solvent, the carrier frequency was placed at the position of the water proton resonance, and the observation pulse was replaced by the jump–return sequence (Plateau & Guēron, 1982) with a delay of 0.118 ms. Proper cycling of the phases of the RF transmitter and receiver was also adopted for the suppression of multiple-quantum coherences and other spectrometer artifacts not eliminated by the homospoil pulse (Wider et al., 1984). Postacquisition delay was set to a relatively short value of 800 ms as a result of more efficient z-magnetization recovery induced by the presence of the enzyme (Andersen et al., 1987). A total of 256 or 512 FIDs of 1K complex data points was acquired with time-proportional phase incrementation (TPPI; Redfield & Kuntz, 1975; Bodenhausen et al., 1980) along the t_1 direction (Marion & Wüthrich, 1983). Typically, 96 or 128 accumulations were collected for each FID. The FID matrices were all premultiplied by the square of a 54° shifted sine bell in both the t_1 and t_2 directions. The pure-phase (Aue et al., 1976) spectra of size 1K × 1K were then calculated by complex Fourier transformations in the t_2 direction and phased in the f_2 direction to pure absorption by using phase parameters obtained from the first slice of the FID matrix (corresponding to zero t_1). The imaginary parts of the f_2-transformed data matrices were set to zero and then complex Fourier transformed along the t_1 direction. A procedure for t_1-ridge suppression was also employed to improve spectral clarity (Otting et al., 1986). Phase adjustments in the f_1 direction were minimal and only zeroth order (typically between −10° and +10°) for the Bessel filter setting of the receiver.

RESULTS

Confirmation of Peptide Sequences and Assignments of Proton Resonances. The amino acid sequences and assignments of sequence-specific proton resonances of peptides F10, F11, and F12 in aqueous solutions at pH 5.3 were established in our previous work (Ni et al., 1988). The sequences of peptides F13, F14, and F15 were confirmed by an analysis of their NOESY spectra at 5 °C, pH 5.3, compared to that of F10 at the same temperature. The chemical shifts of the proton resonances of each residue in peptide F13 are similar to those in F10 under identical solvent conditions, with the exception of the NH proton of Val(20), which appears at 8.16 ppm compared to 8.26 ppm in peptide F10 (Ni et al., 1988). Side-chain proton chemical shifts for residues in F14 and F15 are also similar to those in F10. The exchange rates of the NH protons of Asp(7) in F14 and F15 are both very large because of their location close to the free N-terminal group, similar to that of Asp(2) in peptides F10 and F11 (Ni et al., 1988). Most NH and $C^{\alpha}H$ proton resonances in F14 and F15 appear practically at the same chemical shifts except for those in the sequences Gly(6)-Asp(7)-Phe(8) and Val(15)-Gly(16)-Gly(17) or Val(15)-Leu(16)-Gly(17), which are easily

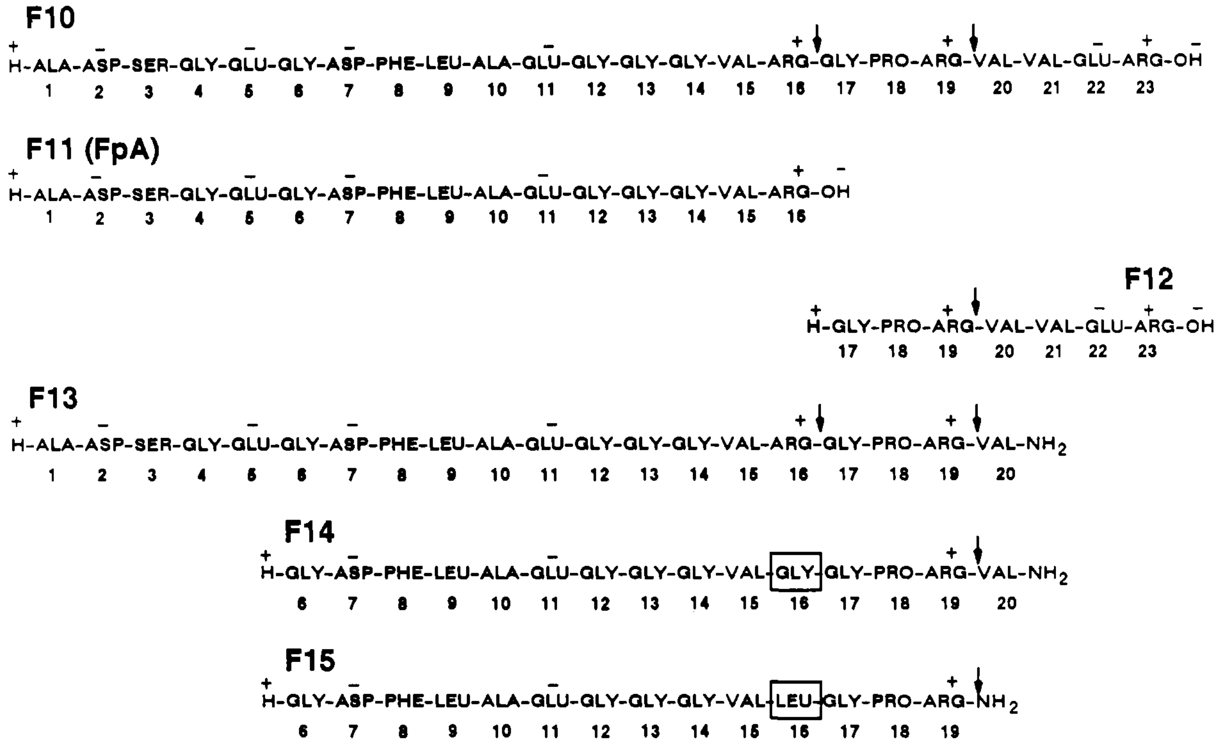

FIGURE 1: Amino acid sequences of peptides F10–F15. The numbering is that of the Aα chain of human fibrinogen, and the charges are those at neutral pH. The arrows in each sequence indicate the peptide bonds cleaved by thrombin. The boxes indicate the residues substituted by other amino acids.

identified when compared with the one- and two-dimensional proton spectra of F10 or F11.

Cleavage of the Arg(16)–Gly(17) and Arg(19)–Val(20) Peptide Bonds by Thrombin at Low pH. The Arg(16)–Gly(17) peptide bonds in peptide substrates are readily cleaved at neutral pH by catalytic amounts of thrombin, as demonstrated with fibrinogen-derived peptides (Hogg & Blombäck, 1978) and with synthetic fibrinogen-like peptides (Liem & Scheraga, 1974; van Nispen et al., 1977; Meinwald et al., 1980; Marsh et al., 1982, 1983). The cleavage of the Arg(19)–Val(20) peptide bond (Figure 1), although not involved in the interaction of thrombin with fibrinogen under physiological conditions, has also been observed with fibrin and degradation products of fibrinogen (Blombäck et al., 1967; Kudryk et al., 1974; Hageman & Scheraga, 1974) and short synthetic fibrinogen-like peptides (van Nispen et al., 1977; Meinwald et al., 1980; Marsh et al., 1983).

It has been demonstrated that thrombin is fully active at low pH, around 5.3, in its interaction with fibrinogen (Ehrenpreis et al., 1958). The thrombin cleavage of peptide bonds in synthetic peptides, however, has not been studied at low pH (pH 5.3), where the proton resonances of peptides F10, F11, and F12 were assigned (Ni et al., 1988). Figure 2 shows the NH region of the proton NMR spectra of peptide F10 at various times after mixing with active thrombin at pH 5.3. Spectral changes of most of the NH proton resonances can be observed within the first few hours after the addition of thrombin. Previously, we have established that the NH proton of Arg(16) in peptide F10 appears at 8.48 ppm at pH 5.3 and 25 °C, whereas it appears at 8.05 ppm in F11 in which it is located at the carboxyl terminus (Ni et al., 1988). At the high concentration of thrombin (~0.6 mM) used to obtain the spectra of Figure 2, the Arg(16)–Gly(17) bond is cleaved *before* spectrum A was obtained. This conclusion is based on the observation that the peak intensity at 8.48 ppm does not change over the entire time course of spectral acquisition. The observed spectral changes (e.g., the increase in peak intensity at around 8.05 ppm) may thus be attributed to the further cleavage of the Arg(19)–Val(20) peptide bond.

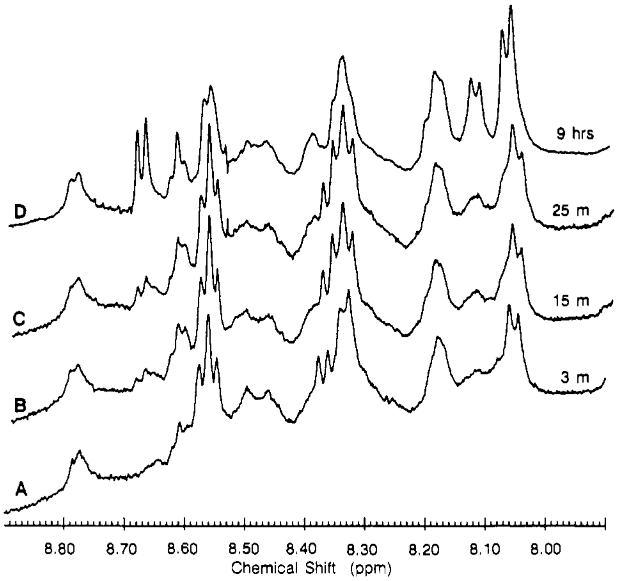

FIGURE 2: NMR spectra for the time course of the interaction of thrombin with peptide F10 in buffered H_2O solution at pH 5.3 and ambient temperature (~21 °C). (A) NH region of the proton NMR spectra taken 3 min after mixing 0.3 mL of the stock thrombin and 0.1 mL of the stock F10 solutions: concentration of thrombin, ~0.6 mM; mole ratio of F10 to thrombin, 10:1; (B) after 15 min; (C) after 25 min; (D) after 9 h.

To confirm this conclusion, we compared the spectra in Figure 2 with spectra obtained after thrombin was added to a premixture of F11 and F12, as shown in Figure 3. We see a close resemblance between trace A in Figure 2 and trace B in Figure 3, especially for the line-broadened NH resonances. The sequence-specific resonance assignments indicated in trace A of Figure 3 were reported previously for F11 and F12 (Ni et al., 1988). The assignments of NH protons in trace D to

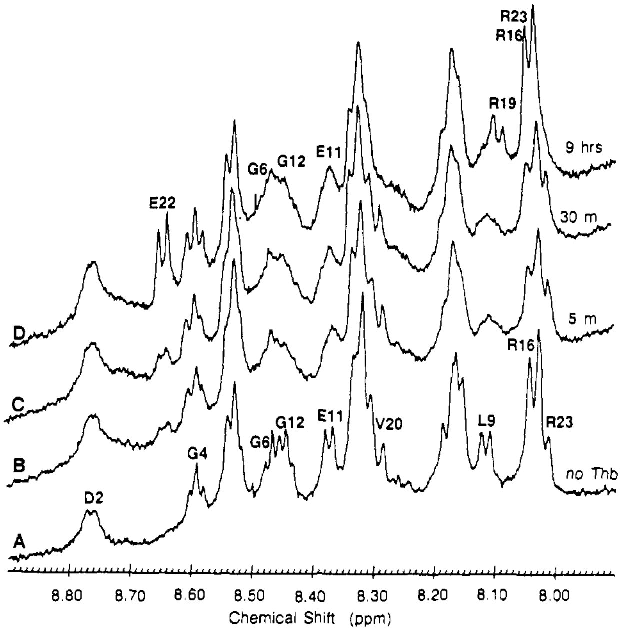

FIGURE 3: NMR spectra for the time course of the interaction of thrombin with premixed peptides F11 and F12 in buffered H_2O solution at pH 5.3 and 25 °C: concentration of F11, ~2 mM; concentration of thrombin, ~0.14 mM. The resonance assignments pertain to the NH proton of the indicated residue. (A) NH region of the proton NMR spectra of an approximately 3:2 (mole ratio) mixture of F11/F12 taken before the introduction of thrombin; (B) 5 min after mixing with thrombin; (C) after 30 min; (D) after 9 h.

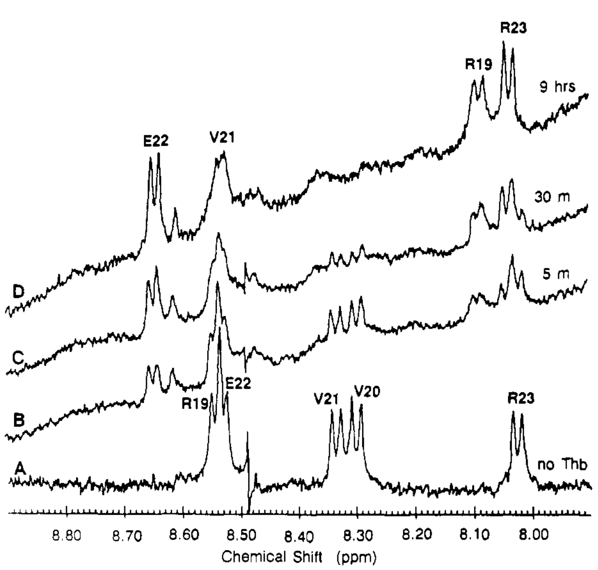

FIGURE 4: NMR spectra for the time course of the interaction of thrombin with peptide F12 in buffered H_2O solution at pH 5.3 and 25 °C: concentration of F12, ~1.3 mM; mole ratio of F12 to thrombin, 10:1. The resonance assignments pertain to the NH proton of the indicated residue. (A) NH region of the proton NMR spectra of peptide F12 taken before the introduction of thrombin; (B) 5 min after mixing with thrombin; (C) after 30 min; (D) after 9 h.

the thrombin cleavage products of F12 were further confirmed by monitoring the time course of thrombin cleavage of F12 as shown in Figure 4. The broad peak at 8.55 ppm was assigned to the NH proton of Val(21) from its fast exchange behavior similar to that of Asp(2) (see Figures 2 and 3) due to the influence of the positively charged free N-terminus in ^+H_3N-Val-Val-Glu-Arg and in F11 (Ni et al., 1988). The doublet at 8.10 ppm was assigned to the NH of Arg(19) from a comparison with the proton spectra of the peptide Gly-Pro-Arg under the same aqueous solvent conditions. Arg(23), being at the carboxyl terminus, always has the most upfield shifted NH proton resonance (Ni et al., 1988). We therefore conclude that the spectral changes within the first few hours after peptide F10 is mixed with thrombin are the result of the cleavage of the Arg(19)–Val(20) peptide band. This conclusion is further supported by the observation that the spectral features in trace D of both Figures 2 and 3 remain unchanged after the samples have been left for a few months in an ice bath.

The interaction of thrombin with peptide F13 follows an identical pattern as with peptide F10. In peptides F14 and F15, Arg(16) has been replaced by Gly and Leu, respectively. It was expected that these replacements would make the peptides resistant to thrombin cleavage at this site. Indeed, the only observed spectral changes after these peptides have been mixed with thrombin were found to arise from thrombin cleavage at the Arg(19)–Val(20) site [Arg(19)–NH_2 in the case of peptide F15, see Figure 1].

The relatively slow cleavage of the Arg(19)–Val(20) peptide bond enabled us to carry out an experiment where the interaction of thrombin with peptide F12 was followed by measuring peak intensities of the NH proton of Glu(22) in peptide product Val-Val-Glu-Arg (Figures 2–4) at various times after mixing with thrombin. With a fixed concentration of peptide F12 (about 1.3 mM) and thrombin (about 0.14 mM), the rate of release of peptide Val-Val-Glu-Arg decreased with increasing concentrations of peptide F11. This indicates that peptide F11 inhibits the thrombin cleavage of the Arg(19)–Val(20) bond in peptide F12.

Interaction of Thrombin with Specific Residues in Synthetic Peptides. NMR spectroscopy can provide a wealth of information about the interactions of ligands with macromolecules such as enzymes or antibodies (Sykes & Scott, 1972; Dwek, 1973). Specifically, resonance peaks of the free ligands are significantly broadened particularly when the off-rate of the ligand binding is fast compared to the NMR time scale and the concentration of the free peptide is higher (>10-fold) than that of the macromolecule (Dwek, 1973). Protein-induced line broadening of peptide resonances has been utilized in various systems such as chymotrypsin plus peptide substrates (Gerig, 1968; Gerig & Reinheimer, 1970) and neurophysin plus peptide hormones (Balaram et al., 1973) to study the mechanism of the interaction processes.

In the interaction of thrombin with fibrinogen-like peptides, some NH proton resonances of the free peptides are strongly affected, as seen in both Figures 2 and 3. Since there is no indication of thrombin-induced line broadening in F12 or its cleavage products (Figure 4), the broadened resonance lines in both Figures 2 and 3 are due solely to the interaction between thrombin and peptide F11. This observation suggests that the interaction between thrombin and peptide F12 is weak compared to that between thrombin and peptide F11. Undoubtedly, the slow cleavage of the Arg(19)–Val(20) peptide bond is due to this weak interaction.

On the basis of the sequence-specific resonance assignments for peptides F11 and F12 (Ni et al., 1988), we were able to study the involvement of individual residues of the peptides in the interaction with thrombin. It is seen in Figure 3 that the NH protons of Asp(2) and Gly(4) are not much affected by thrombin binding compared to those of the *free* peptide, whereas the NH protons of Gly(6), Glu(11), and Gly(12) are severely broadened. In Figure 5, the NH regions of the one-dimensional spectra are displayed along with the NH–$C^{\alpha}H$ correlation (NOE and zero-quantum coherence transfer) maps of peptide F11 (in a mixture of F11 and other thrombin cleavage products of F10) in the presence of different con-

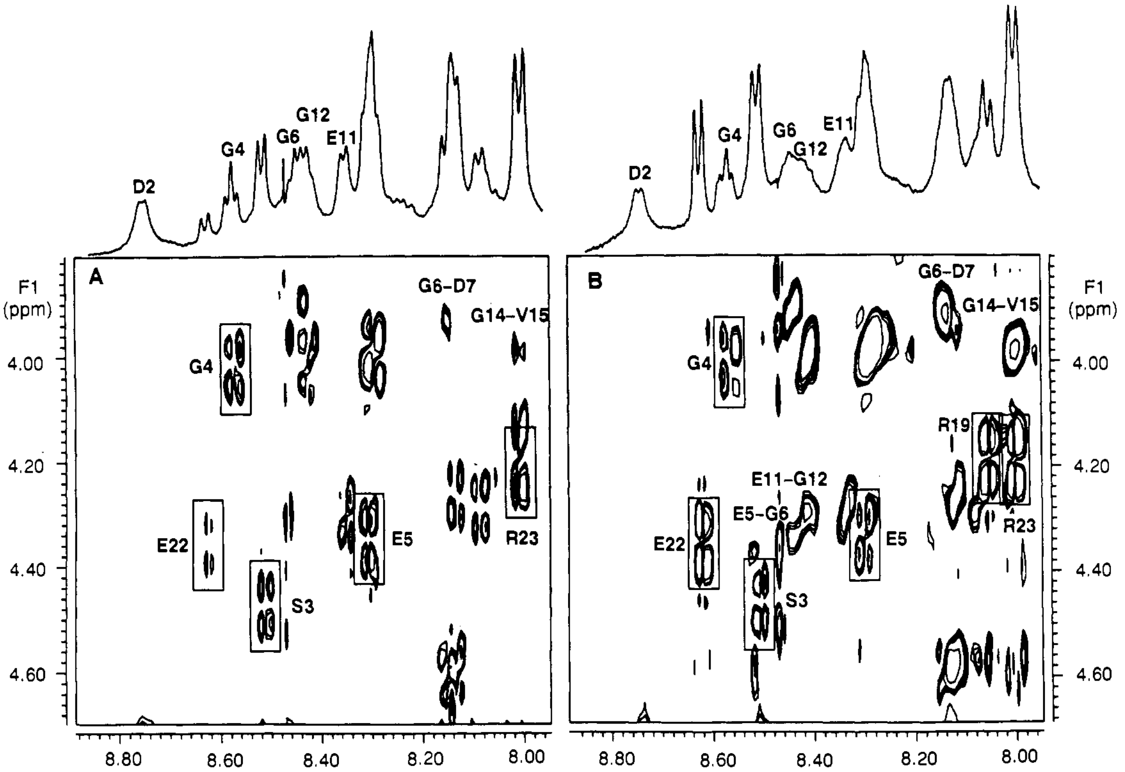

FIGURE 5: NOE and zero-quantum C$^\alpha$H–NH correlations (with a mixing time of 200 ms) of peptide F11 and thrombin cleavage products of F12 in a mixture of F11 and F12 in buffered H_2O solution at pH 5.3 and 25 °C with different concentrations of thrombin: (A) Thrombin concentration, 0.14 mM; mole ratios, F11:thrombin ~40:1, F11:F12 6:1. (B) Thrombin concentration, 0.6 mM; mole ratios, F11:thrombin 10:1, F11:F12 1:1 [arising as a result of the thrombin cleavage of the Arg(16)–Gly(17) peptide bond in F10]. Boxed cross peaks are from the combination of NOE interactions and zero-quantum J coherence transfers between the C$^\alpha$H and NH protons of the residues indicated. Interresidue sequential NOEs are also indicated by the residues involved.

centrations of thrombin. Overlapping peaks are resolved in the two-dimensional correlation spectra. With a lower thrombin concentration (~0.14 mM) and a short mixing time (200 ms), the intraresidue NH–C$^\alpha$H cross peaks are dominated by two-dimensional antiphase multiplets arising from zero-quantum coherence transfers (Macura et al., 1981) (Figure 5A). As the thrombin concentration increases (Figure 5B), zero-quantum coherence transfers are attenuated if there is thrombin-induced resonance line broadening or if exchange-enhanced intraresidue NH–C$^\alpha$H NOEs exist (transferred NOEs, see the next section). We see that most antiphase intraresidue NH–C$^\alpha$H cross peaks collapse into single peaks at the higher thrombin concentration, except for those from Ser(3), Gly(4), Glu(5), Arg(19), Glu(22), and Arg(23). Cross peaks between the C$^\alpha$H and NH protons from Asp(2) and Val(21) are missing because of the aforementioned fast NH exchange of the two residues. The cross peak from Arg(16) still overlaps with that of Arg(23), but an analysis of this two-dimensional multiplet pattern indicates that it has a similar intensity to that of a *single* set of antiphase multiplet between the NH and C$^\alpha$H protons of Arg(19) or Glu(22). This means that Arg(16) contributes very little to the intensity of the overlapped cross peak. Thus, the NH peak of Arg(16) may also be broadened upon thrombin binding.

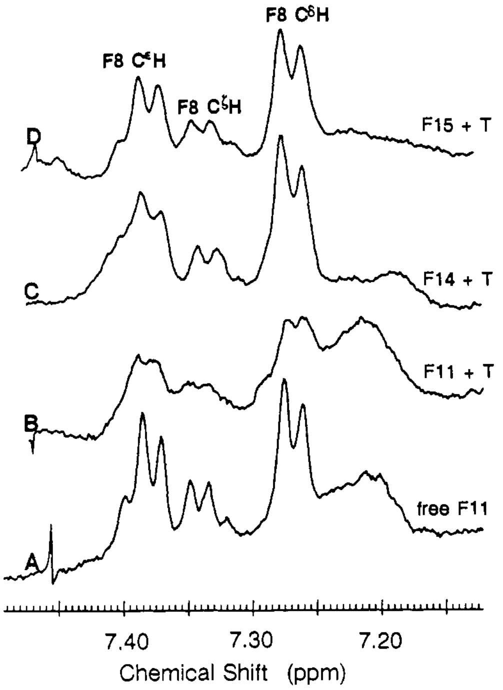

FIGURE 6: Resonances of the ring protons of Phe(8) in peptides F11, F14, and F15 in the presence and absence of thrombin in buffered H_2O solution at pH 5.3 and 25 °C: (A) Free F11 (2 mM); (B) F11 (2 mM) in presence of 0.14 mM thrombin; (C) F14 (6 mM) in the presence of 0.6 mM thrombin; (D) F15 (6 mM) in the presence of 0.6 mM thrombin.

The side-chain proton resonances are also affected by thrombin binding. The most pronounced effects are on the aromatic protons of Phe(8), as seen when a comparison is made between parts B (peptide plus thrombin) and A (peptide alone) of Figure 6. Figure 7 shows the side-chain aliphatic region of the proton spectra of F10 or of a mixture of F11 and F12 with thrombin. The C$^\beta$H protons of Asp(7) are slightly broadened at higher concentrations of thrombin. The C$^\beta$H protons of Phe(8) and Ala(10) and the C$^\delta$H protons of Leu(9) are severely affected (line broadening and reduction of peak intensities) by thrombin. The side-chain proton resonances of Glu(11), Val(15), and Arg(16) are not resolved from those

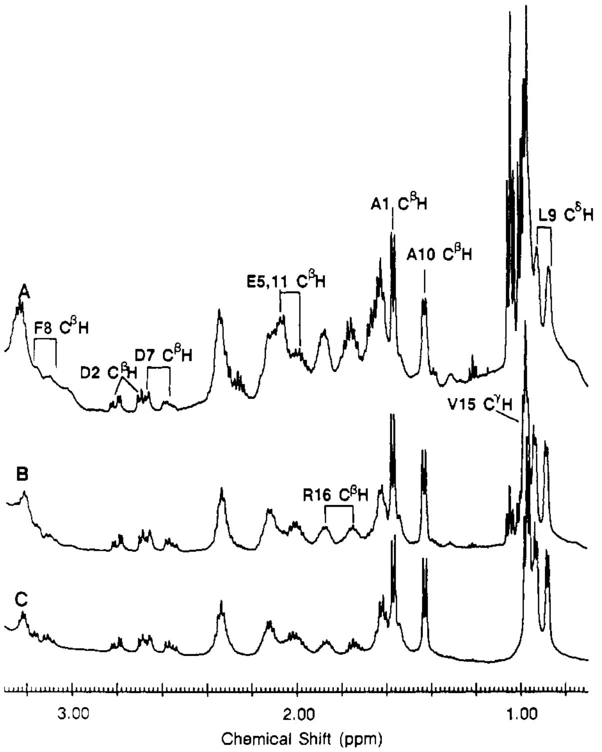

FIGURE 7: Thrombin-induced resonance line broadening in mixtures of peptides F11 and F12 in buffered H$_2$O solution at pH 5.3 and 25 °C: (A) Thrombin–F10 interaction (mole ratios, F11:F12 1:1, F10:thrombin 10:1; thrombin concentration, 0.6 mM); (B) thrombin–F11/F12 interaction (mole ratios, F11:F12 6:1, F11:thrombin 40:1; thrombin concentration, 0.14 mM); (C) mixture of F11 and F12 (mole ratio, F11:F12 6:1) without thrombin.

of Glu(5), Pro(18), Arg(19), Val(20), Val(21), Glu(22), and Arg(23) in the mixture of F10 with thrombin [Figure 7A; see also Ni et al. (1988)]. In Figure 7B, C, where the concentration of F11 is about 6 times that of F12 [and hence that of Gly-Pro-Arg and Val-Val-Glu-Arg after thrombin cleavage of the Arg(19)–Val(20) peptide bond], thus reducing interferences from other arginines and valines, we see that peaks from the C$^{\gamma}$H protons of Val(15) and the C$^{\beta}$H protons of Arg(16) are broadened even at a large ratio (40:1) of peptides to thrombin (Figure 7B), compared to those in the absence of thrombin (Figure 7C). The side-chain proton resonances of Glu(5) and Glu(11) do not appear to be affected, but due to their overlap and overlap with C$^{\beta}$H of Val(15) (Ni et al., 1988), it is difficult to observe possible small broadening of the C$^{\beta}$H and C$^{\gamma}$H peaks of Glu(11). On the other hand, it is clear that the C$^{\beta}$H protons of Ala(1) and Asp(2) are not affected by thrombin binding. As noted above, none of the NH protons in peptide F12 or in its thrombin cleavage products appears to be affected by thrombin (Figures 2–4). Furthermore, no line broadening of the side-chain protons of the residues in these peptides could be detected.

Thrombin also binds to peptides F14 and F15 in which Arg(16) of the natural sequence is replaced by Gly or Leu, respectively. Since the Gly(16)–Gly(17) and the Leu(16)–Gly(17) peptide bonds are not cleaved by thrombin, the sequence Gly(17)-Pro(18)-Arg(19) is present in the bound peptides. Analysis of the one-dimensional spectra (not shown) of the F14–thrombin complex and the F15–thrombin complex revealed that the side-chain protons of Pro(18) and Arg(19) in both F14 and F15 are broadened upon thrombin binding. Overall, however, there seems to be less pronounced line broadening in F14 and F15 compared to F11 with a similar thrombin concentration and with the same or lower concentrations of peptides F14 and F15 [see, e.g., the resonances of the ring protons of Phe(8) in these peptides (Figure 6C,D)].

Transferred NOEs of Thrombin-Bound Peptides. In a previous study, it was found that, at 500 MHz, peptide F11 exhibits very weak negative NOEs in aqueous solutions at 25 °C and only with a very long mixing time (500 ms) (Ni et al,. 1988). This is presumably due to the fact that the tumbling correlation time of this peptide, with a molecular weight of ~1500, is such that interproton nuclear Overhauser effects (NOEs) barely depart from the null region between the extreme narrowing limit (positive NOEs) and the spin-diffusion limit (negative NOEs) (Balaram et al., 1973). Under this condition, the NOE buildup rates would be vanishingly small and, thus, a long mixing time is required for the magnetization to be exchanged between spatially nearby nuclei (Macura & Ernst, 1980).

When the peptide binds to thrombin (molecular weight 36000), however, the internuclear rotational correlational times for the bound peptide are determined principally by the tumbling of the thrombin–peptide complex; i.e., binding to thrombin shifts the peptide more toward the "spin diffusion regime" of the internuclear motion (Balaram et al., 1973). The fast exchange (compared to the T_1 time scale of the proton resonances of both the free and bound peptide) of the peptide between the free and bound states serves to establish a nonequilibrium spin distribution (NOEs) in the *free* peptide molecules that is characteristic of the *bound-state* conformation (Clore & Gronenborn, 1982, 1983). Transferred NOEs have been measured by use of both one-dimensional and two-dimensional NMR techniques for the determination of bound-state substrate and inhibitor conformations in various systems involving enzyme–substrate interactions (James, 1976; Albrand et al., 1979; Levy et al., 1983; Clore & Gronenborn, 1982, 1983; Clore et al., 1986; Andersen et al., 1987; Meyer et al., 1988).

Figure 5 demonstrates the observation of NOEs in peptide F11 as induced by thrombin. With a ~40:1 ratio of peptide to thrombin (Figure 5A), the transferred NOEs are from the i to $i+1$ interresidue C$^{\alpha}$H–NH interactions between residues Gly(6) and Asp(7) and between residues Gly(14) and Val(15). As the thrombin concentration increases, the sequential C$^{\alpha}$H–NH NOEs between Glu(5) and Gly(6) and between Glu(11) and Gly(12) begin to be observable (Figure 5B).

To eliminate possible NOE cross peaks from thrombin itself, the experiment was repeated at various temperatures, since the resonance peaks from the peptides shift in a known manner (Ni et al., 1988). Furthermore, efforts have been made to locate cross peaks from thrombin alone. It was found that NOE peaks arising from thrombin diminish as the temperature is decreased (≤15 °C). An analysis of the NOE maps at 15 and 25 °C revealed strong sequential C$^{\beta}$H–NH interactions between residues Ala(10) and Glu(11) (Table I). Other NOEs include strong sequential C$^{\alpha}$H–NH interactions between residues Gly(6) and Asp(7), Asp(7) and Phe(8), and Glu(11) and Gly(12) and weak sequential C$^{\alpha}$H–NH interactions between residues Glu(5) and Gly(6), Phe(8) and Leu(9), Leu(9) and Ala(10), and Ala(10) and Glu(11). There are also intraresidue NH–C$^{\beta}$H interactions in residues Glu(5), Asp(7), Phe(8), Leu(9), Ala(10), Glu(11), and Val(15). No other NOEs were observed in the region from residue Ala(1) to residue Gly(6).

In the previous study, NH–NH NOEs were not observed for F10 or F11 at short mixing times (<300 ms) until the temperature was lowered to 5 °C or less (Ni et al., 1988). In the presence of thrombin, the sequential NH–NH NOEs

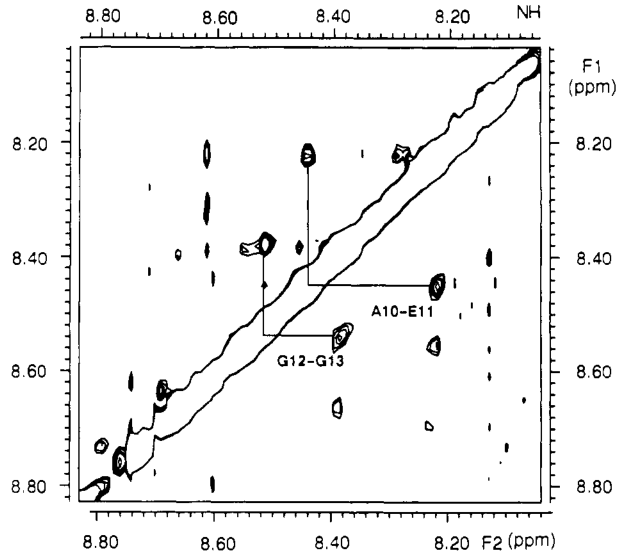

FIGURE 8: Transferred NH–NH NOE map obtained in buffered H_2O solution of the interaction of thrombin with peptide F11 (in mixture of F11 and F12) at pH 5.3: thrombin concentration, 0.6 mM; mole ratios, F11:thrombin 10:1, F11:12 1:1 [arising as a result of the thrombin cleavage of the Arg(16)–Gly(17) peptide bond in F10]. The NOESY spectrum was acquired with a mixing time of 200 ms at 15 °C.

between Ala(10) and Glu(11) and between Gly(12) and Gly(13) are selectively enhanced at 15 °C (or 25 °C) even at a mixing time of 200 ms (Figure 8). More interestingly, in the same spectrum (Figure 9A), NOE cross peaks were also found between the ortho ring protons (C^{δ}Hs) of Phe(8) and the C^{δ}H protons of Leu(9) and between the meta ring protons (C^{ϵ}Hs) of Phe(8) and the C^{α}H protons of Gly(13) or Gly(14) (their C^{α}Hs are not resolved). We have temporarily assigned the Phe–Gly interactions to that between Phe(8) and Gly(14) on the basis of the presence of NOEs between the meta ring protons (C^{ϵ}Hs) of Phe(8) and the C^{γ}H protons of Val(15) (Figure 9A). This assignment is confirmed by further experiments, involving another peptide, in the accompanying paper (Ni et al., 1989). A further analysis of the NOE spectrum of the *isolated* peptide F10, acquired with a mixing time of 500 ms at -8 °C (Ni et al., 1988), revealed the presence of long-range NOEs in the *free* peptide (Figure 9B) similar to those in the thrombin-bound peptide F11 (Figure 9A). This finding confirms the assignment of the long-range NOEs in the thrombin–F11 complex discussed above. More importantly, it also suggests that residues Gly(17)–Arg(23) might not contribute much to the stability of structure within residues Asp(7)–Arg(16) when the peptide is bound to thrombin (see Discussion).

Some transferred NOEs were also observed for the interaction of thrombin with peptides F14 and F15. With F15 in particular, the higher solubility of which allowed the acquisition of NOE spectra of better quality, in addition to the sequential and intraresidue NOEs discussed above for F11, there are transferred sequential C^{β}H–NH NOEs between Asp(7) and Phe(8), between Phe(8) and Leu(9), and between Leu(9) and Ala(10). There are also sequential NH–NH NOEs between Phe(8) and Leu(9) and between Gly(16) and Gly(17). However, long-range NOEs between Phe(8) and Gly(14) and Val(15), as seen in the complex of peptide FpA with thrombin, were absent in the complex of peptide F14 with thrombin.

Titration Shifts of Amide Proton Resonances. It has been established in the previous sections that thrombin binds preferentially to residues Asp(7)–Arg(16) within fibrinopeptide A. Further experiments involving peptides F14 and F15 demonstrated that, if Arg(16) is replaced by Gly(16) or Leu(16), residues Pro(18) and Arg(19) also interact with thrombin as indicated by the thrombin-induced line broadening of the side-chain proton resonances of these two residues (spectra not shown). To define the spatial relationship between residues in fibrinopeptide A and residues Gly(17)–Arg(19), particularly to identify possible ionic interactions between Asp(7) and Arg(16) or Arg(19), we have studied the pH titration shifts of various proton resonances, especially those of the amide protons, of all the residues in peptide F13 with which possible complicating interferences from Gly(22) and Arg(23) of F10 have been eliminated. The experiments were carried out at both 25 and -8 °C, where *isolated* peptide F10 exhibits a long-range NOE pattern similar to that of fibrinopeptide A bound to thrombin.

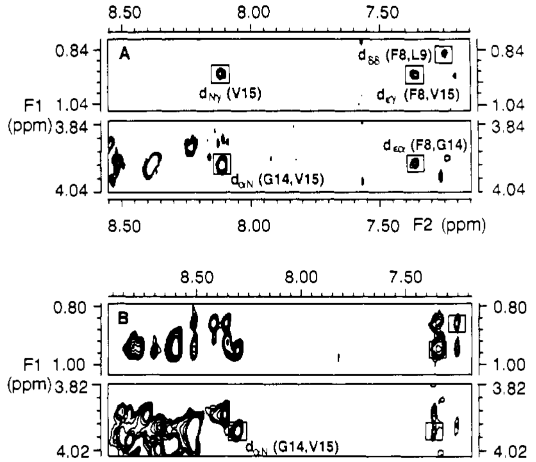

FIGURE 9: Long-range NOEs obtained in buffered H_2O solution in peptide F11 bound to thrombin at 15 °C and in *free* peptide F10 at -8 °C: (A) Thrombin–F11 complex, pH 5.3, 15 °C, mixing time 200 ms, F11 concentration 6 mM, mole ratio F11:thrombin 10:1; (B) F10, pH 5.3, -8 °C, mixing time 500 ms, F10 concentration 20 mM. The boxed cross peaks are the corresponding long-range interactions indicated in (A).

Figure 10 is the plot of the pH variation of the resolved NH chemical shifts of F13 at 25 (part A) and -8 °C (part B). The NH protons of Asp(2) and Asp(7) both experience sizable upfield shift with the increase of pH, as expected from the more pronounced shielding (induction through chemical bond) effects of the negatively charged carboxylate groups at pH values above their pK_as (Bundi & Wüthrich, 1979). From the pH variations of both the NH and the C^{β}H signals of Asp, the pK_a of the carboxyl group of Asp(2) was estimated to be 3.3 (lower than normal) at both 25 and -8 °C (temperature variation of the pHs of the samples were not corrected). Similar decreased pK_as of Asp have been observed in a small protein, bovine pancreatic trypsin inhibitor (BPTI) (Richarz & Wüthrich, 1978), where a salt bridge exists between Asp(50) and Arg(53) in the high-resolution crystal structure (Deisenhofer & Steigemann, 1975). It is thus possible that, in peptide F13, there is a salt bridge between Asp(2) and the positively charged free amino group of Ala(1), in this portion of peptide F13 that does not bind to thrombin. The pK_a of Asp(7), on the other hand, is around 3.9, a value close to that of Asp in nonstructured peptides (Bundi & Wüthrich, 1979). This value of pK_a for the carboxylate group of Asp(7) is also the same as that in the terminally blocked aspartic acid *N*-acetyl-Asp-NHMe, determined here under the same solvent conditions. Because the NH proton of Asp(7) was poorly resolved at -8 °C (Figure 10B), the titration was followed only

Table I: Thrombin-Induced Line Broadening and Transferred NOEs within Synthetic Peptides Derived from Residues Ala(1)–Arg(23) of the Aα Chain of Human Fibrinogen[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	A	D	S	G	E	G	D	F	L	A	E	G	G	G	V	R	G	P	R	V	V	E	R
L_{NH}						S	S	S	S	S	S	S	S	S	S	?	W*	X	W*				
$L_{\beta H}$						X	W	S	?	S	?	X	X	X	?	S	X	?	?				
L_{CH}						X	X	S	S	X	?	X	X	X	S	?	X	W*	W*				
$d_{N\beta}$					W	X	S	S	S	S	S	X	X	X	S		X	X	S*				
$d_{\alpha N}$					W	S	S	W	W	W	S	?	?	S	?	S*	X	S*	X				
$d_{\beta N}$						X		W*	W*	S		X	X	X		X	X	S*	X				
d_{NN}							?	S*	?	S		S	?		?	S*	X	X	X				
$d_{\phi\gamma}$								├─	─	─	─	─	─	─	─┤								
$d_{\phi\delta}$								├─	─┤														
$d_{\phi\alpha}$								├─	─	─	─	─	─	─┤									

[a] L_{NH}, $L_{\beta H}$, and L_{CH}, line-broadening effects on the NH, C$^\beta$H, and other side-chain CH protons by thrombin binding; W, line broadening observed; S, pronounced line broadening observed. $d_{N\beta}$, intraresidue NH–C$^\beta$H NOE; W, weak; S, strong. $d_{\alpha N}$, sequential C$^\alpha$H–NH NOE; W, weak; S, strong. $d_{\beta N}$, sequential C$^\beta$H–NH NOE; W, weak; S, strong. d_{NN}, sequential NH–NH NOE; W, weak; S, strong. $d_{\phi\gamma}$, NOEs between the ring protons of Phe(8) and C$^\gamma$H protons of Val(15). $d_{\phi\delta}$, NOEs between the ring protons of Phe(8) and the C$^\delta$H protons of Leu(9). $d_{\phi\alpha}$, NOEs between the ring protons of Phe(8) and the C$^\alpha$H protons of Gly(14). Similar long-range NOEs are also present in *free* F10 at a subzero temperature of −8 °C (Figure 9B). These NOEs, however, are absent in the peptide–thrombin complex if Arg(16) is replaced by Gly(16) (peptide F14; see Figure 1). In the table, X indicates that this particular NMR parameter does not apply to that residue; a question mark indicates that the resonances could not be assigned because of spectral overlap with those from other residues. The asterisk indicates that this parameter was obtained from the study of the interaction of thrombin with peptide F14. All other parameters pertain to the interaction of thrombin with fibrinopeptide A [residues Ala(1)–Arg(16)]. Thrombin does not induce line broadening in peptide F12 [residues Gly(17)–Arg(23)], although further cleavage of the Arg(19)–Val(20) peptide bond by thrombin was observed (Figure 4).

by the C$^\beta$H proton resonances of Asp(7) and compared to that in *N*-acetyl-Asp-NHMe. The observed pK_a was the same as that at 25 °C (the pH values of the samples were not corrected for temperature variation). It thus appears that Asp(7) is not involved in any strong ionic (or hydrogen bonding) interaction with positively charged side chains such as those from Arg(16) or Arg(19).

There are also other interesting features in the titration data of Figure 10. The NH protons of Glu(5) and Glu(11), for example, exhibit *downfield* shifts upon the increase of pH, indicating that the carboxylate groups of both residues are hydrogen bonded to their own NH protons as established previously (Bundi & Wüthrich, 1979). More interestingly, the NH proton of Gly(12) also experiences sizable *downfield* shift similar to that of Glu(11) at both 25 and −8 °C. This NH titration behavior of Gly(12) suggests that a hydrogen bond also exists between the carboxylate oxygen of Glu(11) and the NH proton of Gly(12) in a significant population of the free peptide at both high and subzero temperatures. Similar downfield shifts can also be seen for the NH proton resonances of Ser(3), Gly(4), and Glu(5), presumably due to hydrogen bonding between their NHs and the carboxylate group of Glu(5).

Around Asp(7), the NH proton resonance of Phe(8) is rather insensitive to pH variations at both high and low temperatures (Figure 10). The NH proton of Leu(9), however, is shifted downfield slightly at both temperatures with the increase of pH. Since the influence of Glu(11) is limited to its own NH proton and that of Gly(12) [no sizable shifts can be seen for the NH protons of Ala(10) and Gly(13)], we attribute this downfield shift to a *weak* hydrogen bond (or a small population of hydrogen bonds) between the carboxylate oxygens of Asp(7) and the NH proton of Leu(9). In fact, such a hydrogen bond between the carboxylate oxygens of Asp and backbone NH protons has recently been observed in a small protein, but seminal inhibitor IIA, by use of amide titration shifts (Ebina & Wüthrich, 1984). The pH titration data thus suggest that the negatively charged carboxylate groups of Asp(2), Glu(5), Asp(7), and Glu(11) are primarily involved in hydrogen-bonding interactions with NH protons *within* fibrinopeptide A.

DISCUSSION

Table I contains a summary of the NMR data discussed in the previous sections. It is not unexpected that fibrinopeptide A binds to thrombin after the cleavage of the Arg–Gly

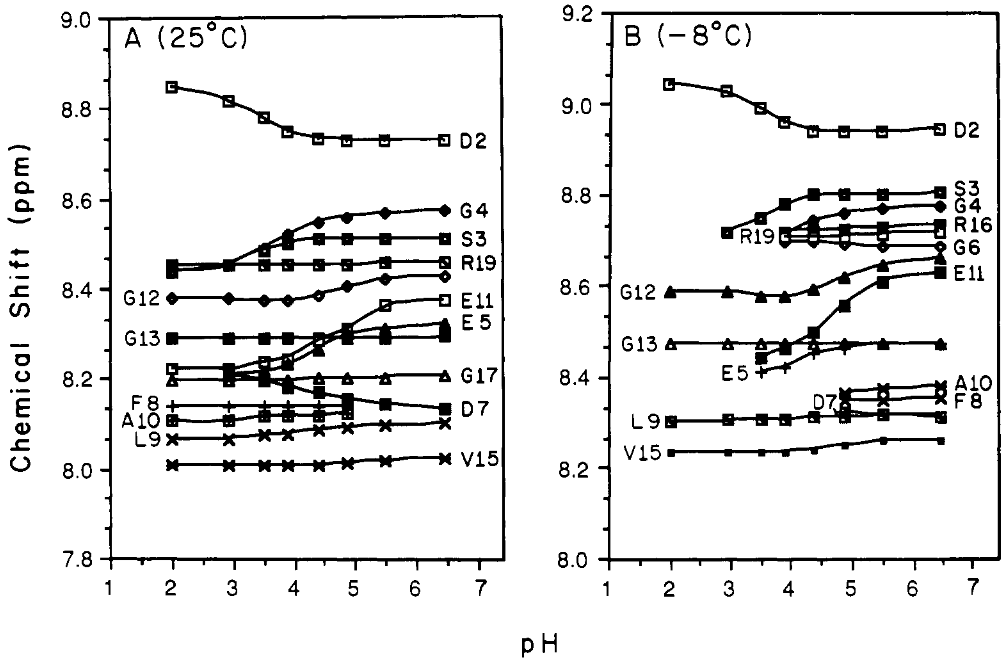

FIGURE 10: Amide proton titration shifts in peptide F13 (Figure 1). Chemical shift values were referenced to DSS (0 ppm). The pH values at −8 °C were readings at 25 °C as in (A). At 25 °C, the resonances of the NH protons of Gly(6) and Arg(16) overlap with that of Arg(19). At −8 °C, the titration curves for some NH protons could not be completed because their resonances are not resolved from one another.

peptide bond since it was previously reported that fibrinopeptide A inhibited the thrombin-induced clotting of fibrinogen (Bettelheim, 1956). In a later study, it was found that a Bzl-nonapeptide methyl ester of residues Phe(8)–Arg(16) also inhibited the interaction of thrombin with fibrinogen (Blombäck et al., 1969). It is very interesting, however, to note that the resonances of the entire region from Ala(1) to Glu(5) in FpA are not affected by thrombin binding. This is the same region of *free* F10 that does not assume any structure in solution (Ni et al., 1988). These results suggest that residues preceding Asp(7) do not contribute significantly to the specificity of the interaction of thrombin with fibrinogen. This conclusion is consistent with the fact that this region contains highly frequent substitutions when comparison is made between human fibrinogen and fibrinogens from other mammalian species (Blombäck, 1967; Henschen et al., 1983). In contrast, within the conserved segment from Asp(7) to Arg(16), proton resonances of all the residues are broadened upon thrombin binding. Furthermore, most of the observed NOEs arise from residues in this region of fibrinopeptide A, suggesting that the flexibility of these residues is significantly reduced upon thrombin binding. In previous studies, it was found that the efficiency of the cleavage of the Arg(16)–Gly(17) peptide bond by thrombin is significantly enhanced by Asp(7) and Phe(8), which are 10 or 9 residues away from the cleavage site (van Nispen et al., 1977; Meinwald et al., 1980; Marsh et al., 1983). These results indicate that this region of the Aα chain of fibrinogen constitutes an essential structural element in the interaction with thrombin.

The finding that the side-chain proton resonances of Phe(8), Leu(9), and Val(15) are broadened by thrombin provides direct evidence for the hypothesis that the specificity of the interaction of thrombin with fibrinogen lies partially in the interactions of nonpolar residues on the thrombin surface with nonpolar residues on the substrate such as Phe(8) in the Aα chain of human fibrinogen (Blombäck et al., 1966). It has been shown that both human and bovine thrombin bind to affinity resins which contain the aromatic, uncharged *p*-chlorobenzyl amino group (Thompson & Davie, 1971; Thompson, 1976). Chemical modifications of thrombin have provided evidence for the involvement of tyrosine and tryptophan in maintaining the activity of thrombin for cleaving fibrinogen (Sokolovsky & Riordan 1969; Lundblad & Harrison, 1971; Lundblad et al., 1973; Uhteg & Lundblad, 1974). Further biochemical studies (Sonder & Fenton, 1984) have demonstrated the importance of a hydrophobic pocket in the active site of thrombin. Studies using spin-labels have indicated that an apolar binding site is located within 4 Å of the primary site, and 8 Å is the upper limit of the diameter of this hydrophobic binding site (Berliner et al., 1981; Berliner, 1984). This hydrophobic pocket in the active site of thrombin would accommodate the nonpolar side chains of the three residues, Phe(8), Leu(9), and Val(15), on the substrate since transferred NOEs indicate distances of less than 5 Å between the ring protons of Phe(8) and the $C^{\delta}H$ protons of Leu(9) and the $C^{\gamma}H$ protons of Val(15). This structural feature accounts for the importance of Phe(8) and Leu(9) in the interaction of synthetic fibrinogen-like peptides with thrombin (van Nispen et al., 1977; Marsh et al., 1982) and the requirement for nonpolar residues at the P2–P4 positions for efficient thrombin cleavages of Arg–X peptide bonds in *other* protons (Chang, 1985). It also explains why the placement of a Phe residue at position P3 [in place of Gly(14)] greatly enhances the inhibitory effects of peptide methyl esters on thrombin action (Blombäck et al., 1969). In fact, it has been demonstrated by NMR measurements (Rae & Scheraga, 1979) that, in a tripeptide with D-Phe (but not L-Phe) at the P3 position, the aromatic ring of the Phe residue is bent directly over the side chain of Val, thus making the D-Phe peptide a much better substrate (Claeson et al., 1977).

The observation of less pronounced line broadening [see, e.g., the ring protons of Phe(8) (Figure 6)] in peptides F14 and F15 may be accounted for by the reduced affinity of these peptides toward thrombin since an Arg residue at the P1 position determines the primary specificity for the thrombin cleavage of peptide bonds in synthetic peptides (Liem & Scheraga, 1974). Furthermore, it was also found that thrombin preferentially cleaves Arg–X peptide bonds in other proteins (Chang, 1985). In fact, the release of fibrinopeptide A has been found to be very slow if Arg(16) is replaced by

His(16), as in some abnormal fibrinogens from patients with bleeding disorders (Henschen et al., 1983). We have discussed earlier that the Arg(16)–Gly(17) peptide bond is cleaved much faster by thrombin than the Arg(19)–Val(20) peptide bond is in both peptides F10 and F13. The highly efficient thrombin cleavage of the Arg(16)–Gly(17) peptide bond may be due to the combined interactions of residues Asp(7), Phe(8), Leu(9), Val(15), and Arg(16) with thrombin. Indeed, when Arg(16) is replaced by Gly(16) (F14) or by Leu(16) (F15), the Gly(16)–Gly(17) or the Leu(16)–Gly(17) peptide bond is no longer cleaved by thrombin. Furthermore, in the complex of thrombin with peptide F14 or F15, where the primary binding site becomes Arg(19), the structure of the bound peptide is different from that of thrombin-bound FpA, as suggested by the absence of long-range NOEs between protons in Phe(8) and those in Gly(14) and Val(15) in the complex of thrombin with peptide F14. These results further support the conclusion that residues Asp(7)–Arg(16) are essential in maintaining a correct conformation for the interaction with thrombin.

In previous kinetic studies, it has been demonstrated that both Pro(18) and Arg(19) are important in the determination of catalytic efficiency of the thrombin cleavage of the Arg(16)–Gly(17) peptide bonds in very short synthetic peptides corresponding to Gly(14)–Val(20) of the Aα chain of human fibrinogen (Liem & Scheraga, 1974). The interaction of residues Pro(18) and Arg(19) with thrombin, on the other hand, is probably very weak, since we could not observe any significant thrombin-induced line broadening on the proton resonances of these residues in peptide F12 (Figures 2–4). With the presence of residues up to Phe(8), residues Arg(19)–Arg(23) were found to contribute only very slightly to the efficiency of the thrombin cleavage of the Arg(16)–Gly(17) peptide bond in synthetic fibrinogen-like peptides (Meinwald et al., 1980). Since no strong interactions were detected between Asp(7) and Arg(16) or Arg(19), there is probably no salt bridge in the biologically active structure for the isolated peptide in solution. The previous observation of the enhanced interaction of thrombin with residues Asp(7)–Val(20) (Marsh et al., 1983) may be explained by the stabilization of the structure of the thrombin–substrate complex, brought about by a hydrogen bond between the carboxylate oxygens of Asp(7) and the NH proton of Leu(9). These results account for the observation that the Arg(19) to Ser(19) mutation does not significantly influence the rate of the thrombin release of fibrinopeptide A from the abnormal fibrinogen (Blombäck et al., 1978), although it results in impaired fibrin polymerization (Blombäck et al., 1968). Nevertheless, the weak interactions of Pro(18) and Arg(19) with thrombin are still important since the mutation of Arg(19) to Asn(19) was reported to result in delayed release of fibrinopeptide A (Henschen et al., 1981) and to produce bleeding disorders (Henschen et al., 1983; Mēnachē, 1983, and references cited therein).

The presence of long-range NOEs in the FpA–thrombin complex and in isolated F10 at low temperature (−8 °C) suggests that there is a chain reversal within residues Leu(9)–Gly(14) of this peptide. Most of the sequential C$^{\alpha}$H–NH and NH–NH NOEs of the bound peptide (Table I) are very similar to those in the free peptide obtained at 0–5 °C with short mixing times (Ni et al., 1988). One difference is the interaction between the NH protons of Gly(12) and Gly(13): no such NOE was observed in the free peptide (Ni et al., 1988), whereas this interaction is especially strong in the complex (Figure 8). Another difference is the NH–NH interaction of residues Gly(14) and Val(15). In the free peptide, a strong NOE cross peak was observed between these two protons, but this NOE is absent in the FpA–thrombin complex (Table I). The NOE data for *isolated* F10 in solution was interpreted as indicative of a type I′ β-turn involving residues Gly(12)–Val(15) (Ni et al., 1988). This local bend structure, however, is not consistent with the data for the bound peptide, for there is no transferred NOE observed between the NH protons of Gly(14) and Val(15) (Figure 8 and Table I). The presence of strong transferred NH–NH NOEs between residues Ala(10) and Glu(11) and between residues Gly(12) and Gly(13) indicates that, instead, a β-turn involving residues Ala(10)–Gly(13) might exist in the complex. A similar backbone structure might also be present within residues Ala(10)–Gly(13) in the isolated peptides in solution since the presence of the local bend structure within residues Gly(12)–Val(15) would not, in itself, account for the reduced temperature coefficients of the amide proton resonances of Gly(13) and Gly(14) observed in peptides F10 and FpA (Ni et al., 1988) and in other smaller fibrinogen-like peptides (Marsh et al., 1985). The peptide backbone of residues Ala(10)–Gly(14) might be very flexible in the isolated peptide, since, as discussed above, there was no NOE between the NH protons of Gly(12) and Gly(13) that is present in the FpA–thrombin complex. In fact, the value of K_{conf} (a measure of the stability of the native conformation) for isolated fibrinopeptide A was found to be less than 2.0×10^{-6}, compared to a value of infinity for fibrinopeptide A when it is part of the fibrinogen molecule (Nagy et al., 1982). Thrombin binding thus seems to selectively stabilize one particular conformation from an ensemble of conformational states in the isolated peptides in solution. Indeed, using peptides corresponding to residues Phe(8)–Pro(18) (F6) and to residues Asp(7)–Val(20) (F8) (Meinwald et al., 1980; Marsh et al., 1982, 1983), we have identified a type II β-turn within residues Ala(10)–Gly(13) of the peptides bound to thrombin (Ni et al., 1989). This structural feature in the bound peptide (which is different from the type I′ bend in the isolated peptide) may serve to bring the nonpolar residues Phe(8) and Leu(9) close to Val(15), which binds to the active site of thrombin.

CONCLUSION

NMR techniques have been applied to study the interactions between human fibrinogen-like peptides and bovine thrombin. The results revealed the direct involvement of residues Asp(7), Phe(8), Leu(9), Val(15), and Arg(16) of the peptides in the complex with thrombin. Furthermore, transferred NOE measurements indicated the existence of a chain reversal within the segment from Asp(7) to Arg(16), so that Phe(8) is brought close to Val(15) in the complex.

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High-Resolution NMR Studies of Fibrinogen-like Peptides in Solution: Structure of a Thrombin-Bound Peptide Corresponding to Residues 7–16 of the Aα Chain of Human Fibrinogen[†]

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ABSTRACT: The interaction of the following human fibrinogen-like peptides with bovine thrombin was studied by one- and two-dimensional NMR techniques in aqueous solution: acetyl-Phe(8)-Leu(9)-Ala(10)-Glu(11)-Gly(12)-Gly(13)-Gly(14)-Val(15)-Arg(16)-Gly(17)-Pro(18)-NHMe (F6), acetyl-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg(16) (tF6), acetyl-Asp(7)-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg(16)-Gly(17)-Pro-Arg(19)-Val(20)-NHMe (F8), and acetyl-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg(16) (tF8). At pH 5.3 and 25 °C, the Arg(16)–Gly(17) peptide bonds in both F6 and F8 were cleaved instantaneously in the presence of 0.5 mM thrombin, producing truncated peptides tF6 and tF8 and other peptide fragments. On the basis of observations of line broadening, thrombin was found to bind to the cleavage products, tF6 and tF8, of peptides F6 and F8. Peptide tF8 may have a higher affinity for thrombin than peptide tF6, as suggested by the more pronounced thrombin-induced line broadening on the proton resonances in peptide tF8. Transferred NOE (TRNOE) measurements were made of the complexes between thrombin and peptides tF6 and tF8. Medium- and long-range NOE interactions were found between the NH proton of Asp(7) and the $C^{\beta}H$ protons of Ala(10), between the $C^{\alpha}H$ proton of Glu(11) and the NH proton of Gly(13), and between the ring protons of Phe(8) and the $C^{\alpha}H$ protons of Gly(14) and the $C^{\gamma}H$ protons of Val(15). Sets of structures of the decapeptide tF8 were deduced by use of distance geometry calculations based on sequential and medium- and long-range TRNOEs from the thrombin-bound peptide. A predominant feature of these structures is the nonpolar cluster formed by the side chains of residues Phe(8), Leu(9), and Val(15) that are directly involved in binding to thrombin. This structural feature is brought about by an α-helical segment involving residues Phe(8)–Ala(10), followed by a multiple-turn structure involving residues Glu(11)–Val(15). These results provide an explanation for the observations that Asp(7), Phe(8), and Gly(12) are strongly conserved in mammalian fibrinogens and that the mutations of Asp(7) to Asn(7) and of Gly(12) to Val(12) result in delayed release of fibrinopeptide A, producing human bleeding disorders.

In this series of papers, we have examined the interactions between thrombin and the N-terminal portion (residues 1–23) of the Aα chain of human fibrinogen. In the accompanying paper (Ni et al., 1989), we reported NMR[1] evidence for the direct involvement of residues Asp(7), Phe(8), Leu(9), Val(15), and Arg(16) of human fibrinogen-like peptides in a complex with bovine thrombin. We also discussed transferred NOE measurements that revealed there is a chain reversal within residues Asp(7)–Arg(16), so that a distant residue, Phe(8), is brought close to Val(15) near the peptide bond between Arg(16) and Gly(17) that is cleaved by thrombin. However, the detailed backbone structure of residues Asp(7)–Arg(16) could not be further defined because of peak overlaps with resonances from residues Ala(1)–Gly(6). In this paper, we present transferred NOE studies of the interaction of bovine thrombin with synthetic peptides corresponding to residues Phe(8)–Pro(18) (F6; Meinwald et al., 1980; Marsh et al., 1982, 1985) and to residues Asp(7)–Val(20) (F8; Marsh et al., 1983). Sets of structures of the thrombin-bound decapeptide corresponding to residues Asp(7)–Arg(16) were derived by use of distance geometry calculations based on the

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; TRNOE, transferred NOE; NOESY, two-dimensional nuclear Overhauser and exchange spectroscopy; COSY, two-dimensional scalar correlation spectroscopy; DQ-COSY, double-quantum filtered COSY; EDTA, ethylenediaminetetraacetic acid; ECEPP, empirical conformational energy program for peptides; FpA, fibrinopeptide A; HPLC, high-performance liquid chromatography.

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