Thrombin Hydrolysis of an N-Terminal Peptide from Fibrinogen Lille: Kinetic and NMR Studies†

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ABSTRACT: Fibrinogen Lille, a congenital dysfibrinogenemia, has been reported to arise from a mutation from Asp to Asn at position 7 of the Aα chain of human fibrinogen, thereby reducing the thrombin-catalyzed rate of hydrolysis of the Arg(16)–Gly(17) peptide bond of this chain. Synthetic peptides of relevant portions of the wild-type and mutant Aα chains were prepared, and the thrombin-catalyzed rates of hydrolysis of their Arg(16)–Gly(17) peptide bonds were determined. In addition, transferred NOE measurements were made to deduce their conformations, when complexed to bovine thrombin. The kinetics data showed little difference in the hydrolysis rates between the wild-type and mutant peptides, and the NMR data indicate no difference in the bound conformation of these two peptides. Therefore, electrostatic (or salt-bridge) interactions between Asp(7) and thrombin do not influence the bound conformations of these peptides. Asp(7) may interact with a remote residue of fibrinogen, not present in these synthetic peptides, or there may be additional mutations beyond Aα(1–20) which have not been detected in fibrinogen Lille. Alternatively, when thrombin binds to fibrinogen at its secondary binding site, its primary (active) site may display different reactivities toward wild-type fibrinogen and fibrinogen Lille.

Transferred NOE[1] measurements of synthetic fibrinogen-like peptides complexed to thrombin (Ni et al., 1989a,b) and studies of the rates of hydrolysis of the Arg(16)–Gly(17) peptide bond (Hageman & Scheraga, 1974; Martinelli & Scheraga, 1980; Marsh et al., 1982, 1983) have provided information about the mechanism of action of thrombin on fibrinogen. In particular, such measurements on a mutant human fibrinogen (Fibrinogen Rouen), in which Gly(12) is replaced by Val in the Aα chain, revealed that a conformational change was responsible for the slow rate of hydrolysis of the Arg(16)–GLy(17) bond of this chain (Ni et al., 1989c). In this paper, we report similar studies of another mutant human fibrinogen, fibrinogen Lille, in which Asp(7) of the Aα chain is replaced by Asn (Denninger et al., 1978; Morris et al., 1981), leading to a much reduced rate of clotting. Such a mutation could change the conformation of this portion of the Aα chain of fibrinogen either by disrupting a possible salt link involving Asp(7) or by removing a salt link between Asp(7) and a positively-charged group of thrombin.

Materials and Methods

Preparation of Bovine Thrombin. Bovine thrombin was prepared and purified as described previously (Ni et al., 1989a). A stock thrombin solution for the kinetics experiments had a pH of 6.5 and a concentration of 0.29 mg/mL, as determined by absorbance at 280 nm, using the value $E_{1\%}$ = 19.5 (Winzor & Scheraga, 1964). The activity of the thrombin stock solution was determined to be 764 NIH units/mL by the FpA release assay of Lewis and Shafer (1984). The stock solution was stored at −85 °C and then thawed, and aliquots were diluted 100-fold prior to use in a buffer of pH 6.5, 0.05 M Na_2HPO_4, 0.1 M NaCl, and 0.1% PEG 6000 solution. The pH of these solutions was kept at 6.5 to retard thrombin autolysis. A clotting assay was used to ensure that no loss of thrombin activity occurred during the course of the experiments.

Table I: FAB Mass Spectroscopic Analysis of Peptide

peptides	exptl av mass (Da)	theor av mass (Da)	exptl mass − theor mass (Da)
Ac-(7–20)-NH_2	1471.2	1471.7	−0.5
Ac-(7–16)-OH	1063.2	1063.2	+0.0
Ac-(7N–20)-NH_2	1470.9	1470.7	+0.2
Ac-(7N–16)-OH	1062.3	1062.2	+0.1

Synthesis of Peptides. The Ac-(7–20)-NH_2 and Ac-(7N–20)-NH_2 peptides were synthesized by solid-phase methodology with a Milligen 9050 peptide synthesizer at the Cornell University Biotechnology Program Analytical and Synthesis Facility. The amino acid sequences were the following: Ac-(7–20)-NH_2, Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-NH_2; and Ac-(7N–20)-NH_2, Ac-Asn-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-NH_2. Except for the acetylated N-terminus, these peptides are identical in amino acid sequence to residues 7–20 of the Aα chain of human fibrinogen (Henschen et al., 1983) and fibrinogen Lille (Morris et al., 1981), respectively. Peptides Ac-(7–16)-OH and Ac-(7N–16)-OH were obtained by thrombin digestion of purified Ac-(7–20)-NH_2 and Ac-(7N–20)-NH_2, respectively.

The peptides were purified by reverse-phase HPLC using a Dynamax-300 C_{18} semipreparative column on an LKB Bromma HPLC system consisting of a 2152 LC controller, a 2150 HPLC pump, and a 2141 wavelength detector. The mobile-phase buffers were A, 0.09% TFA in water; and B,

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[1] Abbreviations: CNBr, cyanogen bromide; FpA, fibrinopeptide A; NOE, nuclear Overhauser effect; NMR, nuclear magnetic resonance; PEG 6000, poly(ethylene glycol) with an average molecular weight of 6000; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; FAB, fast atom bombardment; NOESY, two-dimensional NOE spectroscopy; FID, free induction decay.

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0.09% TFA in acetonitrile. The purified peptides were lyophilized to fluffy powders. Each peptide was subjected to amino acid analysis and fast atom bombardment mass spectroscopic analysis. The amino acid composition for each peptide agreed with that deduced from its sequence. The results of FAB mass spectroscopic analysis are listed in Table I.

Each of the four peptides eluted as a single peak in HPLC chromatograms. Mixtures of Ac-(7–20)-NH_2 and Ac-(7N–20)-NH_2 and of Ac-(7–16)-OH and Ac-(7N–16)-OH were injected onto the HPLC column used in the kinetics experiments and eluted under the same conditions used to analyze kinetic reaction mixtures. In both cases, two resolved peaks appeared on the chromatogram.

Kinetics Experiments. The kinetics experiments included the acquisition of standard peptide calibration curves and the measurement of initial velocities at various substrate concentrations. HPLC analyses of reaction products were carried out with a Brownlee Aquapore RP-300, 7-μm, C_{18} column (Applied Biosystems, Foster City, CA), on the LKB Bromma HPLC system described above, with the addition of a 2157 autosampler and a 2221 integrator. The mobile-phase buffers were the same as those used in the purification of the peptides.

Serial dilutions were prepared from stock solutions of peptides whose concentrations were established by amino acid analysis. Aliquots from each diluted solution were injected onto the column and eluted with a gradient of 15–20% buffer B at a flow rate of 0.5 mL/min. Eluting peaks were detected at 205 nm. Peptide concentrations vs peak areas were plotted as calibration curves and used to determine the peptide concentrations of injected samples from their peak areas in the chromatograms. Calibration curves for Ac-(7–20)-NH_2 and Ac-(7N–20)-NH_2 were thus prepared over the range of 0–600 μM, and those for Ac-(7–16)-OH and Ac-(7N–16)-OH were over the range of 0–70 μM. All of the calibration curves were linear.

Initial velocity (kinetics) experiments for the thrombin-induced cleavage of Ac-(7–20)-NH_2 and Ac-(7N–20)-NH_2 were carried out under identical conditions, i.e., at 25 °C in a buffer of 25 mM Tris, pH 8.0, 0.3 M NaCl, and 0.01% PEG 6000. The addition of a small amount of thrombin solution at pH 6.5 did not affect the pH of the reaction mixture. In a typical kinetics run, the substrate peptide sample was dissolved in the above buffer, and its pH was adjusted to 8.0. A portion of it was injected onto the HPLC column to determine the initial concentration from the peak area, using the substrate standard curve (see below). Two hundred microliters from the remaining substrate solution was mixed with 10 μL of a thrombin solution (7.64 NIH units/mL); aliquots of 20 μL were withdrawn from the mixture after timed intervals and added to 10 μL of a 5% phosphoric acid solution to quench the reaction. Complete quenching was indicated by a constant concentration of product in the reaction mixture over a long period of time. Twenty-microliter aliquots of the quenched reaction mixtures were injected by the autosampler onto the HPLC column to resolve the cleavage products and determine their peak areas, which were converted into product concentration. In each experiment, only two peaks were observed, corresponding to the 10-residue cleavage product and the unreacted substrate. The other product, peptide Gly-Pro-Arg-Val, was eluted in the void volume. Plots of product concentration vs time yielded straight lines, and the slopes were used as the initial velocities of peptide cleavage.

In the analysis of the reaction mixture from mutant substrate Ac-(7N–20)-NH_2, the identity of the cleavage product peak in the chromatogram as Ac-(7N–16)-OH was supported by the enhancement of this peak upon addition of standard Ac-(7N–16)-OH to the reaction mixture. In contrast, addition of standard Ac-(7–16)-OH resulted in a new peak eluting after the product peak. As an additional check, another cleavage product peak, Gly-Pro-Arg-Val, was monitored under low-acetonitrile elution conditions; addition of a solution of purified Gly-Pro-Arg-Val to the reaction mixture did not produce any additional peaks in the chromatogram.

In the kinetics experiments with wild-type peptides, the substrate concentration was varied from 0.3 K_M to 3 K_M; with mutant peptides, the concentrations ranged from 0.5 K_M to 4 K_M. The data in the kinetics runs were acquired only in the range of substrate cleavage up to 10% of the original substrate concentration.

The Michaelis–Menten parameters for thrombin cleavage of the Arg(16)–Gly(17) bond and their standard errors were determined by a nonlinear least-squares fit to the Michaelis–Menten equation, using the program HYPER (Cleland, 1979).

NMR Measurements. The NMR samples were prepared by the procedure described previously (Ni et al., 1989a,b) and contained peptides at concentrations with a 10–20-fold excess over that of thrombin, at pH 5.3. Transferred NOE spectra were acquired with a relatively short mixing time of 200 ms for peptides Ac-(7–20)-NH_2 and Ac-(7N–20)-NH_2 with a peptide:thrombin mole ratio at 20:1 and 10:1, respectively. This large excess of the peptides ensured that the peptides spend most of the time in the free state during the NOE buildup so that undesirable spin-diffusion effects are minimized. To improve the quality of the NOE spectra, enhanced processing strategies were incorporated into the postacquisition transformation steps using an extension of the FTNMR program (Ni et al., 1992b). Briefly, the NOESY FID matrices were premultiplied by cosine-square windows along both the t_1 and t_2 directions and Fourier-transformed and phase-corrected *first* along the t_1 dimension. The first five data points of each t_2 interferogram were then corrected using a maximum-entropy (backward) extrapolation procedure (Ni & Scheraga, 1986) before Fourier transformation and phase correction. The residual water proton signals were further suppressed by subtraction of the water signal reconstructed by time–domain convolution and linear prediction (F. Ni, unpublished results). These treatments significantly reduced base-plane distortions commonly observed in NOESY spectra of peptides in aqueous solutions.

It has been established that fibrinopeptide A and its analogues show negligible NOE's in the uncomplexed (free) state (Ni et al., 1988, 1992a). A large number of NOE peaks (transferred NOE's) were observed for these peptides in the presence of a less than stoichiometric amount of active thrombin (Ni et al., 1989a–c). For both peptides Ac-(7–20)-NH_2 and Ac-(7N–20)-NH_2, transferred NOE's were observed only for the fragments Ac-(7–16)-OH and Ac-(7N–16)-OH since active thrombin cleaves the Arg(16)–Gly(17) peptide bonds, releasing peptide fragments ^+H_3N-Gly-Pro-Arg-OH and ^+H_3N-Val-NH_2 that do not bind to thrombin (Ni et al., 1989a). These transferred NOE spectra enabled us to assign all the proton resonances of peptides Ac-(7–16)-OH and Ac-(7N–16)-OH (Table II) on the basis of a comparison with the previous assignments of the proton resonances of fibrinopeptide A and its analogues (Ni et al., 1988).

Results and Discussion

Kinetics Results. Lineweaver–Burk plots for the thrombin-catalyzed hydrolysis of Ac-(7–20)-NH_2 and Ac-(7N–

Table II: Proton Resonance Assignments of the Fibrinogen-like Peptides in Aqueous Solution at 25 °C and pH 5.3

residue	chemical shift (ppm)[a] NH	αCH	βCH	others
		(A) Ac-(7–16)-OH		
Ac		2.01		
Asp(7)	8.22	4.55	2.53, 2.63	
Phe(8)	8.15	4.62	3.09, 3.18	δCH_2 7.27; ϵCH_2 7.39; ζCH 7.36
Leu(9)	8.10	4.32	1.64	γCH 1.52; δCH_3 0.88, 0.94
Ala(10)	8.18	4.30	1.43	
Glu(11)	8.39	4.32	2.01, 2.12	γCH_2 2.34
Gly(12)	8.46	4.00, 4.07		
Gly(13)	8.32	4.02		
Gly(14)	8.32	4.02		
Val(15)	8.04	4.18	2.12	γCH_3 0.97, 0.97
Arg(16)	8.03	4.26	1.75, 1.89	γCH_2 1.62; δCH_2 3.21; ϵNH 7.21
		(B) Ac-(7N–16)-OH		
Ac		2.00		
Asn(7)	8.29	4.67	2.67, 2.74	δNH_2 6.90, 7.57
Phe(8)	8.19	4.65	3.06, 3.19	δCH_2 7.26; ϵCH_2 7.39; ζCH 7.35
Leu(9)	8.12	4.33	1.62	γCH 1.55; δCH_3 0.89, 0.95
Ala (10)	8.20	4.31	1.43	
Glu(11)	8.40	4.32	2.02, 2.12	γCH_2 2.33
Gly(12)	8.47	3.99, 4.07		
Gly(13)	8.32	4.01		
Gly(14)	8.32	4.01		
Val(15)	8.05	4.17	2.12	γCH_3 0.97, 0.97
Arg(16)	8.03	4.25	1.75, 1.88	γCH_2 1.62; δCH_2 3.22; ϵNH 7.21

[a] All of the chemical shifts were determined from the NOESY spectra of the peptides in the presence of thrombin. The values of the shifts were calibrated against the solvent proton resonance that was set to 4.80 ppm.

Table III: Kinetics Constants for the Hydrolysis of Arg–Gly Bonds by Thrombin at pH 8 and 25 °C

substrates	$K_M \times 10^6$ (M)	$k_{cat} \times 10^{11}$ [M/[(NIH unit/L) s]]	$k_{cat}/K_M \times 10^7$ [[(NIH unit/L) s]$^{-1}$]	ref
human fibrinogen Aα chain	9.5 ± 0.5	45.0 ± 5.0	475 ± 50	Hanna et al. (1984)
CNBr Aα fragment	47 (20–500)[a]	48 (38–330)[a]	100 (60–200)[a]	Hageman et al. (1974)
Ac-F-L-A-E-G-G-G-V-R-G-P-R-V-$NHCH_3$	633 ± 386	20 ± 8	3.2 ± 0.6	Meinwald et al. (1980)
Ac-D-F-L-A-E-G-G-G-V-R-G-P-R-V-$NHCH_3$	310 ± 99	31 ± 7	10 ± 5	Marsh et al. (1983)
Ac-D-F-L-A-E-G-G-G-V-R-G-P-R-V-NH_2	373 ± 52	47 ± 3	13 ± 3	this work
Ac-N-F-L-A-E-G-G-G-V-R-G-P-R-V-NH_2	430 ± 37	53 ± 2	12 ± 2	this work

[a] Range is in parentheses.

20)-NH_2 were linear from 86 to 1650 μM. The final results from the program HYPER were K_M = 373 ± 52 μM, k_{cat} = 47 (±3) × 10^{-11} M/[(NIH unit/L) s] for the wild-type peptide Ac-(7–20)-NH_2; and K_M = 430 ± 37 μM, k_{cat} = 53 (±2) × 10^{-11} M/[(NIH unit/L) s] for the mutant peptide Ac-(7N–20)-NH_2. The results from this study, as well as those from related studies previously undertaken in this laboratory, are listed in Table III.

Kinetic studies of the hydrolysis of a series of synthetic fibrinogen-like peptides (Marsh et al., 1983) have provided much information about the mechanism of thrombin action on fibrinogen. For example, the importance of Phe(8) was demonstrated in such studies (Marsh et al., 1982) and was later confirmed by NMR conformational studies (Ni et al., 1989a). Involvement of Asp(7) was suggested from the occurrence of this residue in a homologous position in other species of fibrinogen (Henschen et al., 1983), and from early kinetic studies (Marsh et al., 1983) using fluorescamine as the probe for determining product concentration. Our present study, using HPLC which enabled us to monitor the product concentration directly and more accurately, again supports this conclusion.

The role of Asp(7) in the hydrolysis of the Arg–Gly bond was also indicated by the reported reduced rate and extent of fibrinopeptide A release from the *whole* fibrinogen Lille molecule, in which Asp(7) was replaced by Asn (Denninger et al., 1978; Morris et al., 1981). If this Asp residue were involved in an ionic interaction with thrombin, we would expect significantly altered kinetics parameters for the mutant peptide, because the substitution of Asn for Asp would destroy the salt link and alter the rate of hydrolysis, thereby accounting for the abnormality of fibrinogen Lille. However, the kinetics data showed that there is little difference in the rate of hydrolysis of the Arg–Gly bond of the wild-type and mutant peptides. These results corroborate the observations of Lord et al. (1990), wherein an Aα fusion protein mutant [Asp(7) → Ala] was as good a substrate as the Aα fusion protein containing the native sequence and was also an equally good competitive inhibitor of the chromogenic substrate spectrozyme TH. This may suggest that an additional mutation might have occurred among the residues after Val(20), which have not been sequenced in fibrinogen Lille or, more likely, that Asp is involved in a long-range interaction with a remote residue of fibrinogen not included in the synthetic peptide.

Comparison of the data from this study with those for the CNBr fragment of fibrinogen (Hageman & Scheraga, 1974) indicates that the differences in hydrolysis rates of the two fragments are reflected mainly in the values of K_M, suggesting poorer substrate binding for the shorter synthetic peptide. Immunochemical studies of the fibrinogen Aα chain (Nagy et al., 1982) have shown that peptide Aα(8–21) contains all the residues required for thrombin binding yet lacks the long-range interactions with the rest of the fibrinogen needed to adopt an optimal conformation. Several reports have shown the importance of residues 23–51 of the Aα chain in the interaction with thrombin (Blombäck et al., 1976; Van Nispen

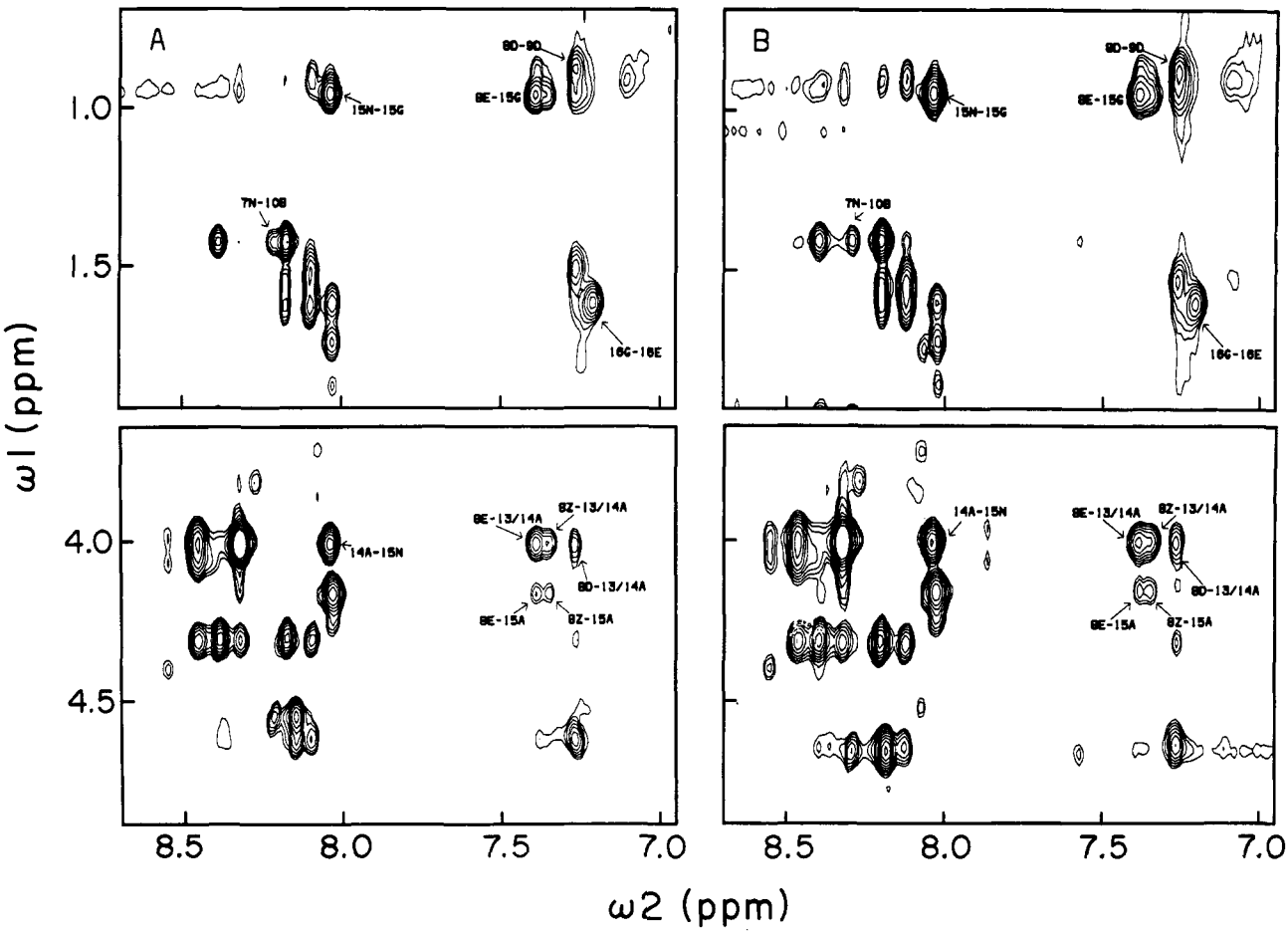

FIGURE 1: Transferred NOE's characteristic of the thrombin-bound conformation of fibrinopeptide A. The spectra were acquired at 25 °C, pH 5.3, with a mixing time of 200 ms. The concentration of Ac-(7–16)-OH was ~10 mM and that of Ac-(7N–16)-OH was ~5 mM. The concentrations of thrombin were all 0.4 mM. The labels indicate NOE's between protons attached to heavy atoms (capital letters) of specific residues (numbers). 8E–13/14A, for example, refers to NOE's between the ϵCH_2 protons of Phe(8) and the αCH_2 protons of Gly(13) or Gly(14). Proton resonance assignments are documented in Table II. (A) Transferred NOE's from the complex of thrombin with Ac-(7–16)-OH. (B) Transferred NOE's from the complex of thrombin with Ac-(7N–16)-OH.

et al., 1977). All of these observations support the suggestion that Asp(7) may be involved in a long-range interaction with a remote residue of fibrinogen.

Alternatively, the secondary binding site on fibrinogen, which is known to participate in binding to thrombin (Hofsteenge & Stone, 1987; Vali & Scheraga, 1988; Kaczmarek & McDonagh, 1988), and which is not present in the peptides investigated here, may be the origin of the observed defect in fibrinogen Lille. An interaction between the secondary binding site of thrombin and the secondary binding site of fibrinogen [or with hirudin or other ligands (Mao et al., 1988; Hogg & Jackson, 1990; Hortin & Trimpe, 1991; Ye et al., 1991)] might alter the structure of the *primary* (*active*) site of thrombin so that it interacts differently with wild-type fibrinogen and fibrinogen Lille.

Transferred NOE's. For peptide Ac-(7–16)-OH, all of the transferred NOE's that were observed in our previous study (Ni et al., 1989b) were again observed in the current study. These include the characteristic NOE's between the side-chain protons of residues Phe(8), Leu(9), and Val(15) and those between the ring protons of Phe(8) and the αCH_2 protons of Gly(13) and/or Gly(14) (Figure 1A). These NOE's were also observed for the mutant peptide Ac-(7N–16)-OH in the complex with thrombin (Figure 1B). The presence of these transferred NOE's demonstrates that there must be a hydrophobic cluster formed by the side chains of Phe(8), Leu(9), and Val(15) in the complex of thrombin with both the normal peptide fragment Ac-(7–16)-OH and the peptide Ac-(7N–16)-OH derived from fibrinogen Lille.

Attempts were made previously (Ni et al., 1989a,b) to resolve the transferred NOE's between the aromatic ring protons of Phe(8) and the αCH_2 protons of Gly(13) and/or Gly(14) (Figure 1). The NOE cross-peak still existed in the transferred NOESY spectrum of a fibrinopeptide with the αCH_2 protons of Gly(13) replaced by deuterium (Ni et al., 1989b). Furthermore, there are NOE's between the ring protons of Phe(8) and the γCH_3 protons of Val(15) (Figure 1), indicating that the aromatic ring of Phe(8) must be close to residue Gly(14). Therefore, only the NOE's between residues Phe(8) and Gly(14) were positively identified, while the distances between residues Phe(8) and Gly(13) were left unconstrained in distance-geometry calculations (Ni et al., 1989b). To refine the thrombin-bound structure of the fibrinopeptides, further experiments are necessary to identify possible NOE's between the ring protons of Phe(8) and the αCH_2 protons of Gly(13) and to resolve the NOE's between the ring protons of Phe(8) and the NH protons of Gly(13) and/or Gly(14) (Figure 2; see the following sections). The resolution of these NOE's would significantly alter the backbone conformations of residues Gly(13) and Gly(14) from those predicted previously on the basis of limited experimental data (Ni et al., 1989b, 1991a).

As a result of the optimized acquisition and processing of the NOESY spectra, new NOE's were observed that were not observed previously for analogues of fibrinopeptide A. These include the NOE's between the ϵCH_2 protons and the ζCH

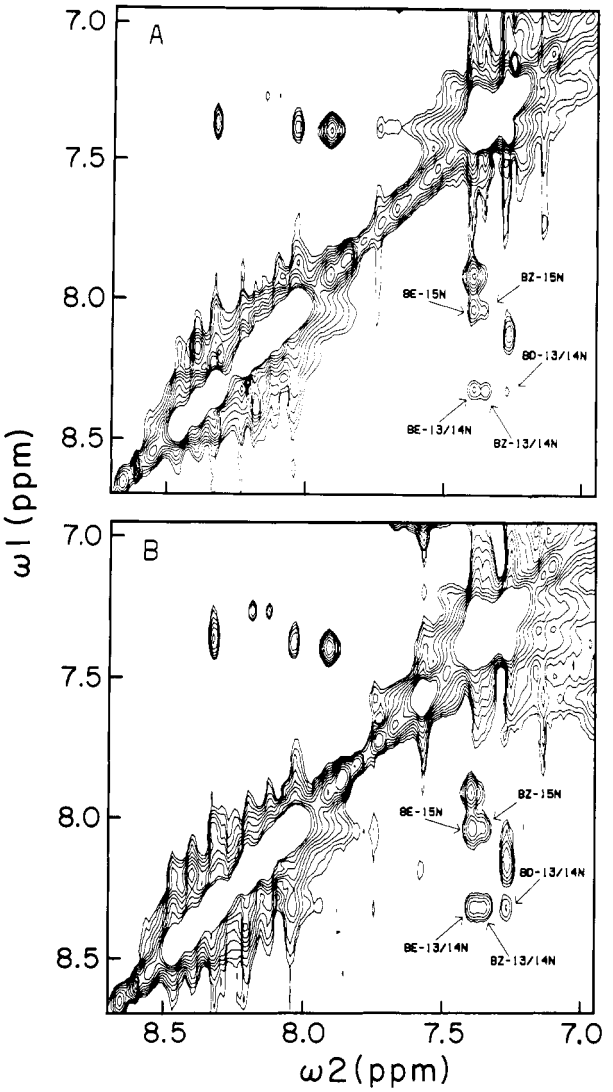

FIGURE 2: New transferred NOE's identified as a result of enhanced spectral acquisition and processing. The labeling follows the same convention as in Figure 1. (A) Transferred NOE's for peptide Ac-(7–16)-OH. (B) Transferred NOE's for peptide Ac-(7N–16)-OH.

proton of Phe(8) and the αCH_2 protons of Val(15) for both peptides Ac-(7–16)-OH and Ac-(7N–16)-OH (Figure 1). Furthermore, there are NOE's from the ring protons of Phe(8) to the NH proton of Gly(13) and/or that of Gly(14) and to the NH proton of Val(15) (Figure 2). Strong NOE's were also observed between the βCH_2 protons and the δCH_2 protons of Arg(16) (Figure 3) and between the γCH_2 protons and the ϵNH proton of Arg(16) (Figure 1), indicating that the side chain of Arg(16) adopts an extended conformation. For both the wild-type and mutant peptides, there exist NOE's between the βCH_2 protons of Asp(7)/Asn(7) and the βCH_3 protons of Ala(10) and NOE's between the βCH_3 protons of Ala(10) and the γCH_2 protons of Glu(11) (Figure 3). More interestingly, the relative intensities of the two sets of cross-peaks between the βCH_2 protons of Asp(7)/Asn(7) and the βCH_3 protons of Ala(10) are approximately the same, as judged by the relative number of contours and by the projection of these peaks onto the horizontal spectral axis (spectra not shown). These observations suggest that the Asn(7) substitution for Asp(7) does not cause significant changes in the orientation of the side chain of this residue and thus may not cause major structural changes around Asp(7) in the complex with thrombin. Also, the identification of the δNH_2 protons of Asn(7) (Table II) and the shift in the 7B–10B NOE's between

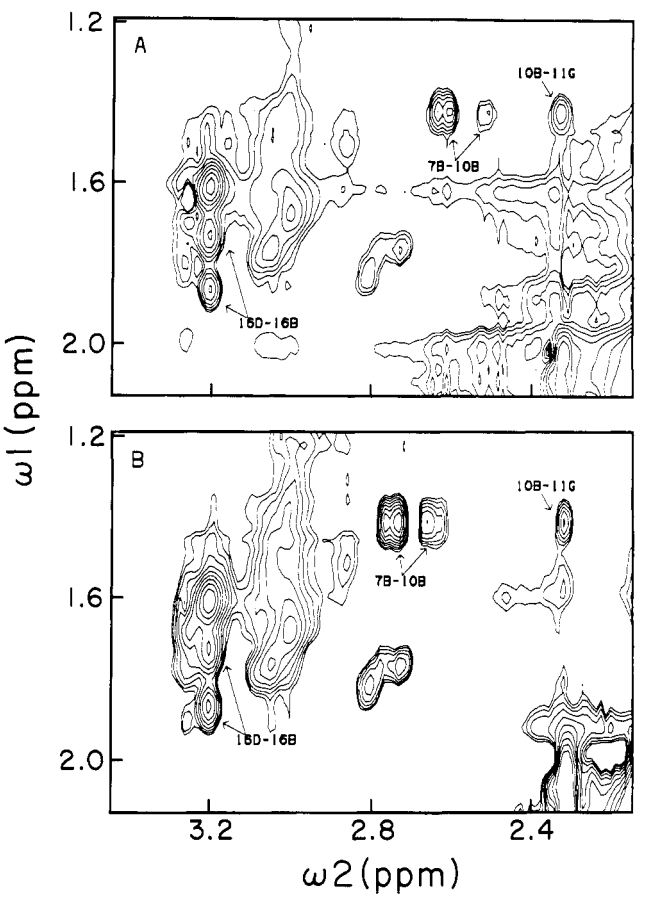

FIGURE 3: Side-chain to side-chain transferred NOE's: (A) peptide Ac-(7–16)-OH in complex with thrombin; (B) peptide Ac-(7N–16)-OH in complex with thrombin. Experimental conditions are the same as in Figures 1 and 2.

the Asp and Asn peptides (Figure 3) demonstrate that Asn(7) was not deamidated in these experiments.

In our previous study, both Ac-(7–16)-OH and Ac-(8–16)-OH were found to bind to thrombin and exhibit similar transferred NOE's (Ni et al., 1989b). Therefore, the absence of Asp(7) in the latter peptide does not significantly alter the conformation of residues Phe(8)–Arg(16) of fibrinopeptide A in the thrombin-bound state. This conclusion is further supported by the observation of transferred NOE's involving the binding of the mutant peptide Ac-(7N–16)-OH to thrombin. However, previous kinetics experiments have shown that Asp(7) is required for the interaction with thrombin since the deletion of this residue resulted in a reduced rate for the thrombin-catalyzed cleavage of the Arg–Gly peptide bond in synthetic fibrinogen-like peptides (Marsh et al., 1983).

It has been proposed that the side chain of Asp(7) may form *intramolecular* hydrogen bonds with the NH protons of Leu(9) and Ala(10) to provide further stabilization of the helical structure of residues Phe(8)–Glu(11) in the bound state (Ni et al., 1989b). It was also predicted that the replacement of Asp(7) by Asn(7) would not disrupt the bound conformation since an Asn side chain is still capable of forming intramolecular hydrogen bonds. Indeed, the transferred NOE's are very similar for both Ac-(7–16)-OH and Ac-(7N–16)-OH, indicating that there is no significant difference between the bound conformations of the two peptides. Kinetics data indicate that both the Asp(7) and the Asn(7) peptides are equally good substrates for thrombin with similar kinetics constants for the hydrolysis of the Arg–Gly peptide bonds (Table III). Therefore, the negative charge of Asp(7) is not important in the interaction of the peptide substrates with

thrombin. It appears that Asp(7) may be involved in a long-range interaction with a remote residue of fibrinogen not included in the synthetic peptide. Alternatively, thrombin may have altered reactivity at its primary (active) site, when bound to fibrinogen at its secondary binding site.

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Dimeric Structure and Conformational Stability of Brain-Derived Neurotrophic Factor and Neurotrophin-3

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ABSTRACT: We have examined the molecular structure of the related neurotrophic factors brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) by physical methods, including gel filtration, velocity sedimentation, sedimentation equilibrium, urea gel electrophoresis, fluorescence spectroscopy, and far-ultraviolet circular dichroism. The results of these studies indicate that at physiologically relevant concentrations both recombinant proteins exist as tightly associated dimers. The dimers are stable even in 8 M solutions of urea. In solutions of guanidine hydrochloride, BDNF and NT-3 undergo slow unfolding between 3 and 5 M concentration of denaturant. Circular dichroism spectroscopy revealed approximately 70% β-sheet and 20% β-turn content in the native structure of both neurotrophic factors. In this respect, BDNF and NT-3 resemble other polypeptide growth factors whose receptors are also integral protein-tyrosine kinases.

Brain-derived neurotrophic factor (BDNF) (Leibrock et al., 1989; Barde et al., 1982; Hofer & Barde, 1988) and neurotrophin-3 (NT-3) (Maisonpierre et al., 1990; Hohn et al., 1990; Rosenthal et al., 1990) in addition to nerve growth factor (NGF) and the recently discovered NT-4 (Hallböök et al., 1991) constitute a family of structurally related neurotrophic factors termed neurotrophins. Mature BDNF and NT-3 are 119 amino acid residue polypeptides with NGF being shorter by 1 amino acid residue. The neurotrophins display about 55% sequence identity including six conserved cysteine residues that,

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