High-Resolution NMR Studies of Fibrinogen-like Peptides in Solution: Structural Basis for the Bleeding Disorder Caused by a Single Mutation of Gly(12) to Val(12) in the Aα Chain of Human Fibrinogen Rouen†

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Received October 24, 1988

ABSTRACT: In human fibrinogen Rouen, which is the origin of a bleeding disorder, a single amino acid is mutated from Gly(12) to Val(12) in the Aα chain. In the previous paper of this series, this mutation was predicted to disrupt the structure of fibrinogen-like peptides bound to bovine thrombin. The structural basis of this bleeding disorder has been further assessed by studying the interaction of the following Val(12)-substituted human fibrinogen-like peptides with bovine thrombin in aqueous solution by use of two-dimensional NMR spectroscopy (including TRNOE): Ala-Asp-Ser-Gly-Glu-Gly-Asp(7)-Phe-Leu-Ala-Glu-Val(12)-Gly-Gly-Val-Arg(16)-Gly(17)-Pro-Arg-Val-NH_2 (F16), Ala-Asp-Ser-Gly-Glu-Gly-Asp(7)-Phe-Leu-Ala-Glu-Val(12)-Gly-Gly-Val-Arg(16) (tF16), Ala-Asp-Ser-Gly-Glu-Cys(Acm)-Asp(7)-Phe-Leu-Ala-Glu-Val(12)-Gly-Gly-Val-Arg(16)-Gly(17)-Pro-Arg-Val-Cys(Acm)-NH_2 (F17), and Ala-Asp-Ser-Gly-Glu-Cys(Acm)-Asp(7)-Phe-Leu-Ala-Glu-Val(12)-Gly-Gly-Val-Arg(16) (tF17). Binding of thrombin to peptides F16 and F17, and hence to tF16 and tF17 as a result of the cleavage of the Arg(16)–Gly(17) peptide bond, broadens the proton resonances of residues Asp(7) to Arg(16), suggesting that thrombin interacts specifically with this sequence of residues. Medium- and long-range TRNOE's were observed between the NH proton of Asp(7) and the $C^{\beta}H$ protons of Ala(10) and between the ring protons of Phe(8) and the $C^{\gamma}H$ protons of Val(12) and Val(15) in complexes of thrombin with both tF16 and tF17. A strong TRNOE, in peptides tF16 and tF17, between the $C^{\beta}H$ protons of Glu(11) and the backbone NH proton of Val(12) was also observed. However, TRNOE's between the ring protons of Phe(8) and the $C^{\alpha}H$ protons of Gly(14) and between the $C^{\alpha}H$ proton of Glu(11) and the NH proton of Gly(13), previously observed in the complex of thrombin with FpA, were absent in both peptides tF16 and tF17. From incorporation of TRNOE information into distance geometry calculations, Val(12) was found to disrupt the type II β-turn involving Glu(11) and Gly(12) that is present in complexes of thrombin with normal fibrinogen-like peptides. The positions of Gly(13) and Gly(14) in the complex are also displaced, relative to the aromatic ring of Phe(8), by the Val(12) substitution. This altered geometry presumably affects the positioning of the Arg(16)–Gly(17) bond in the active site of thrombin. This suggestion was supported by the observation that the cleavage of the Arg(16)–Gly(17) peptide bond in both F16 and F17 by thrombin is much slower than the cleavage of the same bond in normal fibrinogen-like peptides. Thus, the bleeding disorder caused by the single mutation of Gly(12) to Val(12) may be due to the structural alteration of human fibrinogen Rouen identified here.

Molecular defects in genetically abnormal human fibrinogens cause delayed formation of fibrin clots, thereby leading to bleeding disorders. In recent years, there has been an increased understanding of the cause of bleeding disorders associated with defective fibrinogens. Errors in the amino acid sequence of some of the abnormal fibrinogens have been identified, and in most of the fibrinogens analyzed so far, mutations of single amino acids have been located in the region of residues Asp(7) to Arg(19) in the Aα chain [Henschen et al., 1983; Mênachê (1983) and references cited therein]. In fibrinogen Detroit, for example, the defect was found to be a replacement of Arg(19) by Ser(19) (Blombäck et al., 1968). Other single mutations include Asp(7) to Asn(7) in fibrinogen Lille (Morris et al., 1981), Arg(19) to Asn(19) in fibrinogen München (Henschen et al., 1981), and Gly(12) to Val(12) in fibrinogen Rouen [Henschen et al., 1983; Mênachê (1983) and references cited therein].

To summarize our understanding of the structural basis for the specificity of the interaction between thrombin and fibrinogen, we have established that residues Asp(7), Phe(8), Leu(9), Val(15), and Arg(16) are involved in the interaction of thrombin with synthetic peptides derived from the N-terminal portion (residues 1–23) of the Aα chain of human fibrinogen (Meinwald et al., 1980; Marsh et al., 1982, 1983; Ni et al., 1989a). On the basis of TRNOE[1] measurements

† This work was supported by research grants from the National Insitute of General Medical Sciences (GM-24893) and the National Heart, Lung and Blood Institute (HL-30616) of the National Institutes of Health. It was carried out with the GN-500 500-MHz NMR spectrometer at the NIH Regional Research Resource for Multinuclear NMR and Data Processing located at Syracuse University (RR-01317). This is paper 19 in a series. Ni et al. (1989b) is paper 18 of this series.

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[1] Abbreviations: Acm, acetamidomethyl; EDTA, ethylenediaminetetraacetic acid; PEG 6000, poly(ethylene glycol) with an average molecular weight of 6000; 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; FpA, fibrinopeptide A; RPLC, reverse-phase high-performance liquid chromatography.

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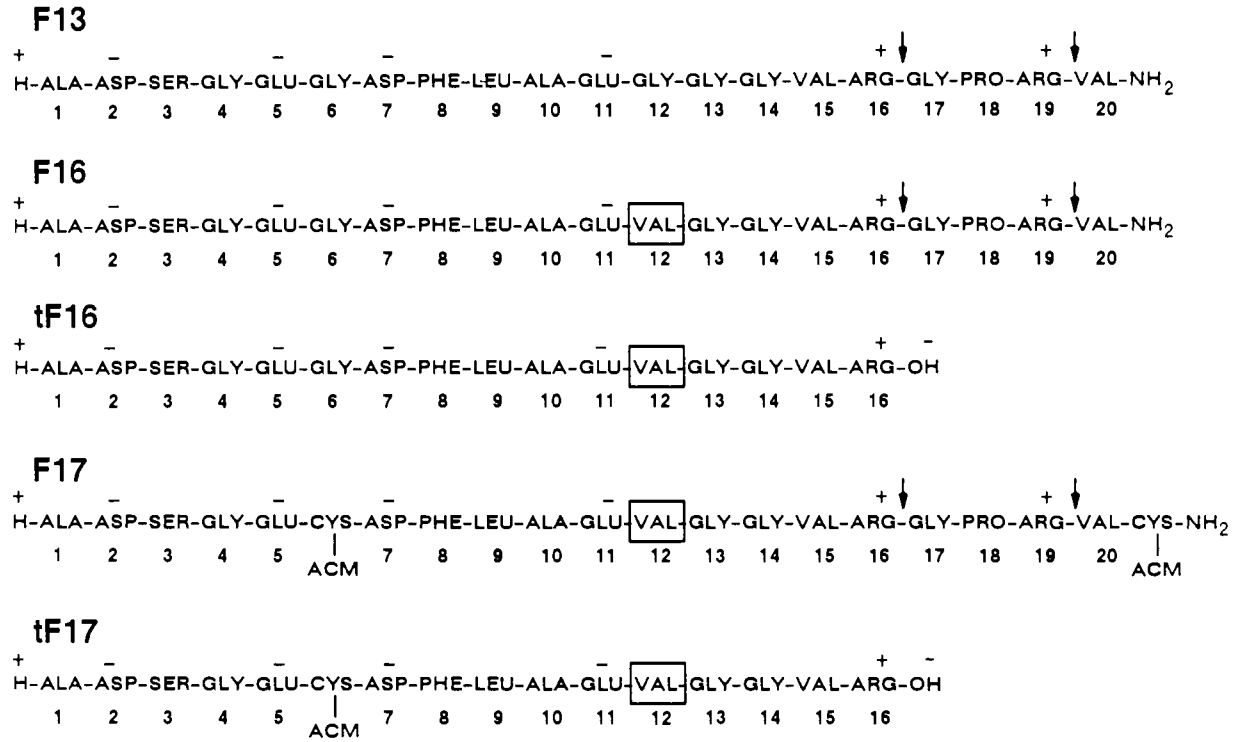

FIGURE 1: Amino acid sequences of the peptides used in the study. The sequence of a peptide (F13) derived from the first 20 residues of the Aα chain of normal human fibrinogen (Ni et al., 1989a) is also shown for comparison. The numbering is that of the Aα chain of human fibrinogen, and the charges are those at neutral pH. Peptide F17 was designed to increase the solubility of the Val(12)-substituted peptide. The arrows in each sequence indicate the peptide bonds cleaved by thrombin. The boxes indicate the positions where residues in the natural sequence have been substituted by other amino acids. Peptides tF16 and tF17 are the thrombin-cleavage products of peptides F16 and F17, respectively. Fibrinogen Rouen, which appeared in a patient with a bleeding disorder, has a single mutation of Gly(12) to Val(12) in the Aα chain [Henschen et al., 1983; Mênaché (1983) and references cited therein].

and distance geometry calculations, the nonpolar side chains of residues Phe(8), Leu(9), and Val(15) within fibrinopeptide A (residues 1–16) were found to form a hydrophobic cluster which presumably would fit in the complementary hydrophobic combining portion of the active site of thrombin (Ni et al., 1989a,b). There is a chain reversal within residues Asp(7) to Arg(16) so that the 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. This chain reversal consists of a type II β-turn at residues Glu(11) and Gly(12) and may also involve a short α-helical segment from residue Phe(8) to residue Ala(10), stabilized by a hydrogen bond between the carbonyl oxygen of Asp(7) and the backbone NH proton of Glu(11) and possibly by another hydrogen bond involving the backbone NH proton of Leu(9) and the carboxylate oxygens of Asp(7) (Ni et al., 1989b). In order to form a stable type II β-turn at residues Glu(11) and Gly(12), the residue at position 12 must be a glycine (Venkatachalam, 1968; Zimmerman & Scheraga, 1978). The substitution of Gly(12) by other amino acid residues would disrupt the structure of the thrombin-bound peptides. The NMR results (Ni et al., 1989a,b) suggest an explanation for the observations that the mutations of Asp(7) to Asn(7) and of Gly(12) to Val(12) result in delayed release of fibrinopeptide A, producing bleeding disorders [Henschen et al., 1983; Mênaché (1983) and references cited therein].

In order to gain further insight into the specificity of the interaction of thrombin with fibrinogen, we have used two-dimensional NMR methods (including TRNOE spectroscopy) to investigate the interaction of thrombin with synthetic peptides derived from residues 1–20 of the Aα chain of abnormal human fibrinogen Rouen. To facilitate the NMR studies of the interaction of thrombin with the Val(12)-substituted peptide, we also synthesized a *more soluble* variant of the abnormal peptide in which Gly(6) is replaced by an Acm-blocked Cys(6), with another Acm-blocked Cys(21) added after Val(20) (Figure 1). In this paper, we report the effect of the Gly(12) to Val(12) substitution on the interaction of bovine thrombin with fibrinogen Rouen like peptides. We also report evidence for a structural change in the Val(12)-substituted peptides in both the free and thrombin-bound states. Structures of residues Asp(7) to Arg(16) of the thrombin-bound abnormal peptides are deduced from transferred NOE measurements and distance geometry calculations. Possible explanations are proposed for the delayed release of fibrinopeptide A from the abnormal human fibrinogen with the Gly(12) to Val(12) substitution in the Aα chain.

Experimental Procedures

Preparation of Bovine Thrombin and Synthetic Peptides. Bovine thrombin was prepared by the procedure of Ghosh and Seegers (1980) and purified as reported previously (Ni et al., 1989a). Peptides F16 and F17 (Figure 1) were prepared by solid-phase methodology (Ni et al., 1989a; Y. Konishi and M. N. Rivetna, unpublished results). The synthesized peptides were purified by RPLC according to a procedure described in our previous papers (Ni et al., 1988, 1989a). The amino acid compositions of the purified peptides were determined 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 compsition of F16 was Asp(1.94,2), Glu(2.01,2), Ser(0.96,1), Gly(5.05,5), Arg(2.09,2), Ala(2.02,2), Pro(1.04,1), Val(2.83,3), Leu(1.04,1), and Phe(1.01,1). That of peptide F17

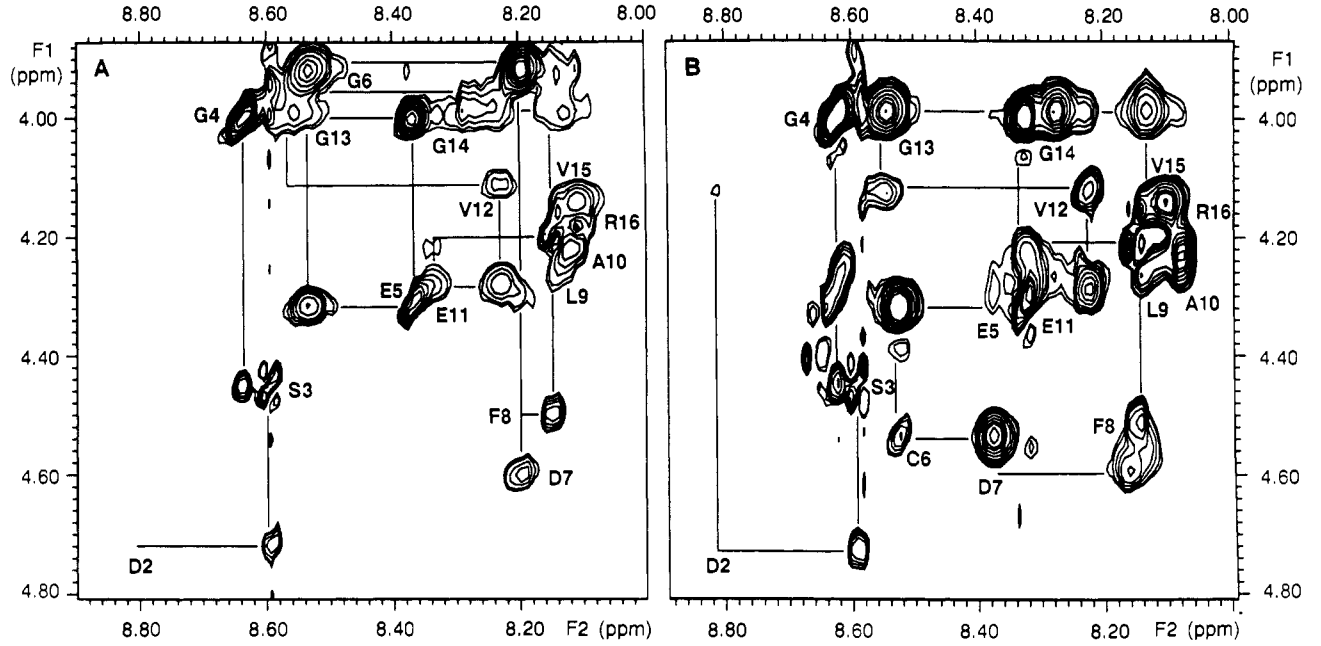

FIGURE 2: Sequential TRNOE connectivities of peptides tF16 (A) and tF17 (B), complexed to thrombin. Every intraresidue NH–C$^{\alpha}$H cross peak is labeled by the corresponding amino acid residues involved (Figure 1). Both spectra were acquired at 15 °C, pH 5.3, with a mixing time of 200 ms. The concentration of thrombin was 0.6 mM in both experiments, and that of peptide tF16 (or F16) was 6 mM, and that of peptide tF17 (or F17) was 8 mM.

was Asp(1.89,2), Glu(2.00,2), Ser(0.97,1), Gly(4.10,4), Arg(2.08,2), Ala(2.01,2), Pro(1.04,1), Val(2.76,3), Cys-(2.04,2), Leu(1.09,1), and Phe(1.00,1). Peptides tF16 and tF17 (Figure 1) were produced by thrombin cleavage of the parent compounds, peptides F16 and F17, and used without further purification [the cleavage products also include Gly-Pro-Arg and Val-NH_2 or Val-Cys(Acm)-NH_2 which do not bind to thrombin].

Assay for the Cleavage of the Arg(16)–Gly(17) Peptide Bond in Synthetic Peptides. Thrombin cleavage of the Arg-(16)–Gly(17) peptide bond at ambient temperature (~21 °C), pH 7.0, was followed by RPLC analysis. A volume of 50 μL of stock solutions of thrombin (0.05 mg/mL or 0.5 mg/mL) was added to 500 μL of solutions that were 8 μM in peptide and 150 mM in NaCl, 50 mM in sodium phosphate, and 0.1% in PEG 6000 with the pH adjusted to 7.0. At various times, 100 μL of the reaction mixture was taken out and mixed with 5 μL of 3 M $HClO_4$ to stop the action of thrombin. The peptides in the reaction mixture were separated by gradient elution on an analytical RPLC system, as described previously (Ni et al., 1988). The times for 50% cleavage of the Arg-(16)–Gly(17) peptide bonds were determined by quantitative estimation of peak areas in the RPLC chromatograms of the peptides in fixed volumes (100 μL) of the reaction mixture. For comparison, a similar assay was carried out for the cleavage of the Arg(16)–Gly(17) peptide bond in peptide F13 (Ni et al., 1989a), which corresponds to residues Ala(1) to Val(20) of the Aα chain of normal human fibrinogen (see Figure 1).

NMR Sample Preparation and Measurements. All NMR experiments were carried out at pH 5.3 in buffered aqueous solution, conditions under which thrombin was previously shown to be active (Ehrenpreis et al., 1958). Solutions of the free peptides were prepared by dissolving peptide F16 (14.8 mg/mL) or F17 (16.4 mg/mL) in an aqueous solution (the NMR solvent) that was 150 mM in NaCl, 50 mM in sodium phosphate, and 0.1 mM in EDTA, with the pH adjusted to 5.3. The samples were centrifuged to remove a small amount of precipitate that was formed during the pH adjustments, especially for peptide F16 which apparently has lower solubility in H_2O.

For thrombin-binding and NOE studies, dilute solutions of the peptides were prepared by dissolving 7.1 mg of F16 or 7.0 mg of F17 in 0.4 mL of H_2O and 0.1 mL of the NMR solvent. This peptide solution was incubated overnight at room temperature in the presence of catalytic amounts (~40 NIH units/mL) of active thrombin at pH 7.0 [this treatment was found to increase the solubility of the peptides, especially for F16, presumably because the cleavage products, tF16 (Figure 1), Gly-Pro-Arg, and Val-NH_2 are more soluble in aqueous solutions]. The pH values of the resulting peptide solutions were then adjusted to 5.3 with trace amounts of 1 M HCl or 1 M NaOH. The pH-adjusted solutions were freeze-dried to increase the peptide concentration. The dried powders (including salt, peptides, and trace amounts of thrombin) were then taken up in a mixture of 60 μL of H_2O and 40 μL of D_2O. The pH values of the aqueous samples were all checked with an Ingold electrode without correction for the presence of D_2O. Finally, for use in the binding and NOE studies, NMR samples of the peptide–thrombin solutions were prepared by mixing 0.3 mL of a concentrated thrombin stock solution (Ni et al., 1989a) and 0.1 mL of the peptide solutions prepared above.

The detailed procedure of the NMR experiments was described previously (Ni et al., 1988, 1989a,b). A brief summary is presented below. The temperature dependence of the chemical shifts of the amide protons was determined from one-dimensional spectra, at various temperatures from 15 to 45 °C, of the *free* peptides in H_2O solutions at pH 5.3. The relative rates of exchange of amide protons were determined by comparing the intensities of the C$^{\alpha}$H–NH cross peaks in the COSY spectra of the peptides that were freshly dissolved in D_2O at pH 3.0 and 0 °C. The involvement of specific residues in binding to thrombin was determined by examining the line broadening of the amide and side-chain proton resonances in the peptides in the presence of about 0.1 equiv (compared to the peptide) of active thrombin. The pulse sequence for two-dimensional transferred NOE measurements was the same as for the regular NOESY procedure with minor

Table I: NMR Chemical Shifts (in ppm) of the Backbone NH Protons[a] of Free Peptides F13, F16, and F17 in H_2O at pH 5.3 and 25 °C

residue	F16	Δ[b]	F17	Δ[b]	F13[c]
D2	8.75	0.34	8.75	0.34	8.76
S3	8.53	0.15	8.52	0.14	8.53
G4	8.59	0.20	8.57	0.18	8.59
E5	8.33	−0.04	8.24	−0.13	8.33
G6 (C6)	8.46	0.07	8.46	0.15	8.48
D7	8.17	−0.24	8.34	−0.07	8.19
F8	8.16	−0.07	8.10	−0.13	8.17
L9	8.11	−0.31	8.09	−0.33	8.12
A10	8.10	−0.15	8.05	−0.20	8.17
E11	8.28	−0.09	8.28	−0.09	8.38
V12 (G12)	8.14	−0.30	8.14	−0.30	8.44
G13	8.54	0.15	8.53	0.14	8.30
G14	8.24	−0.15	8.23	−0.16	8.32
V15	8.05	−0.39	8.05	−0.39	8.04
R16	8.49	0.22	8.49	0.22	8.48
G17	8.24	−0.15	8.23	−0.16	8.22
R19	8.48	0.21	8.48	0.21	8.46
V20	8.17	−0.27	8.20	−0.24	8.16

[a] Residues Ala(1) and Pro(18) do not have amide protons and thus are not listed. [b] Deviations from values of nonstructured peptides in H_2O (Bundi & Wüthrich, 1979). [c] Resonances were assigned in our previous paper (Ni et al., 1989a).

modifications for the improvement of spectral clarity and for more efficient suppression of the water proton resonance. Possible NOE cross peaks from thrombin were located by a combined analysis of the TRNOE spectra of the complex of thrombin with different fibrinogen-like peptides (Ni et al., 1989a).

Results

Confirmation of Amino Acid Sequences and Assignments of Proton Resonances. The amino acid sequences of peptides F16 and F17 (Figure 1) were confirmed by an analysis of the sequential $C^{\alpha}H$–NH NOE connectivities of these peptides at 5 °C and pH 5.3, compared to that of peptide F10 (residues 1–23; Ni et al., 1988) and F13 (residues 1–20; Ni et al., 1989a) at the same temperature and solvent conditions. The backbone NH proton resonances (Table I) of both F16 and F17 were assigned in aqueous solution at pH 5.3 by use of the NMR techniques discussed in our previous paper (Ni et al., 1988). The $C^{\alpha}H$ and side-chain proton resonances of residues Ala(1) to Glu(5), Asp(7) to Glu(11), and Gly(13) to Val(20) in the free peptides do not appear to be shifted by the Gly(6) to Cys(6), Gly(12) to Val(12), and Val(21) to Cys(21) substitutions. The most affected resonances are those of the backbone NH protons of the residues around the sites of substitution, as seen from Table I where the backbone NH chemical shifts of peptides F16 and F17 at pH 5.3 and 25 °C are compared with those of peptide F13 (Figure 1) under identical experimental conditions. Also listed are the deviations of the NH chemical shifts in peptides F16 and F17 from the values of the corresponding amino acid residues in nonstructured peptides (Bundi & Wüthrich, 1979).

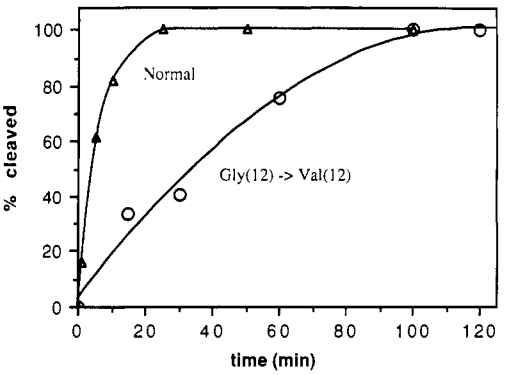

FIGURE 3: Rates of the thrombin cleavage of the Arg(16)–Gly(17) peptide bonds in the normal and the Val(12)-substituted peptides. The normal peptide was peptide F13. The substituted peptide was peptide F16 (Figure 1). For assay with peptide F16, the concentration of thrombin used was 10 times that used with peptide F13. Experimental conditions are described in the text.

The assignments of the NH and $C^{\alpha}H$ resonances of peptides tF16 and tF17 are illustrated in Figure 2, where the transferred $C^{\alpha}H$–NH NOE's of these two peptides are displayed with the sequential NOE connectivities indicated. Compared to the uncleaved peptides F16 and F17, the only significantly shifted resonance is that of the NH proton of Arg(16), which now appears at about 8.10 ppm (at 15 °C) because of its location at the carboxyl terminus (Ni et al., 1988).

Effect of the Gly(12) to Val(12) Substitution on the Con-

Table II: Survey of the NMR Parameters for F16 and F17 (or tF17 and tF16) in the Free and Thrombin-Bound States[a]

	1 A	2 D	3 S	4 G	5 E	6 G	7 D	8 F	9 L	10 A	11 E	12 V	13 G	14 G	15 V	16 R	17 G	18 P	19 R	20 V
Δ		B	A	A			B		B	A		B	A	A	B	A	A		A	B
k_{ex}								A	B	A	A	B	A		A	A		X	A	B
L_{NH}						S	S	S	S	S	S	S	S	S	S	S				
$L_{\beta H}$						X	W	S	S	S	W	S	X	X	S	S				
L_{CH}						X	X	S	S	X	W	S	X	X	S	S				
$d_{N\beta}$					S	X	S	S	S	S	S	S	X	X	S	S				
$d_{\alpha N}$	W	W	W	W	S	S	S	W	?	W	W	W	?	S	S	X				
$d_{\beta N}$					W	X	W	S	S	S	S	W	X	X	?	X				
d_{NN}				W	W	W		?	?	S	S	S	W		?	X				
$d_{\phi\delta}$								⌊	⌋											
$d_{N\beta}$							⌊			⌋										
$d_{\phi\gamma}$								⌊				⌋								
$d_{\phi\gamma}$								⌊							⌋					

[a] Δ, residues in the *free* peptide F16 (Figure 1) with deviations of the NH chemical shifts from those of nonstructured peptides in H_2O [B, very large (≥±0.22); A, large (<±0.22; >±0.09)]. k_{ex}, exchange rate of NH protons in both peptides F16 and F17 in the free state (B, very slow; A, slow). L_{NH}, $L_{\beta H}$, and L_{CH}, line-broadening effects on the NH, $C^{\beta}H$, and other side-chain CH protons of peptides tF16 and tF17 by thrombin binding: *W*, line broadening observed; *S*, pronounced line broadening observed. The boldface S under Phe(8) indicates that its ring proton resonances are severely broadened upon thrombin binding (Figure 4). $d_{N\beta}$, intraresidue NH–$C^{\beta}H$ NOE: W, weak; S, strong. It also indicates the medium-range NOE between the NH proton of Asp(7) and the $C^{\beta}H$ protons of Ala(10). $d_{\alpha N}$, 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. $d_{\phi\delta}$, NOE's between the ring protons of Phe(8) and the $C^{\delta}H$ protons of Leu(9). $d_{\phi\gamma}$, NOE's between the ring protons of Phe(8) and $C^{\gamma}H$ protons of Val(12) or Val(15). 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. All NOE's pertain to the interaction of thrombin with tF16 or tF17.

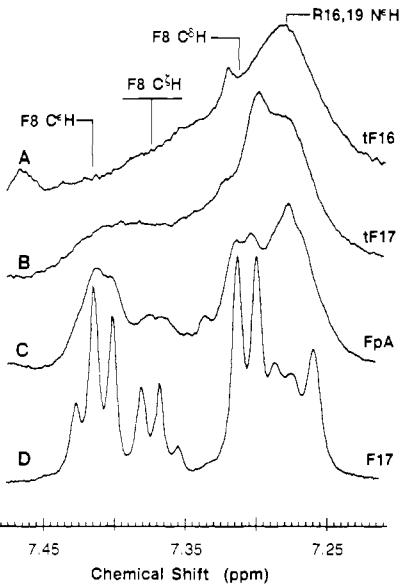

FIGURE 4: Resonances of the ring protons of Phe(8) in one-dimensional proton NMR spectra of peptide tF16 (A), peptide tF17 (B), and fibrinopeptide A (C) in the presence of thrombin and of the *free* peptide F17 (D) in buffered H_2O solution at pH 5.3 and 25 °C. Peptide concentration: tF16, 6 mM; tF17, 8 mM; FpA, 6 mM; F17, 8 mM. The concentration of thrombin in spectra A–C was 0.6 mM. The ring proton resonances of Phe(8) in peptides tF16 (A) and tF17 (B) are seen to be much more broadened compared to those in fibrinopeptide A (C) in the presence of similar concentrations of thrombin.

formational Properties of the Free Peptides. In our previous study, it was shown that both the exchange rates of backbone NH protons and deviations of the chemical shifts (from those of nonstructured peptides) of the backbone amide proton resonances indicate the formation of structure within the free linear fibrinogen-like peptides (Ni et al., 1988, 1989a). Furthermore, both Gly(13) and Gly(14) possess abnormal temperature coefficients of the NH chemical shifts in peptides F6 (residues 8–18), F8 (residues 7–20), FpA (residues 1–16), and F10 (residues 1–23) (Marsh et al., 1985; Ni et al., 1988), indicating that their NH protons are possibly involved in hydrogen bonds (Ni et al., 1989b).

As seen in Table I, there are significant deviations of the backbone amide proton chemical shifts from reference values for residues from Asp(2) to Gly(4), Asp(7), Leu(9), Ala(10), and Val(12) to Val(20), as observed for peptide F10 derived from the first 23 residues of Aα chain of normal human fibrinogen. However, the backbone conformations of residues Val(12) to Gly(14) are probably different from those of the corresponding residues in the normal peptides since, in the substituted peptides F16 and F17, the NH chemical shifts of these residues are different from those in nonstructured peptides, a phenomenon not observed in the normal peptides (Ni et al., 1988). We have also studied the exchange behavior of the backbone NH protons in peptides F16 and F17. Both peptides have the same pattern of slowly exchanging protons, as listed in Table II. Similar to the normal peptides, the NH protons of residues Ala(1) to Asp(7) in both peptides F16 and F17 exchange very fast, suggesting that the Gly(12) to Val(12) substitution does not affect the backbone conformations of the residues in this region of the molecule. The NH proton of Val(12), however, exchanges slowly, and the NH proton of Gly(14) exchanges fast in contrast to what was observed for the corresponding residues in the normal peptides (Ni et al., 1988). Another interesting finding is that the amide proton resonances of residues Gly(13) and Gly(14) no longer show abnormal temperature coefficients in either peptide F16 or

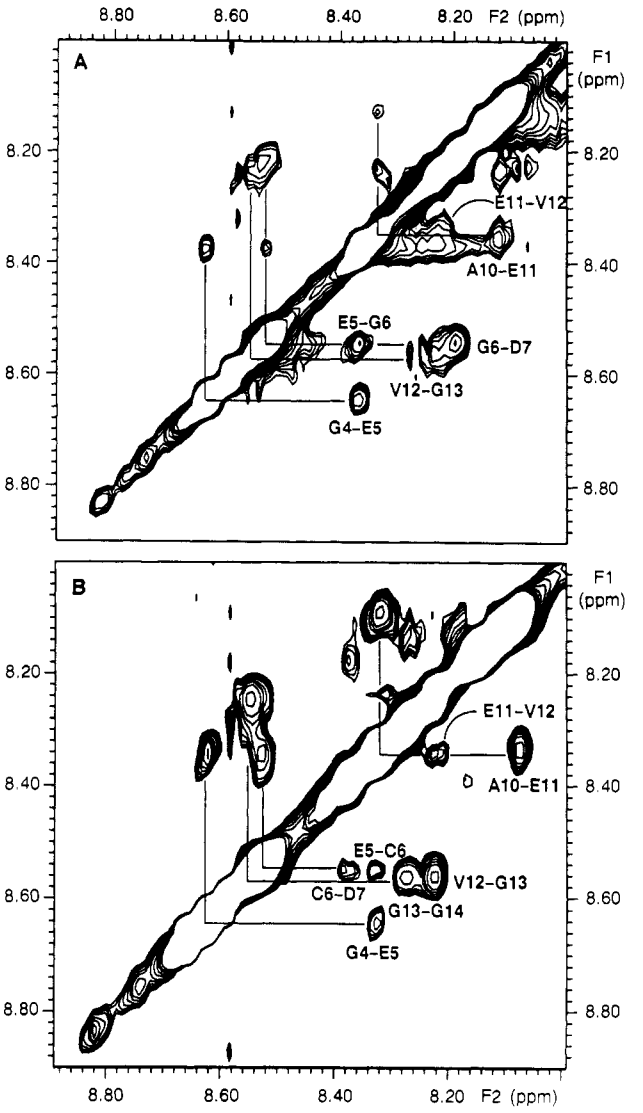

FIGURE 5: NH–NH region of the TRNOE spectra obtained in buffered H_2O solution of the interaction of thrombin with peptide F16 (A) and F17 (B) (resulting in a mixture of peptide tF16 or tF17 and other thrombin cleavage products) at pH 5.3. The NOE's are labeled by the amino acid residues involved. Experimental conditions are the same as indicated in Figure 2.

peptide F17. The Val(12) substitution thus probably alters the backbone conformations of the residues at positions 12, 13, and 14 of the free peptides significantly.

Interaction of Thrombin with Peptides F16 and F17. The Arg(16)–Gly(17) peptide bonds in both peptides F16 and F17 are cleaved by thrombin at a much slower rate than those in normal fibrinogen-like peptides F6 (residues 8–18), F8 (residues 7–20), F10 (residues 1–23), and F13 (residues 1–20) studied in our previous papers (Ni et al., 1989a,b). For example, the time for 50% cleavage of the Arg(16)–Gly(17) peptide bond in F13 is about 5 min (Figure 3) at pH 7.0 and ambient temperature (~21 °C), at a peptide concentration of 8 μM and a thrombin concentration of 70 nM. For peptide F16 or F17, less than 1% cleavage of the Arg(16)–Gly(17) peptide bonds was observed, under identical conditions, within the first hour of incubation of the peptides with thrombin. At similar peptide concentrations and under identical solvent conditions, the time for 50% cleavage is over half an hour for the substituted peptides, with 10 times more concentrated thrombin (700 nM) (Figure 3).

At pH 5.3, the N-terminal cleavage products tF16 and tF17

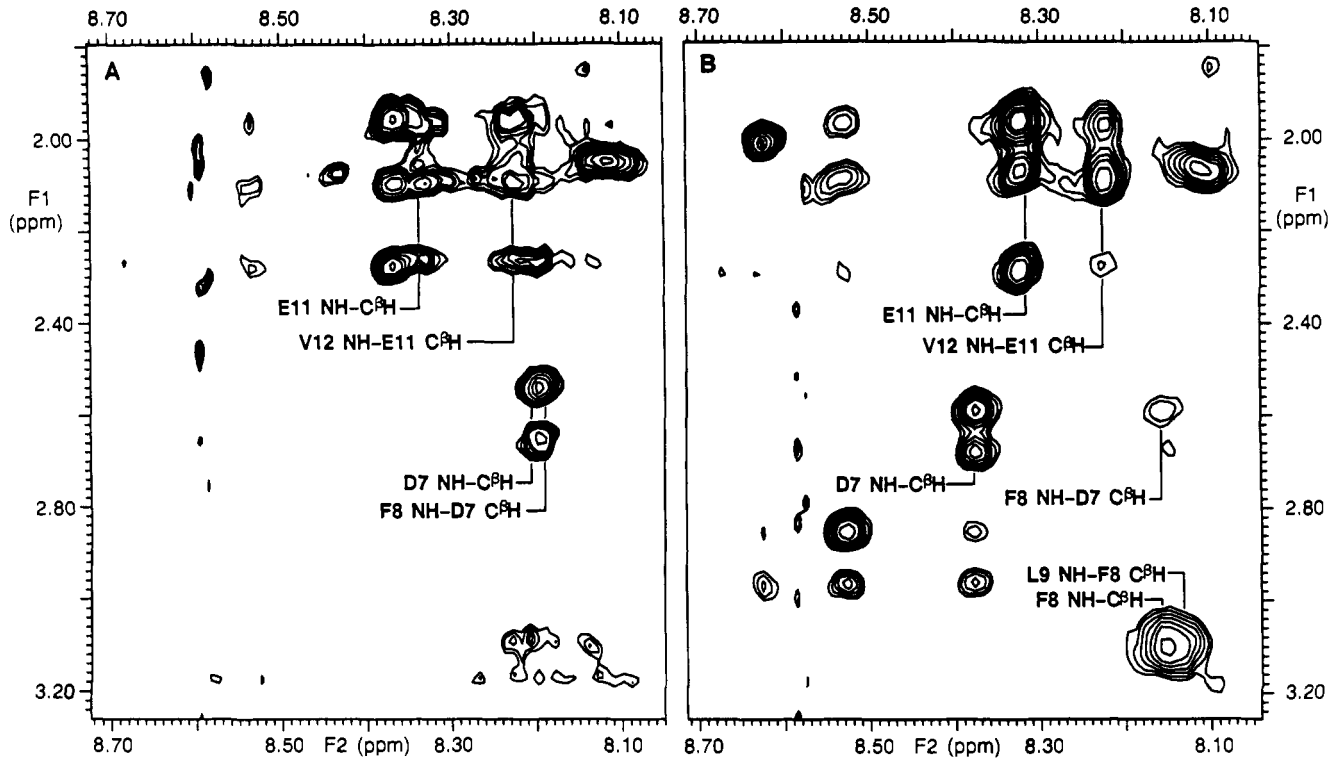

FIGURE 6: NH–C^{β}H regions of the TRNOE spectra of peptide tF16 (A) and tF17 (B) (see also Figures 2 and 5). The indicated cross peaks are intraresidue (single amino acid) or sequential (two amino acids) NOE's between the NH and C^{β}H protons of the residues involved. The C^{β}H–NH NOE between residues Glu(11) and Val(12) in both tF16 and tF17 was not observed for residues Glu(11) and Gly(12) in normal fibrinogen-like peptides (Ni et al., 1989a,b). In peptide tF17 (B), the NH proton resonance of Asp(7) is shifted downfield by the Gly(6) to Cys(6) substitution (see also Table I), leaving the C^{β}H–NH NOE between residues Asp(7) and Phe(8) clearly resolved. The higher solubility of peptide tF17 makes the NOE's belonging to residues Phe(8) and Leu(9) also observable.

remain bound to thrombin as indicated by line broadening of the proton resonances in one-dimensional spectra of these peptides in the presence of about 0.1 equiv (compared to the peptides) of thrombin, as observed similarly for the interaction of thrombin with the unsubstituted peptides (Ni et al., 1989a,b). Upon binding to thrombin, the affected resonances in the substituted peptides are those of all the NH protons of residues Gly(6) to Arg(16), those of the C^{β}H protons of Asp(7), Phe(8), Leu(9), Ala(10), Glu(11), Val(12), Val(15), and Arg(16), the C^{δ}H protons of Leu(9), and the C^{γ}H protons of Glu(11), Val(12), and Val(15), and those of the aromatic ring protons of Phe(8) (Table II and Figure 4). Again, there is no significant thrombin-induced line broadening of the resonances of residues Ala(1) to Glu(5), as observed for the binding of thrombin to normal fibrinopeptide A (Ni et al., 1989a). This observation suggests that residues Ala(1) to Glu(5) are not involved in the interaction with thrombin. However, thrombin induces a much more pronounced line broadening of the ring proton resonances of Phe(8) in both peptides tF16 and tF17 than in fibrinopeptide A. This is seen in Figure 4, where the aromatic regions of the proton spectra of tF16, tF17, and fibrinopeptide A in the presence of thrombin are shown along with the same region of the proton spectra of the *free* F17 at the same pH and at identical salt concentrations.

Transferred NOE's of Thrombin-Bound Peptides. Transferred NOE's have been observed in the interaction of thrombin with normal and other fibrinogen-like peptides with Arg(16) replaced by Gly(16) or Leu(16) (Ni et al., 1989a,b). Transferred NOE's were also observed in the interaction of thrombin with peptides tF16 and tF17, as illustrated in Figure 2. For the *free* peptides, sequential C^{α}H–NH NOE's were found to be very weak at 5 °C with a mixing time of 200 ms (spectra not shown). In Figure 2, the NOE spectra were acquired at 15 °C and contain primarily transferred NOE's from the thrombin-bound peptides, since, as the temperature of the sample increases, the intensities of regular NOE's tend to decrease because of the rapid tumbling of the small linear peptides in solution, as discussed previously (Ni et al., 1989a).

For both peptides tF16 and tF17, most of the sequential and intraresidue transferred NOE's are within residues Asp(7) to Arg(16) (Table II), as observed similarly in normal fibrinogen-like peptides (Ni et al., 1989a,b). Some transferred sequential C^{α}H–NH NOE's in the region of residues Ala(1) to Glu(5) are also clearly visible in the TRNOE spectra of the complexes between thrombin and peptides tF16 and tF17 (Figure 2). Furthermore, weak sequential NH–NH TRNOE's exist within residues Gly(4) to Gly(6) [or Cys(6)] for peptides tF16 and tF17 (Figure 5). Under identical experimental conditions, the same sequential C^{α}H–NH and NH–NH TRNOE's were also found within residues Ala(1) to Gly(6) for normal fibrinopeptide A in a complex with thrombin (spectra not shown), although these NOE's were not observed for the same complex in TRNOE experiments with shorter (800 ms instead of 1.5 s used here) postacquisition delays (Ni et al., 1989a). However, proton resonances (especially those of the side chains) of residues Ala(1) to Glu(5) in both tF16 and tF17 are not much affected by thrombin binding (Table II), indicating that these residues do not contribute significantly to the specificity of the interaction of thrombin with fibrinogen. Peptide tF17, which contains a second substitution of Gly(6) to an Acm-blocked Cys(6), has similar thrombin-induced line broadening as peptide tF16 and gives rise to a similar pattern of TRNOE's (Figures 2 and 5 and Table II). This observation suggests that Gly(6) may also be unimportant in binding to thrombin.

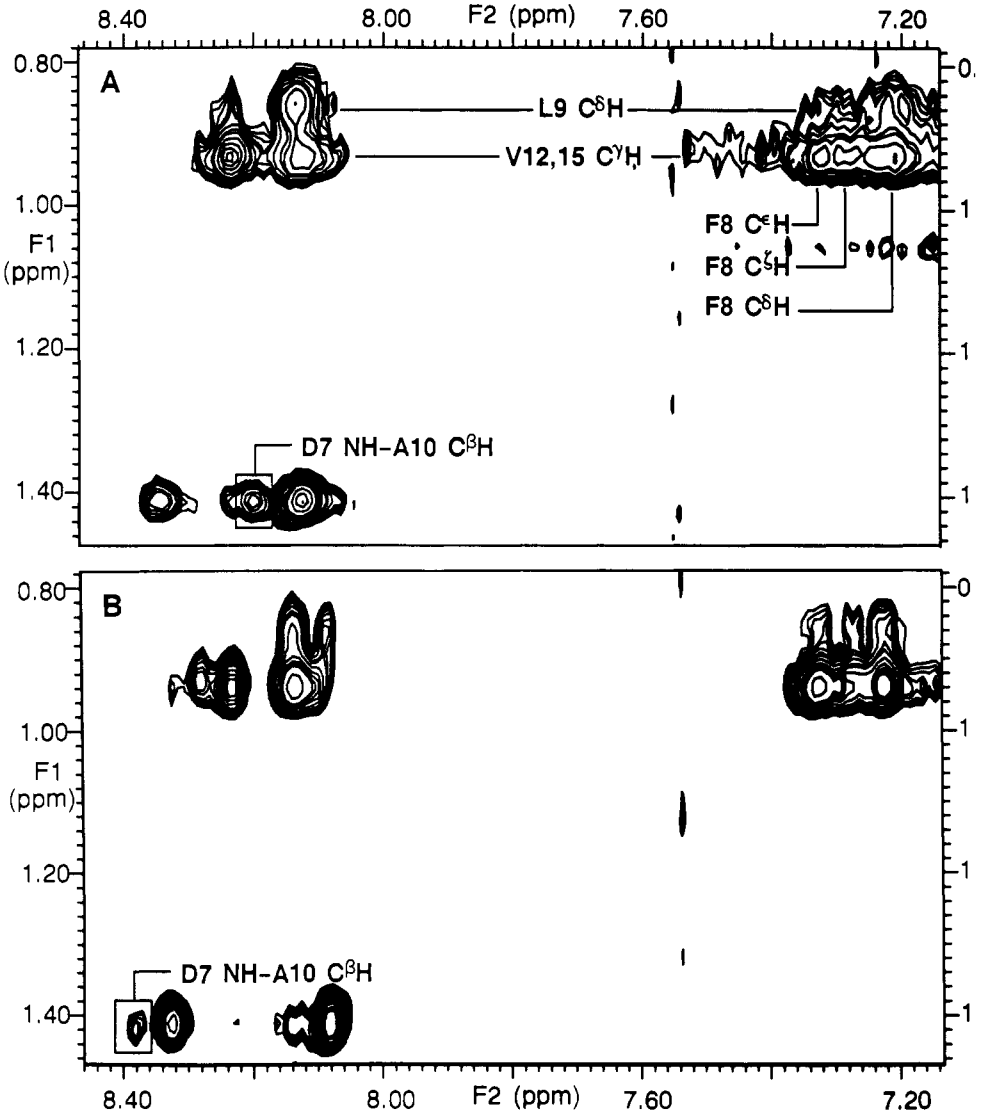

FIGURE 7: Selected region of the transferred NOE spectra of the complexes of thrombin with peptides tF16 (A) and tF17 (B). The NOESY spectra were acquired with experimental conditions as indicated in Figure 2. The medium-range NOE's between Asp(7) and Ala(10) and the long-range NOE's between Phe(8) and Val(12) and/or Val(15) are indicated by the protons involved. In (B), the NOE between the NH proton of Asp(7) and $C^{\beta}H$ protons of Ala(10) is shifted downfield due to the Gly(6) to Cys(6) substitution. Furthermore, the quality of the spectrum is better than that in (A), especially for the NOE cross peaks between the ring protons of Phe(8) and the $C^{\gamma}H$ protons of Val(12) and Val(15). This is because of the less-pronounced line broadening in the ring protons of Phe(8) of peptide tF17 (see Figure 4). The intensities of the NOE cross peaks originating from the $C^{\gamma}H$ protons of Val(12) and/or Val(15) were analyzed further by taking a sum of all the slices between 0.90 and 1.00 ppm on the F_1 frequency axis. The resulting one-dimensional spectra are presented in Figure 8A,B.

For the specificity sequence, Asp(7) to Arg(16), NOE patterns within residues Gly(14) to Arg(16) were not affected by the Val(12) substitution. For example, no TRNOE was observed between the NH protons of Gly(14) and Val(15) in the complex of thrombin with fibrinopeptide A (Ni et al., 1989a). There is still no TRNOE between the NH protons of Gly(14) and Val(15) in both tF16 and tF17 (Figure 5). Major differences in TRNOE's of the peptides are within residues Glu(11) to Gly(13), indicating possible conformational changes brought about by the Gly(12) to Val(12) substitution. Particularly, the previously observed NOE interaction between the $C^{\alpha}H$ proton of Glu(11) and the NH proton of Gly(13) (Ni et al., 1988b) is absent in the complex of thrombin with the Val(12)-substituted peptides, tF16 and tF17 (Figure 2). On the other hand, the NOE cross peaks between the NH protons of Glu(11) and Val(12) are present in the TRNOE spectra of these peptides (Figure 5), whereas no such NOE could be found between residues Glu(11) and Gly(12) in the normal peptides (Ni et al., 1989a,b). Furthermore, the interaction between the $C^{\beta}H$ protons of Glu(11) and the NH proton of Val(12), previously not observed for residues Glu(11) and Gly(12) in the normal peptides (Ni et al., 1989a,b), is very strong in the TRNOE spectra of peptides tF16 and tF17 (Figure 6). A weak sequential NH–NH NOE can also be seen between residues Gly(13) and Gly(14) (Figure 5), because of the shift of the NH proton resonance of Gly(13) (Table I), caused by the substitution of Val(12) for Gly(12) in peptides tF16 and tF17.

In the previous study (Ni et al., 1989b), it was found that an NOE between the resonances of the NH proton of Asp(7) and the $C^{\beta}H$ protons of Ala(10) produced one of the most important distance constraints for the determination of the structure of the decapeptide derived from residues Asp(7) to Arg(16) of the Aα chain of normal human fibrinogen. The same NOE interaction was also found in the substituted peptides, as seen in both panels A and B of Figure 7. The replacement of the natural Gly(6) by Cys(Acm) shifts the NH proton resonance of Asp(7) from about 8.20 ppm in tF16 to

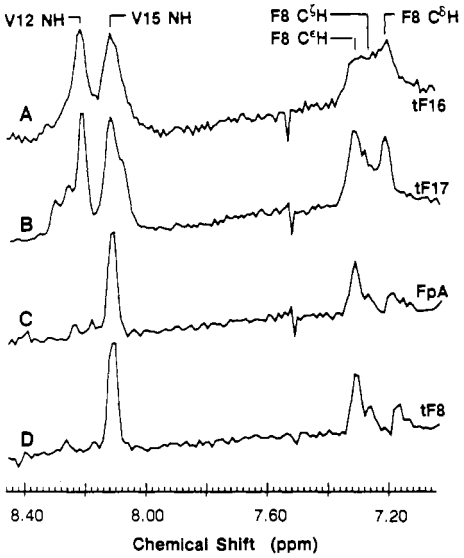

FIGURE 8: Integrated TRNOE cross peaks between the NH and C$^{\gamma}$H protons of Val(12) and/or Val(15) and between the ring protons of Phe(8) and the C$^{\gamma}$H protons of Val(12) and/or Val(15) in peptides tF16 (A) and tF17 (B) (taken from Figure 7), in peptide FpA (C) [residues Ala(1) to Arg(16); Ni et al., 1989a], and in peptide tF8 (D) [residues Asp(7) to Arg(16); Ni et al., 1989b] acquired here under identical experimental conditions (see Figure 2). The integration procedure is described in the text and in Figure 7. The intensity of the NOE peak between the C$^{\zeta}$H proton of Phe(8) and the C$^{\gamma}$H protons of the Val residue(s) for peptides tF16 and tF17 is seen to be almost twice that in FpA or peptide tF8 where only one Val residue [Val(15)] interacts with Phe(8) (Ni et al., 1989a,b). In (A) and (B), the NOE peaks between the C$^{\zeta}$H and C$^{\epsilon}$H protons of Phe(8) and the C$^{\gamma}$H protons of the Val residue(s) are lower in intensity because of the severe thrombin-induced line broadening of these proton resonances of Phe(8) (Figure 4). Nevertheless, those NOE peaks between Phe(8) and Val in peptide tF17 are still more intense than the corresponding peaks in FpA or tF8.

a larger chemical shift of 8.38 ppm in tF17 (Figures 2 and 6). The NOE cross peak between Asp(7) and Ala(10) indicated in Figure 7A is also shifted accordingly as seen in Figure 7B. The presence of this cross peak in the TRNOE spectra of different peptides confirms the assignment of a medium-range NOE between the NH proton of Asp(7) and the C$^{\beta}$H protons of Ala(10) in the normal peptide (Ni et al., 1989b) and in the substituted peptides studied here. Furthermore, it also indicates that the substitution of Cys(Acm) for Gly(6) does not affect the binding of the peptides with thrombin in agreement with results from line-broadening studies discussed in previous sections.

In Figure 7, intense cross peaks can be seen between the resonances of the ring protons of Phe(8) and the C$^{\gamma}$H protons of Val(12) or Val(15). Unfortunately, the C$^{\gamma}$H protons of Val(12) resonate at exactly the same frequency as those of Val(15). To resolve this ambiguity, we integrated these NOE peaks along the F_1 frequency axis by taking a sum of the individual slices of the two-dimensional spectra (a procedure later referred to as a sum projection onto the F_2 frequency axis) between 0.90 and 1.00 ppm on the F_1 frequency axis (Figure 7). The results are presented in Figure 8 along with similar sum projections from the TRNOE spectra of normal fibrinogen-like peptides (Ni et al., 1989a,b) acquired here under identical experimental conditions. The intensities of the cross peaks between Phe(8) and Val in the substituted peptides (Figure 8A,B) are unusually large compared to those in the normal peptides [with only Val(15) present], considering the pronounced thrombin-induced reduction of peak intensities of the ring proton resonances of Phe(8) in peptides tF16 and tF17 (Figure 4). We thus conclude that the ring protons of Phe(8) interact with C$^{\gamma}$H protons of *both* Val(12) and Val(15).

In the interaction of thrombin with normal fibrinogen-like peptides, long-range TRNOE's were also observed between the ring protons of Phe(8) and the C$^{\alpha}$H protons of Gly(14) (Ni et al., 1989a,b). These NOE's, however, are absent in the TRNOE spectrum of complexes involving peptide tF16 at 15 °C and peptide tF17 at both 5 and 15 °C with mixing times in the range of 50–200 ms. Thus, the Gly(12) to Val(12) substitution causes structural changes around residues Gly(13) and Gly(14) in the bound peptides in agreement with the observations with the free peptides discussed in the previous sections.

TRNOE Distance Constraints. To obtain a more precise assessment of the conformation of the thrombin-bound peptide, we have converted the NOE data (Table II) into distance information for use in distance geometry calculations, as described previously (Ni et al., 1989b). In principle, accurate interproton distances can be obtained from the nuclear cross-relaxation rates (Solomon, 1955), which, in turn, can be measured by use of the initial buildup rates of the intensities of the NOE cross peaks between dipolar-coupled protons (Macura & Ernst, 1980). Because of the low signal-to-noise character of the transferred NOE's, we estimated only upper bounds (and some lower bounds) of interproton distances (Table III) on the basis of an analysis of the NOE cross-peak intensities in TRNOE spectra of tF17 (used because of its higher solubility) acquired with mixing times of 50, 100, and 200 ms, respectively. In each spectrum, the intensities of NOE cross peaks were determined by an inspection of the contour plots or by comparison of the peak heights in the sum projections of the cross peaks onto the F_2 frequency axis (a procedure for partial peak integration, as shown in Figure 8). Upper bounds of 2.5 Å were assigned to the distances between protons with strong TRNOE cross peaks at a mixing time of 50 ms. These strong NOE's arise, for example, from the intraresidue interactions between the NH and C$^{\beta}$H protons of Phe(8), Leu(9), Ala(10), Glu(11), Val(12), Val(15), and Arg(16). The upper bounds were taken to be 3.0 Å if, with a mixing time of 50 ms, the NOE cross peaks were weak, but were strong with a mixing time of 100 ms. These include, e.g., the sequential interactions between the NH protons of Ala(10), Glu(11), Val(12), and Gly(13). Weak cross peaks in the spectra of 100-ms mixing time indicate distances with upper bounds of 3.5 Å. Upper bounds of 4 Å were assigned to all the weak NOE's in the spectra of 200-ms mixing time. Lower bounds of 3.5 Å were also assumed for proton pairs on the basis of the absence of sequential TRNOE's between, e.g., the NH protons of Gly(14) and Val(15) (Figure 5) and between the C$^{\beta}$H protons of Val(15) and the NH proton of Arg(16), as established previously for the normal peptides (Ni et al., 1989b).

Errors in the distance upper bounds could arise especially for protons with reduced resonance intensities (peak heights) brought about by thrombin binding (Table II). For sequential NOE's, differences in intensities because of differences in line broadening can lead to inconsistencies between the distance upper bounds for the NH and C$^{\beta}$H protons of the same residue and for the C$^{\alpha}$H and NH, the C$^{\beta}$H and NH, and the NH and NH protons of adjacent residues. In principle, if there is no limitation on the solubility of the peptides, line broadening can be reduced if the concentration of the peptide is increased, since the enzyme-induced line broadening decreases with an increase in the ratio of the concentrations of the peptide and the enzyme (Dwek, 1973), as observed for the binding of thrombin to fibrinogen-like peptides (Ni et al., 1989a). Unfortunately, the

Table III: Distance Constraints Derived from the TRNOE Experiments

Sequential with Lower Bounds Set to 3.5 Å	
HN Gly(14) to HN Val(15)	
HB Val(15) to HN Arg(16)[a]	
Intraresidue with Upper Bounds Set to 2.5 Å	
HN to HB, HB to HD	Phe(8)
HN to HB	Leu(9)
HN to HB	Ala(10)
HN to HB, HN to HG	Glu(11)
HN to HB, HN to HG	Val(12)
HN to HA	Gly(13)
HN to HB, HN to HG	Val(15)
HN to HB, HB to HE	Arg(16)
Intraresidue with Upper Bounds Set to 3.0 Å	
HN to HB	Asp(7)
HA to HD	Phe(8)
HA to HD, HN to HD	Leu(9)
Sequential with Upper Bounds Set to 2.5 Å	
HA Asp(7) to HN Phe(8)	HB Ala(10) to HN Glu(11)
HB Phe(8) to HN Leu(9)	HA Gly(14) to HN Val(15)
Sequential with Upper Bounds Set to 3.0 Å	
HB Asp(7) to HN Phe(8)	HN Ala(10) to HN Glu(11)
HD Phe(8) to HB Leu(9)	HB Glu(11) to HN Val(12)
HD Phe(8) to HD Leu(9)	HN Glu(11) to HN Val(12)
HN Phe(8) to HN Leu(9)[b]	HN Val(12) to HN Gly(13)
HB Leu(9) to HN Ala(10)	HA Val(15) to HN Arg(16)
HN Leu(9) to HN Ala(10)[a]	
Sequential with Upper Bounds Set to 3.5 Å	
HA Phe(8) to HN Leu(9)	HA Glu(11) to HN Val(12)
HD Phe(8) to HN Leu(9)	HG Glu(11) to HN Val(12)
HE Phe(8) to HD Leu(9)	HA Val(12) to HN Gly(13)
HA Ala(10) to HN Glu(11)	HB Val(12) to HN Gly(13)
Sequential with Upper Bounds Set to 4.0 Å	
HG Val(12) to HN Gly(13)	
HN Gly(13) to HN Gly(14)	
Medium- and Long-Range Upper Bounds	
HN Asp(7) to HB Ala(10)	3.5 Å
HD Phe(8) to HG Val(12)	3.5 Å
HE Phe(8) to HG Val(12)	3.0 Å
HZ Phe(8) to HG Val(12)	3.5 Å
HD Phe(8) to HG Val(15)	3.5 Å
HE Phe(8) to HG Val(15)	3.0 Å
HZ Phe(8) to HG Val(15)	3.5 Å

[a] These distances were those for a peptide corresponding to residues Asp(7) to Arg(16) of the Aα chain of normal human fibrinogen (Ni et al., 1989b). [b] This distance was derived from the complex of thrombin with a peptide corresponding to residues Gly(6) to Arg(19) of the Aα chain of the normal human fibrinogen, with the active site Arg(16) replaced with Gly(16) (Ni et al., 1989a). These distances were used because the corresponding ones in the substituted peptides could not be specified due to resonance overlaps (see text).

replacement of Gly(12) by Val(12) makes the peptide less soluble in aqueous solutions. With about 6 mM of peptide tF16, the sequential TRNOE cross peak between the $C^{\alpha}H$ proton of Val(12) and the NH proton of Gly(13) is absent (Figure 2A) whereas it is clearly visible in the NOE spectra of the complex of thrombin with peptide tF17 (Figure 2B) where the second replacement of Gly(6) by Cys(6) makes the peptide more soluble (8 mM) in H_2O. Thus, only peptide tF17 was used in determining the distance bounds as discussed in the previous section. Even with peptide tF17, differences in line broadening still exist (Table II); therefore, the intraresidue and sequential distance upper bounds were checked for consistency by use of the distribution of these distances based on the amino acid sequence of the peptide chain (Leach et al., 1977; Billiter et al., 1982). If any inconsistency were found, the more often violated distances were reset to a value larger by 0.5 Å. This procedure should represent a more pessimistic interpretation of the NOE data and would lead to less well-defined backbone conformations of some of the residues.

Table IV: Typical Backbone Conformations[a] and Values of RMS Deviations (RMSD) of the Backbone Atoms of the Computer-Generated Structures of the Substituted and the Normal Decapeptide

	7 Asp	8 Phe	9 Leu	10 Ala	11 Glu	12 Val	13 Gly	14 Gly	15 Val	16 Arg	RMSD (Å) (backbone)[b]	
S_I	F	A	A	A	A	A	C*	C*	A	A	1.5	(0.6)
S_{II}	F	A	A	A	C	A*	A*	A	B	C	2.2	(1.7)

[a] The one-letter codes for backbone conformations were defined in Zimmerman et al. (1977). Letter A, for example, refers to the α-helical region; A*, the region of the left-handed α-helix; C and F, the regions of the extended conformation. It should be noted that only Gly and Ala can adopt low-energy backbone conformations in the region of positive values of ϕ in the ϕ–ψ map (indicated by asterisks). Structure S_I is one of the set of structures (12 with a 1.5-Å RMSD) for the Val(12) decapeptide generated from the TRNOE distance constraints (Table III). Structure S_{II} is one of the set of structures (12 with a 2.2-Å RMSD) of the normal decapeptide computed from the TRNOE distance constraints, as described previously (Ni et al., 1989b). The TRNOE between the NH protons of residues Val(15) and Arg(16) is not observable because of resonance overlaps of these protons in peptides tF16 and tF17 (Figures 2 and 5, Table II) and in normal fibrinogen-like peptides (Ni et al., 1989b). Furthermore, the ψ dihedral angle of Arg(16) is not constrained because peptides tF16 and tF17 both have a free C-terminus as a result of the thrombin cleavage of the Arg(16)–Gly(17) peptide bond in the parent peptides F16 and F17. Therefore, the backbone conformations of Val(15) and Arg(16), particularly that of Arg(16), have large variations in the computed structures of both the normal and the substituted decapeptide. [b] The values of the RMS deviations correspond to averages of *all* pairwise comparisons between individual structures (Vásquez & Scheraga, 1988). Arg(16) was not included in the computation of the RMS deviation because of the large variation of the backbone conformation of this residue. The values of the RMS deviations in parentheses are those for the backbone atoms of residues Asp(7) to Val(12) [Gly(12) for the normal peptide] between the converged structures.

In both peptides tF16 and tF17, the Val(12) substitution resulted in upfield shifts for the NH proton resonances of both Leu(9) and Ala(10) leading to significant resonance overlaps between the NH protons of Leu(9), Ala(10), Val(15), and Arg(16) (Figure 2). For residues Phe(8), Leu(9), and Ala(10), we retained the NOE distance constraints deduced for the normal peptides (Ni et al., 1989a,b) whenever ambiguity arose as a result of resonance overlaps. This is justified since the Val(12) substitution does not seem to affect the backbone conformations of residues Asp(7) to Ala(10) in free peptides, as indicated by the similarity of the conformationally sensitive NMR parameters of the substituted and the normal peptides (Table II; Ni et al., 1988). Nor does this substitution seem to change the thrombin-bound structure of the chain segment of residues Asp(7) to Ala(10) since, in both the normal and the substituted peptides, there is a medium-range TRNOE between the NH proton of Asp(7) and the $C^{\beta}H$ protons of Ala(10) (Table II; Ni et al., 1989b). Furthermore, there exists a strong NOE between the NH protons of Ala(10) and Glu(11) in all of those peptides, indicating that Ala(10) assumes similar backbone conformations in the normal and substituted peptides (Billeter et al., 1982).

Distance Geometry Calculations and Molecular Modeling. An implementation of a variable-target-function procedure (Braun & Gō, 1985; Vásquez & Scheraga, 1988; Ni et al., 1989b) was used for the generation of sets of terminally blocked decapeptide (*N*-acetyl-Asp-Phe-Leu-Ala-Glu-Val-Gly-Gly-Val-Arg-NHMe) conformations that were free of steric overlaps, and that were consistent with the TRNOE distance information in Table III. Briefly, a contact energy of 20 kcal/mol was first used to define steric overlaps (a larger value of the contact energy is equivalent to assigning a lower weight to the steric-contact penalty term of the object function being optimized). Distance constraints were incorporated sequentially (Braun & Gō, 1985), starting from 200 conformations randomly sampled from the single-residue-based

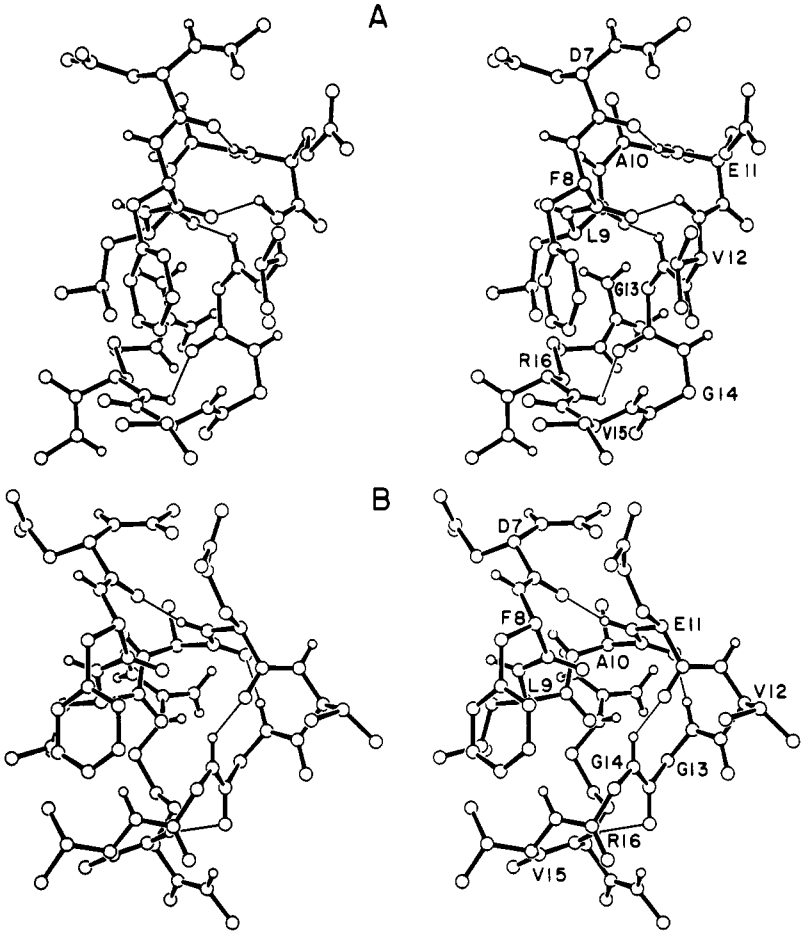

FIGURE 9: (A) Proposed structure (stereo drawings) of the Val(12) peptide [residues Asp(7) to Arg(16)] in the thrombin-bound state. The C^{α} atoms are labeled by the corresponding amino acid residues (Figure 1). The nonpolar cluster formed by the side chains of Phe(8), Leu(9), Val(12), and Val(15) is clearly seen. Residue Val(12) assumes an α-helical conformation in this model. Since there were no TRNOE constraints on the relative orientation of the Arg side chain, it adopted different conformations in the set of structures generated by distance geometry calculations (S_I in Table IV). (B) A hypothetical structure of the Val(12) peptide constructed from the model for the normal peptide (S_{II} in Table IV; Ni et al., 1989b) by replacing Gly(12) with Val(12). Residue Val(12) has an incorrect backbone conformation in the region of positive ϕ in the ϕ–ψ map (Zimmerman et al., 1977). In both structures, a thin line connecting an oxygen atom to a hydrogen atom indicates that those two atoms are separated by less than 2.0 Å (possible hydrogen bonds). The difference in the relative distances between the aromatic ring of Phe(8) and the C^{α} atom of Gly(14) in the two structures in (A) and (B) should be noted.

backbone dihedral angle distributions (Zimmerman et al., 1977; Vásquez & Scheraga, 1988). Structures with values of the distance part of the objective function (Vásquez & Scheraga, 1988) smaller than 1.0 (arbitrary units) were then selected, and these were subjected to a second optimization, by using a contact energy of 10 instead of 20 kcal/mol to define steric overlaps and incorporating all the distance constraints at once (in contrast to the sequential incorporation used above). This same procedure was repeated with a contact energy of 5 kcal/mol and, finally, with a contact energy of 3 kcal/mol to define steric overlaps.

Structures thus generated were checked for the satisfaction of distance constraints by use of two criteria: a structure should have a maximum distance violation of less than 0.5 Å, *and* all other violations should be less than 0.3 Å. These criteria were considered to be satisfactory since errors of ±0.5 Å for the distance upper bounds could arise as a result of differences in line broadening and uncertainties in estimation of peak intensities. By use of these criteria, a total of 12 structures was found with an average RMS deviation of 1.5 Å among them, when all heavy backbone atoms of residues from Asp(7) to Val(15) were considered (structure set S_I in Table IV). The chain segment from Asp(7) to Val(12) is well defined, as indicated by an average deviation of 0.6 Å for the heavy backbone atoms of these residues among the 12 converged structures. This lower value of the RMS deviation among the structures (compared to that of structure set S_{II} in Table IV) reflects the fact that distance constraints in this region of the molecule were a combination of all TRNOE information from both normal and substituted peptides, as discussed in the previous section and as indicated in Table III.

As seen in Table IV, the Val(12) substitution results in changes in backbone conformations of the surrounding residues Glu(11), Gly(13), and Gly(14). For the normal peptide, TRNOE data constrain Gly(12) to have backbone conformations in the region of positive values of ϕ in the ϕ–ψ map (structure set S_{II} in Table IV) (Ni et al., 1989b). It is known that only a Gly or an Ala residue can occur, with high probability, in the region of positive values of ϕ in the ϕ–ψ map (Zimmerman et al., 1977; Némethy & Scheraga, 1977; Richardson, 1981; Iijima et al., 1987). Indeed, in the abnormal peptide, Val(12) can adopt backbone conformations only in the region of negative values of ϕ in the ϕ–ψ map, since there exist strong NOE's between the NH and the $C^{\beta}H$ protons of Val(12) and between the NH protons of Val(12) and Gly(13) (Table II). Furthermore, both Glu(11) and Val(12) are forced to assume α-helical backbone conformations by the requirement that the $C^{\gamma}H$ protons of Val(12) interact characteristically with the ring protons of Phe(8) (Figures 7 and 8). [It is known that it is sterically possible for a Val residue to take

Table V: Atomic Coordinates of the Structure of the Decapeptide (CH_3CO-Asp-Phe-Leu-Ala-Glu-Val-Gly-Gly-Val-Arg-$NHCH_3$)

atom	residue	sequence no.	x (Å)	y (Å)	z (Å)	atom	residue	sequence no.	x (Å)	y (Å)	z (Å)
1 H3	CH_3	1	−2.609	−1.978	0.262	80 CD	Glu^-	6	−1.337	−4.933	1.824
2 H3	CH_3	1	−2.597	−0.346	−0.447	81 OE2	Glu^-	6	−2.204	−4.233	1.258
3 H3	CH_3	1	−2.266	−0.565	1.288	82 OE1	Glu^-	6	−1.128	−6.149	1.622
4 C	CH_3	1	−2.136	−0.997	0.295	83 N	Val	7	1.446	−4.083	4.605
5 O	CO	1	−0.193	−2.242	−0.222	84 HN	Val	7	1.641	−3.751	3.682
6 C	CO	1	−0.682	−1.136	0.000	85 CA	Val	7	2.341	−5.123	5.084
7 N	Asp^-	2	0.000	0.000	0.000	86 HA	Val	7	1.724	−5.933	5.473
8 HN	Asp^-	2	−0.423	0.906	0.000	87 CB	Val	7	3.172	−5.674	3.924
9 CA	Asp^-	2	1.453	0.000	0.000	88 C	Val	7	3.199	−4.567	6.223
10 HA	Asp^-	2	1.739	−0.085	−1.048	89 O	Val	7	3.790	−5.327	6.987
11 CB	Asp^-	2	2.004	1.291	0.609	90 HB	Val	7	3.836	−4.881	3.580
12 C	Asp^-	2	1.959	−1.173	0.842	91 CG2	Val	7	2.276	−6.078	2.751
13 O	Asp^-	2	1.226	−1.705	1.675	92 CG1	Val	7	4.040	−6.849	4.381
14 HB	Asp^-	2	1.168	1.887	0.976	93 HG1	Val	7	4.761	−6.501	5.121
15 HB	Asp^-	2	2.617	1.035	1.473	94 HG1	Val	7	3.406	−7.618	4.823
16 CG	Asp^-	2	2.836	2.151	−0.344	95 HG1	Val	7	4.570	−7.264	3.524
17 OD2	Asp^-	2	3.094	1.668	−1.467	96 HG2	Val	7	1.606	−5.254	2.506
18 OD1	Asp^-	2	3.197	3.273	0.073	97 HG2	Val	7	2.895	−6.314	1.886
19 N	Phe	3	3.207	−1.542	0.596	98 HG2	Val	7	1.689	−6.954	3.028
20 HN	Phe	3	3.796	−1.103	−0.083	99 N	Gly	8	3.239	−3.245	6.299
21 CA	Phe	3	3.820	−2.642	1.322	100 HN	Gly	8	2.755	−2.633	5.673
22 HA	Phe	3	3.212	−3.528	1.138	101 CA	Gly	8	4.014	−2.578	7.331
23 CB	Phe	3	5.253	−2.777	0.803	102 HA	Gly	8	4.574	−1.751	6.894
24 C	Phe	3	3.858	−2.354	2.824	103 HA	Gly	8	3.344	−2.150	8.076
25 O	Phe	3	3.172	−3.014	3.603	104 C	Gly	8	4.981	−3.553	8.007
26 HB	Phe	3	5.229	−2.868	−0.282	105 O	Gly	8	6.173	−3.561	7.704
27 HB	Phe	3	5.799	−1.862	1.034	106 N	Gly	9	4.430	−4.351	8.910
28 CG	Phe	3	6.015	−3.969	1.385	107 HN	Gly	9	3.459	−4.338	9.150
29 CD1	Phe	3	6.868	−3.787	2.428	108 CA	Gly	9	5.228	−5.328	9.631
30 CD2	Phe	3	5.839	−5.211	0.858	109 HA	Gly	9	5.030	−5.248	10.700
31 HD1	Phe	3	7.009	−2.792	2.850	110 HA	Gly	9	4.939	−6.334	9.328
32 CE1	Phe	3	7.576	−4.894	2.968	111 C	Gly	9	6.721	−5.119	9.367
33 CE2	Phe	3	6.547	−6.317	1.398	112 O	Gly	9	7.450	−4.651	10.240
34 HD2	Phe	3	5.155	−5.356	0.022	113 N	Val	10	7.131	−5.477	8.159
35 HE1	Phe	3	8.260	−4.749	3.804	114 HN	Val	10	6.531	−5.858	7.455
36 CZ	Phe	3	7.400	−6.136	2.441	115 CA	Val	10	8.523	−5.335	7.769
37 HE2	Phe	3	6.406	−7.312	0.976	116 HA	Val	10	9.133	−5.764	8.564
38 HZ	Phe	3	7.943	−6.985	2.855	117 CB	Val	10	8.791	−6.127	6.487
39 N	Leu	4	4.667	−1.369	3.185	118 C	Val	10	8.862	−3.849	7.635
40 HN	Leu	4	5.221	−0.837	2.544	119 O	Val	10	9.638	−3.462	6.763
41 CA	Leu	4	4.803	−0.986	4.580	120 HB	Val	10	7.907	−6.051	5.855
42 HA	Leu	4	5.034	−1.889	5.145	121 CG2	Val	10	9.021	−7.608	6.796
43 CB	Leu	4	5.981	−0.026	4.759	122 CG1	Val	10	9.974	−5.538	5.716
44 C	Leu	4	3.472	−0.426	5.083	123 HG1	Val	10	10.454	−6.323	5.131
45 O	Leu	4	3.148	−0.551	6.263	124 HG1	Val	10	9.617	−4.754	5.048
46 HB	Leu	4	6.497	0.067	3.803	125 HG1	Val	10	10.693	−5.117	6.419
47 HB	Leu	4	5.587	0.960	5.006	126 HG2	Val	10	9.897	−7.714	7.437
48 CG	Leu	4	7.007	−0.415	5.825	127 HG2	Val	10	8.147	−8.013	7.306
49 HG	Leu	4	6.499	−0.463	6.788	128 HG2	Val	10	9.184	−8.152	5.866
50 CD1	Leu	4	7.583	−1.806	5.550	129 N	Arg^+	11	8.262	−3.057	8.512
51 CD2	Leu	4	8.103	0.646	5.945	130 HN	Arg^+	11	7.632	−3.380	9.218
52 HD1	Leu	4	7.312	−2.479	6.363	131 CA	Arg^+	11	8.491	−1.622	8.502
53 HD1	Leu	4	7.179	−2.187	4.612	132 HA	Arg^+	11	7.881	−1.248	7.680
54 HD1	Leu	4	8.669	−1.742	5.478	133 CB	Arg^+	11	8.049	−0.986	9.822
55 HD2	Leu	4	9.060	0.160	6.139	134 C	Arg^+	11	9.973	−1.322	8.270
56 HD2	Leu	4	8.165	1.210	5.014	135 O	Arg^+	11	10.314	−0.370	7.570
57 HD2	Leu	4	7.866	1.322	6.765	136 HB	Arg^+	11	7.308	−1.621	10.306
58 N	Ala	5	2.735	0.180	4.163	137 HB	Arg^+	11	8.901	−0.917	10.499
59 HN	Ala	5	3.006	0.278	3.206	138 CG	Arg^+	11	7.462	0.408	9.589
60 CA	Ala	5	1.446	0.760	4.499	139 HG	Arg^+	11	8.094	0.962	8.895
61 HA	Ala	5	1.612	1.515	5.268	140 HG	Arg^+	11	6.480	0.319	9.124
62 CB	Ala	5	0.853	1.438	3.263	141 CD	Arg^+	11	7.341	1.179	10.905
63 C	Ala	5	0.532	−0.331	5.060	142 HD	Arg^+	11	8.247	1.049	11.496
64 O	Ala	5	−0.050	−0.168	6.132	143 HD	Arg^+	11	7.242	2.245	10.703
65 HB	Ala	5	1.531	2.218	2.916	144 NE	Arg^+	11	6.168	0.698	11.668
66 HB	Ala	5	0.716	0.699	2.473	145 HE	Arg^+	11	6.208	−0.222	12.059
67 HB	Ala	5	−0.110	1.881	3.517	146 CZ	Arg^+	11	5.054	1.417	11.866
68 N	Glu^-	6	0.434	−1.420	4.312	147 NH2	Arg^+	11	4.516	1.494	13.091
69 HN	Glu^-	6	0.911	−1.545	3.442	148 NH1	Arg^+	11	4.479	2.058	10.840
70 CA	Glu^-	6	−0.400	−2.537	4.721	149 HH1	Arg^+	11	3.677	1.664	10.390
71 HA	Glu^-	6	−1.090	−2.129	5.458	150 HH1	Arg^+	11	4.851	2.930	10.523
72 CB	Glu^-	6	−1.198	−3.088	3.538	151 HH2	Arg^+	11	3.532	1.367	13.214
73 C	Glu^-	6	0.457	−3.631	5.361	152 HH2	Arg^+	11	5.099	1.680	13.882
74 O	Glu^-	6	0.209	−4.033	6.497	153 N	NH	12	10.813	−2.152	8.869
75 HB	Glu^-	6	−2.172	−3.435	3.882	154 H	NH	12	10.527	−2.924	9.437
76 HB	Glu^-	6	−1.380	−2.292	2.815	155 C	CH_3	12	12.251	−1.987	8.736
77 CG	Glu^-	6	−0.451	−4.239	2.860	156 H3	CH_3	12	12.529	−2.061	7.685
78 HG	Glu^-	6	0.449	−3.859	2.378	157 H3	CH_3	12	12.542	−1.010	9.121
79 HG	Glu^-	6	−0.130	−4.960	3.612	158 H3	CH_3	12	12.760	−2.767	9.302

on the α-helical backbone conformation (Ooi et al., 1967; Epand & Scheraga, 1968).]

A prominent feature in the set of structures computed for the thrombin-bound normal decapeptide is the hydrophobic cluster formed by the nonpolar side chains of Phe(8), Leu(9), and Val(15) (Ni et al., 1989b) and, in the case of the substituted peptide, also by the side chain of Val(12) as required by the existence of TRNOE's between the side chains of these residues (Figures 7 and 8; Table II). In most of the 12 converged structures for the Val(12) decapeptide, the $C^{\alpha}H$ protons of Gly(14) are far away from the ring protons of Phe(8) in agreement with the finding that the TRNOE's between those protons, previously observed for the complex of fibrinopeptide A with thrombin, are absent in the complexes of thrombin with both tF16 and tF17 (see the previous section). Since no distance constraints were incorporated between the ring protons of Phe(8) and the $C^{\alpha}H$ protons of Gly(14) in the distance geometry calculation (Table III) because no TRNOE's were observed between these protons, it is possible that the side chain of Val(12) provides enough steric hindrance (in the distance geometry algorithm) so that Gly(14) can no longer approach the aromatic ring of Phe(8) in the presence of a Val residue at position 12.

In all of the 12 converged structures for the Val(12) peptide, the backbone carbonyl oxygen of Asp(7) was found to be hydrogen bonded to the backbone NH proton of Glu(11). An inspection of all the converged structures revealed that the backbone carbonyl oxygen of Phe(8) is invariably close to the NH proton of Val(12) and the backbone carbonyl oxygen of Leu(9) is close to the NH proton of Gly(13). These hydrogen bonds are expected since the whole chain segment of residues Phe(8) to Val(12) is constrained to the α-helical region (Zimmerman et al., 1977) by strong TRNOE's (thus small distances) (Tables II and III) between the NH proton and the $C^{\beta}H$ protons of the ith residue, between the $C^{\beta}H$ protons of the ith residue and the NH proton of the $(i+1)$th residue, and between the NH protons of the ith and $(i+1)$th residues (Leach et al., 1977; Billeter et al., 1982). The existence of these hydrogen bonds is further supported by the observation that the exchange rates of the NH protons of residues Glu(11), Val(12), and Gly(13) are all significantly reduced compared to, e.g., that of Gly(14) (Table II). In the rest of the molecule, there is a type II′ β-turn involving residues Gly(14) and Val(15), as supported by the absence of a TRNOE between the NH protons of Gly(14) and Val(15) and by the presence of strong $C^{\alpha}H$–NH NOE's between Gly(14) and Val(15) and between Val(15) and Arg(16) (Table II) (Wagner et al., 1986; Wüthrich, 1986). In such a β-turn structure, the backbone carbonyl oxygen of Gly(13) would be hydrogen bonded to the NH proton of Arg(16). This hydrogen bond would account for the reduced exchange rate of the NH proton of Arg(16) (Table II).

Further refinement of the structure of the bound peptide by energy minimization was not carried out since it is not possible to take into account the contribution of the interaction of the peptide with surface residues on thrombin. However, optimal orientation of the hydrogen bonds cannot be ensured in the structures on the basis of TRNOE distances alone since no information regarding hydrogen bonds was included in the distance geometry calculation. To refine the structure of the peptide, we selected the structure with the most optimal hydrogen-bonding distances from the set of structures (S_I in Table IV) generated from TRNOE distances and used it as the starting conformation for further distance geometry optimization incorporating *both* the TRNOE information (Table III) and the hydrogen bonds discussed above, using an NH···O distance of 2.0 Å between the proton and the oxygen. The resulting structure (coordinates can be found in Table V) is presented in Figure 9A. This structure is compared with a hypothetical structure (Figure 9B) of the Val(12) peptide constructed from a model of the normal peptide [S_{II} in Table IV; coordinates can be found in Table III of Ni et al. (1989b)] by simply inserting the side chain of a Val residue at position 12. As a further check, we have also used this hypothetical structure [with Val(12) in the *incorrect* backbone conformation] as the starting point for the aforementioned optimization. The resulting structure is very similar to the one in Figure 9A, indicating that the Val(12) substitution indeed has brought about the structural changes as shown between structures A and B of Figure 9.

Discussion

It was reported that a bleeding disorder is caused by the delayed release of fibrinopeptide A from fibrinogen Rouen in which there is a single mutation of Gly(12) to Val(12) in the Aα chain (Mênachê, 1983). In the present study, we found that the rate of cleavage of the Arg(16)–Gly(17) peptide bond is reduced in the synthetic peptides derived from residues Ala(1) to Val(20) of the Aα chain of the abnormal fibrinogen, compared to that of a similar peptide derived from normal human fibrinogen (Figure 3). The replacement of Gly(12) by Val(12) results in conformational changes within the specificity sequence of Asp(7) to Arg(16), which probably impairs the adequate interaction of the peptide with thrombin. For free peptides in solution, the Val(12) substitution seems to affect the conformation of only residues at positions 12, 13, and 14 compared to that of normal fibrinogen-like peptides since conformationally sensitive NMR parameters for these residues are significantly altered by the substitution (Table II; Ni et al., 1988).

The substituted peptides tF16 and tF17 still bind to thrombin, on the basis of observation of line broadening of proton resonances of the free peptides in the presence of thrombin. Similar to those of the normal peptides (Ni et al., 1989a,b), the resonances of the nonpolar side chains of Phe(8), Leu(9), Val(12), and Val(15) are most severely broadened upon thrombin binding, indicating their direct involvement in the complex of the Val(12) peptide with thrombin. This observation further supports the previous conclusion 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 (Ni et al., 1989a). The complementary hydrophobic binding pocket at the thrombin active site has been shown by biochemical studies (Sonder & Fenton, 1984) and by studies involving spin-labeling techniques (Berliner, 1984) to be limited in size. Furthermore, tolerated substitutions within Asp(7) to Arg(16) in fibrinopeptide A of many mammalian species have all been from residues with polar side chains to residues with side chains equally or more polar in character (Blombäck, 1967; Henschen et al., 1983). The only known case of a polar-to-nonpolar amino acid substitution is that of Gly(12) to Val(12) found in fibrinogen Rouen which, however, results in a bleeding disorder (Henschen et al., 1983; Mênachê, 1983). Thus, the addition of a nonpolar residue at position 12 may also affect the way the peptide interacts with thrombin, apart from possible conformational changes of the residues around the site of substitution.

On the basis of transferred NOE studies, the aromatic ring of Phe(8) was found to be close to the nonpolar side chain of Val(15) in the complex of thrombin with fibrinopeptide A and

other peptides derived from the N-terminal region of the Aα chain of normal human fibrinogen (Ni et al., 1989a,b). Such a structural feature still exists in the complex of thrombin with the Val(12)-substituted fibrinopeptide A (tF16 in Figure 1), as indicated by the TRNOE's between ring protons of Phe(8) and the $C^{\gamma}H$ protons of Val(15) (Figures 7 and 8). In the normal peptides, the chain reversal within residues Leu(9) to Gly(14) is brought about partially by a type II β-turn involving residues Glu(11) and Gly(12), which places the backbone conformation of Gly(12) in the region of positive values of ϕ in the ϕ–ψ map (Ni et al., 1989b). Upon a Gly(12) to Val(12) substitution, such a turn structure is disrupted because of the strong steric hindrance (Figure 9B) between the carbonyl oxygen of Glu(11) and the branched side chain of Val(12) (Venkatachalam, 1968; Zimmerman & Scheraga, 1978). Indeed, as illustrated in Figure 9A, Val(12) invariably adopts a backbone conformation in the region of *negative* values of ϕ in the ϕ–ψ map in the set of structures of the thrombin-bound peptide (Table IV) computed from distance constraints based on TRNOE measurements of peptides tF16 and tF17 in a complex with thrombin. In Figure 9, it is seen that an immediate consequence of the Val(12) substitution is that the two hydrogen bonds between the backbone carbonyl oxygen of Ala(10) and the NH proton of Gly(13) and between the backbone carbonyl oxygen of Glu(11) and the NH proton of Gly(14) in the thrombin-bound structure of the normal peptide (Figure 9B) are replaced by two hydrogen bonds between the backbone carbonyl oxygen of Phe(8) and the NH proton of Val(12) and between the backbone carbonyl oxygen of Leu(9) and the NH proton of Gly(13) (Figure 9A). The NH proton of Gly(14) is no longer involved in any hydrogen bonding in the proposed structure, which would account for its relatively fast exchange with solvent protons compared to that of Gly(13) in the free peptides F16 and F17 (Table II). Furthermore, the $C^{\alpha}H$ protons of Gly(14) are more than 4 Å away from the ring protons of Phe(8) (Figure 9A) as indicated by the absence of TRNOE's between residues Phe(8) and Gly(14) which is observed in the complex of thrombin with normal fibrinogen-like peptides (Ni et al., 1989a,b). This displacement of Gly(14) relative to the aromatic ring of Phe(8) presumably arises because of the existence of strong steric hindrance between the side chain of Val(12) and the backbone atoms of Gly(13) and Gly(14).

In the Val(12) peptide, residues Phe(8), Leu(9), Val(12), and Val(15) all participate in the interaction with thrombin, as discussed in the previous section. In Figure 9A, we can easily see the hydrophobic cluster formed by the nonpolar side chains of these residues. On the basis of sequence homology, a model structure of the B chain of bovine thrombin has been constructed from crystal structures of trypsin and chymotrypsin (Bing et al., 1981, 1986). The primary binding site of thrombin was identified with the catalytic triad, residues His(43), Asp(99), and Ser(205), combined with residue Asp(199) which forms an ion pair with positively charged amino acid side chains, such as that of Arg(16) in fibrinogen-like peptides. The active site of thrombin extends to an apolar region which includes Leu(96), Trp(227), and Tyr(237). Similarly, in a model structure of human thrombin constructed from the crystal structure of elastase (M. J. Dudek and H. A. Scheraga, unpublished results), residues Leu(96), Trp(227), Tyr(237), and Phe(239) are all located near the catalytic residues His(43), Asp(99), and Ser(205). The existence of such an apolar region in the active site of thrombin has been confirmed by competitive binding experiments and spin-labeling studies (Berliner, 1984; Sonder & Fenton, 1984). An inspection of the model structures of fibrinogen-like peptides (Figure 9) suggested that the nonpolar side chains of Phe(8), Leu(9), and Val(15) may interact with the nonpolar cluster of residues Leu(96), Trp(227), and Phe(239) in the active site of thrombin. This is supported by the observation that chemical modification of tryptophan residues significantly decreased the activity of thrombin (Lundblad et al., 1982). In the abnormal peptide, the cluster of Phe(8), Leu(9), Val(12), and Val(15) might still fit in the apolar binding pocket of thrombin. However, we may expect that the presence of Val(12) in the hydrophobic pocket would interfere with the optimal binding of the peptide with thrombin since the positions of Gly(13) and Gly(14) are displaced compared to those of the normal peptide (Figure 9). This observation suggests that the presence of Gly residues at positions 13 and 14 is also important for the adequate interaction of thrombin with fibrinogen in agreement with the fact that, along with Gly(12), both Gly(13) and Gly(14) are strongly conserved in mammalian fibrinogens (Blombäck, 1967; Henschen et al., 1983). The coordinates in Table III of Ni et al. (1989b) may serve as a basis for the design of thrombin-specific inhibitors.

It should be noted, however, that Figure 9 represents only minimal differences between the thrombin-bound structures of the normal and abnormal peptides since, as already discussed, some of the distance constraints were derived from the study of the interaction of thrombin with normal fibrinogen-like peptides (Table III). The current NMR study could not provide further information about the relative orientation of the side chain of Arg(16) in the thrombin-bound state for either the normal or the substituted peptides. The Val(12) substitution may displace the side chains of Val(15) or Arg(16) from their normal binding sites since the rate of the thrombin cleavage of the Arg(16)–Gly(17) peptide bond in the substituted peptides is much slower than that in normal fibrinogen-like peptides (Figure 3). Alternative mechanisms will be resolved by a detailed steady-state kinetic analysis of the cleavage of the Arg(16)–Gly(17) peptide bond in the substituted peptides, by use of the methodology established for the study of the interaction of thrombin with normal fibrinogen-like peptides (Meinwald et al., 1980; Marsh et al., 1982, 1983).

Conclusions

The structural basis of a bleeding disorder was assessed by means of an NMR study of the interaction of thrombin with synthetic peptides derived from residues 1–20 of the Aα chain of abnormal human fibrinogen Rouen. The delayed release of fibrinopeptide A in abnormal fibrinogen Rouen may be caused by a change in structure within residues Val(12), Gly(13), and Gly(14) upon a single amino acid mutation of Gly(12) to Val(12). Val(12) disrupts a type II β-turn conformation involving residues Glu(11) and Gly(12) in normal human fibrinogen-like peptides. Furthermore, in the complex of thrombin with substituted peptides, Gly(13) and Gly(14) were found to be displaced from the hydrophobic cluster formed by the nonpolar side chains of residues Phe(8), Leu(9), Val(12), and Val(15). These findings may provide further insight into the specificity of the interaction of thrombin with fibrinogen.

Acknowledgments

We thank Y. C. Meinwald and R. W. Ashton for helpful discussions and T. W. Thannhauser and R. W. Sherwood of the Cornell University Biotechnology Program Facility for carrying out the amino acid analyses. We are grateful to M.

Vásquez for help with the distance geometry computations which were carried out on the Cornell National Supercomputer Facility, a resource of the Center for Theory and Simulation in Science and Engineering, which is funded in part by the National Science Foundation, New York State, and the IBM Corp. Thanks are due to the staff members of the Syracuse NIH Regional Resource Facility for Multinuclear NMR and Data Processing, particularly to G. Heffron and A. Lipton for maintaining the spectrometer and for their technical assistance. New Methods Research, Inc. (Syracuse), is gratefully acknowledged for providing the NMR1/NMR2 software system for the Sun workstation.

Registry No. F16, 118921-29-2; tF16, 118921-30-5; F17, 118921-31-6; tF17, 118921-32-7; thrombin, 9002-04-4.

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