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# Mechanism of Action of Thrombin on Fibrinogen. Kinetic Evidence for Involvement of Aspartic Acid at Position $P_{10}$†

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ABSTRACT: The following peptide was synthesized by classical methods in solution: Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$ (F-8). The Michaelis–Menten parameters for the hydrolysis of the Arg–Gly bond in F-8 by thrombin were determined to be $k_{cat} = 31 \times 10^{-11}$ M [(NIH unit/L) s]$^{-1}$ and $K_M = 310 \times 10^{-6}$ M. Comparison of these values with those determined previously for native fibrinogen and for a series of similar synthetic peptides, together with information about the amino acid sequences of this portion of the A$\alpha$ chain of abnormal fibrinogens, suggests an important role for Asp at position $P_{10}$. Differences in the Michaelis–Menten parameters between F-8 and the 51-residue N-terminal CNBr fragment of the A$\alpha$ chain of fibrinogen correspond to only 1–2 kcal/mol in binding affinity.

From the observation that the amino acid sequence in a portion of the fibrinogens of many species is strongly conserved, Blombäck (1967) had suggested that Phe at position $P_9$[1] of the A$\alpha$ chain of fibrinogen is essential for normal thrombin action. This suggestion was confirmed by subsequent kinetic experiments involving a series of synthetic peptide substrates (Van Nispen et al., 1977; Meinwald et al., 1980; Marsh et al., 1982). To explain the large effect of a single amino acid residue nine residues distant in the linear sequence from the site of enzyme action, it was proposed that the intervening residues might accommodate a feature such as a $\beta$-bend that would allow the Phe at position $P_9$ to be brought into close spatial proximity to the Arg–Gly bond that is hydrolyzed. The existence of a $\beta$-bend is supported by the high reactivity of D-Phe-Val-Arg-*p*-nitroanilide (but not the corresponding L-Phe compound) toward thrombin (Claeson et al., 1977) and by NMR observations (Rae & Scheraga, 1979) that indicate that the D-Phe residue is folded back over the Val residue but that this arrangement is not found in the L-Phe peptide.

While peptides containing Phe at $P_9$ (but not Asp at $P_{10}$) are substantially better substrates than those lacking this residue, they are poorer substrates of thrombin than even CNBr A$\alpha$, primarily because of differences in binding affinities reflected in the values of $K_M$. On the basis of immunochemical studies, Nagy et al. (1982) concluded that it is possible that these substrates contain all the residues that interact directly at the active site of thrombin yet lack residues that provide the long-range interactions necessary to stabilize the native conformation required for normal binding at the active site.

Direct involvement of Asp at $P_{10}$ can be inferred from the abnormal rate of release of fibrinopeptide A from fibrinogen Lille, in which Asn replaces Asp at $P_{10}$ (Morris et al., 1981). Furthermore, an acid residue (Asp or Glu) is present at position $P_{10}$ in most fibrinogen species (Dayhoff, 1972, 1973, 1976). The only exceptions among the 56 reported species are Thr (for rabbit, kangaroo, and lizard), Ser (for wombat), and Gly (for slow loris).

Recognizing the importance of Asp (or Glu) at position $P_{10}$ and its possible effect in improving the binding affinity (i.e., value of $K_M$) for a Phe($P_9$)-containing synthetic peptide substrate, we have synthesized the peptide Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$ (F-8); this sequence is identical with that of this portion of

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[1] Abbreviations: Ac, acetyl; AcONP, 4-nitrophenyl acetate; Boc, *tert*-butyloxycarbonyl; CNBr A$\alpha$, the N-terminal CNBr fragment of the A$\alpha$ chain of fibrinogen; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, *N*,*N*′-dimethylformamide; HOAc, acetic acid; HOBT, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; OBu$^t$, *tert*-butyloxy; TFA, trifluoroacetic acid; TLC, thin-layer chromatography. The abbreviations used for the amino acid residues and the notation of peptides are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (1972). The positions of residues in peptide substrates are described by the nomenclature of Schechter & Berger (1967) wherein residues on the N-terminal side of the Arg–Gly bond are designated as $P_1$, $P_2$, etc. and those on the C-terminal side are designated as $P_1'$, $P_2'$, etc. (see Table I).

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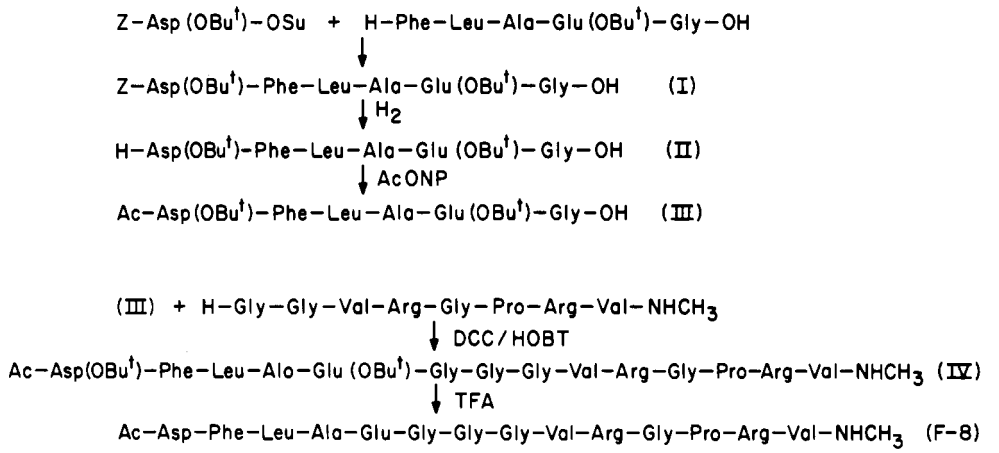


FIGURE 1: Schematic representation of synthesis of peptide F-8.

the A$\alpha$ chain of normal human fibrinogen. We then carried out kinetic studies of the thrombin-induced hydrolysis of the Arg–Gly bond of this peptide to determine whether the addition of Asp would increase the rate of hydrolysis over that of the corresponding peptide (F-5) lacking this Asp residue.

## Materials and Methods

All of the amino acids (except glycine) were of the L configuration. AcONP, HOSu, HOBT, and DCC were purchased from Aldrich Chemical Co. Z-Asp(OBu$^t$)-OH was obtained from Bachem, Inc., and was recrystallized before use. Solvents and inorganic salts were reagent grade or better and were used without further purification. Dowex 50W-X2 was purchased from Sigma Chemical Co.; CM-52 was from Whatman Ltd.

The purity of the amino acid derivatives and peptides was routinely checked by TLC on Merck silica gel plates (F-254, 0.25 mm) in the following solvent systems: (a) chloroform–methanol–acetic acid, 95:20:3; (b) 1-butanol–acetic acid–water, 4:1:1; (c) 1-butanol–pyridine–acetic acid–water, 4:1:1:2; (d) 2-propanol–formic acid–water, 20:1:5.

Methods used for detection of components on TLC plates were ultraviolet light, ninhydrin reagent for free amino groups, and chlorine/potassium iodide–starch reagent for NH groups.

NMR spectra of intermediates were obtained with a Varian EM-90 or a Brüker WM-300 spectrometer and were found to be in full agreement with proposed covalent structures. Amino acid analyses were carried out with a Technicon TSM-1 autoanalyzer. Melting points (uncorrected) were determined with a Thomas–Hoover apparatus.

*Synthetic Methods.* $AcP_{1-14}NHCH_3$ (F-8) was synthesized according to the scheme in Figure 1. The hexapeptide Ac-Asp(OBu$^t$)-Phe-Leu-Ala-Glu(OBu$^t$)-Gly-OH (III) was obtained by the reaction of Z-Asp(OBu$^t$)-OSu (Wünch & Zwick, 1966) with H-Phe-Leu-Ala-Glu(OBu$^t$)-Gly-OH·HOAc (Van Nispen et al., 1977), followed by hydrogenation and acetylation. III was coupled with H-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$·3HCl (Meinwald et al., 1980) with DCC/HOBT to give the fully protected tetradecapeptide IV. After treatment with TFA to remove the *tert*-butyl groups, the final product F-8 was purified by chromatography on a Dowex 50W-X2 column. F-8 showed the following properties: $R_f$ 0.42 (c), 0.33 (d); amino acid analysis (12 N HCl, 110 °C, 24 h) $Asp_{1.01}Glu_{1.05}Pro_{1.01}Gly_{3.99}Ala_{1.02}Val_{1.90}Leu_{1.00}Phe_{0.96}Arg_{2.03}$.

The tetrapeptide intermediate VI was prepared by the reaction of Boc-Gly-OSu (Anderson et al., 1964) with H-Pro-Arg-Val-$NHCH_3$·HOAc (Meinwald et al., 1980), followed by the removal of the Boc group by TFA as depicted in Figure 2. The detailed experimental procedure and the properties of the intermediates are described in the supplementary material (see paragraph at end of paper regarding supplementary material).

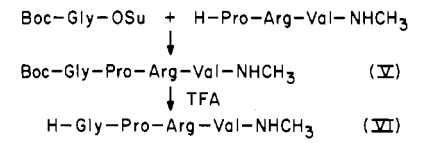


FIGURE 2: Schematic representation of synthesis of tetrapeptide VI.

*Thrombin and Fibrinogen.* Bovine prothrombin (lot 65C-8400) was obtained from Sigma Chemical Co., and thrombin was prepared from this material as described earlier (Hageman et al., 1975), employing the chromatographic procedures of Lundblad (1971). A molecular weight of 41 000 (Heldebrant et al., 1973) and an extinction coefficient $E^{1\%}_{1cm}$ at 280 nm of 19.5 (Heldebrant et al., 1973; Winzor & Scheraga, 1964) were used to determine thrombin concentrations. The purified thrombin had a specific activity of 2200 NIH units/mg and was stored frozen in solution at −70 °C prior to use. Thrombin activities were determined by clotting assays according to the procedure of Fenton & Fasco (1974) and Fenton et al. (1977). Clotting times were averages of five trials, and thrombin concentrations were adjusted from stock by dilution to yield clotting times between 14 and 25 s. A standard curve of reciprocal clotting time vs. thrombin activity (in NIH units/mL) was constructed with an in-house thrombin standard; the in-house standard activity was determined in a similar manner by use of standard NIH thrombin (lot J). Bovine fibrinogen (Sigma, type 1, fraction I) was used in all clotting assays. Highly purified human $\alpha$-thrombin was obtained as a gift from Dr. John W. Fenton, II. It was used as the in-house thrombin standard and was prepared as a series of identical aliquots of the original material; these aliquots were lyophilized and sealed under vacuum in glass ampules.

*Kinetic Methods.* The concentrations of peptide stock solutions were determined by semiautomated micro Kjeldahl nitrogen analysis (Horwitz, 1980). Peptide solutions used in the kinetics experiments were prepared by dilution from these stocks.

The procedure used to determine the rates of hydrolysis of this peptide was essentially the same as described previously (Van Nispen et al., 1977; Meinwald et al., 1980; Marsh et al., 1982). The kinetic experiments were carried out at 25 °C in 0.15 M KCl and 0.05 M sodium borate buffer, pH 8.0. Aliquots of the reaction mixture were withdrawn at timed intervals after the addition of thrombin and assayed for the presence of free amino termini with the fluorogenic reagent

fluorescamine (Weigele et al., 1972; Udenfriend et al., 1972; Böhlen et al., 1973), obtained from Sigma Chemical Co. Since the peptide substrate F-8 does not contain primary amines, the fluorescent product of the reaction with fluorescamine arises from the hydrolysis of peptide bonds.

Fluorescence was measured on a Perkin-Elmer MPF-44B fluorescence spectrophotometer at an excitation wavelength of 390 nm and an emission wavelength of 480 nm. Fluorescence values were corrected for inner filter effects (Parker & Rees, 1960; Parker, 1968) as before (Marsh et al., 1982). Fluorescence intensities were converted to concentrations of product with a calibration curve constructed by reacting fluorescamine with varying concentrations of H-Gly-Pro-Arg-Val-$NHCH_3$ or H-Val-$NHCH_3$ under the same conditions used in the hydrolysis of peptide substrates. Proper fluorescence base-line values were determined by reacting fluorescamine with appropriate control samples that contained borate buffer, borate buffer and thrombin, or borate buffer and peptide F-8 at varying concentrations.

The extent of hydrolysis of the Arg($P_3'$)–Val($P_4'$) peptide bond was estimated from separate semiquantitative TLC experiments. Aliquots (14–16 $\mu$L) of the thrombin hydrolysis products of F-8 at various reaction times were spotted along with standard samples of H-Val-$NHCH_3$ of known concentration. Appropriate controls [starting material (F-8), H-Gly-Pro-Arg-Val-$NHCH_3$ (at varying concentrations), thrombin, and buffer alone] aided in the identification of TLC spots. The plates were developed in solvent system b. Initial substrate concentrations were high, $[S]_0$ = 7.5 mM, so that small extents of Arg($P_3'$)-Val($P_4'$) hydrolysis (0.1% reaction) could be detected on TLC by the presence of H-Val-$NHCH_3$.

The extent of hydrolysis of the Arg–Gly bond was always less than 12% in the determination of initial hydrolysis rates. The Michaelis–Menten parameters were determined from Lineweaver–Burk plots of $1/V_0$ vs. $1/[S]_0$, where $V_0$ is the initial hydrolysis rate and $[S]_0$ is the initial substrate concentration. The values of $k_{cat}$ and $K_M$ represent the averages of three experiments, each employing five different initial concentrations of peptide.

## Results

Calibration curves of fluorescence vs. concentration of peptide were linear with intercepts equal to the fluorescence of borate buffer control samples. The slopes of such calibration curves for H-Val-$NHCH_3$ were an average of 4.27 times greater than the slopes of similar calibration curves for the tetrapeptide H-Gly-Pro-Arg-Val-$NHCH_3$; i.e., the single residue had a higher fluorescence yield than the tetrapeptide, when reacted with fluorescamine.

Control samples containing only peptide F-8 in borate buffer yielded a small but significant background fluorescence (when reacted with fluorescamine), which was directly proportional to the peptide concentration. This background fluorescence was equivalent to that that would be obtained from hydrolysis of 0.7% of the peptide at Arg–Gly or from hydrolysis of 0.2% of the peptide at Arg–Val. This background fluorescence quantitatively accounted for the nonzero intercepts observed in the fluorescence vs. time plots for the hydrolysis of peptide F-8 by thrombin (see Figure 3). No significant hydrolysis of peptide F-8 in buffer alone (i.e., without added thrombin) was observed on the time scale of the kinetic experiments (1 h).

A typical fluorescence vs. time plot is shown in Figure 3. If one assumes, as in previous work (Van Nispen et al., 1977; Meinwald et al., 1980), that contributions to the fluorescence from hydrolysis of the Arg–Val bond are negligible, the Michaelis–Menten parameters for hydrolysis of the Arg–Gly bond could be obtained directly from Lineweaver–Burk plots of the initial velocities. The mean values of three such determinations are $k_{cat} = 31 \times 10^{-11}$ M [(NIH unit/L) s]$^{-1}$ and $K_M = 310 \times 10^{-6}$ M, with standard deviations of 23 and 32%, respectively.

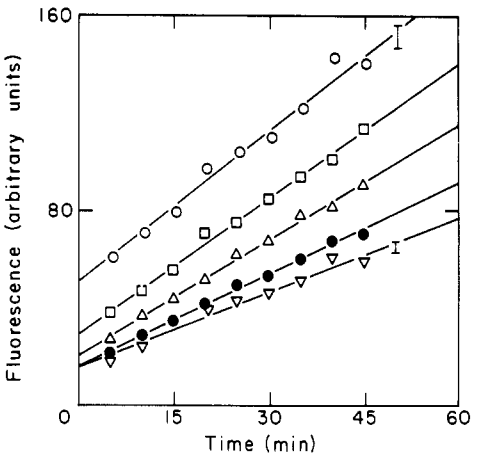


FIGURE 3: Time dependence of fluorescence for hydrolysis of peptide F-8 by thrombin (0.067 NIH unit/mL) at five different peptide concentrations: (▽) 0.16, (●) 0.22, (△) 0.33, (□) 0.49, and (○) 1.1 mM. The error symbols indicate the estimated maximum contributions from hydrolysis of the Arg($P_3'$)–Val($P_4'$) peptide bond.

Separate TLC experiments demonstrated that contributions from hydrolysis of the Arg–Val bond can reasonably be ignored. At concentrations of substrate and enzyme at which 10% of the Arg–Gly bonds would be hydrolyzed, a maximum of 0.2% of the Arg–Val bonds were hydrolyzed, as indicated by the presence of the H-Val-$NHCH_3$ product on TLC. However, since this product has a higher fluorescence yield than the tetrapeptide product (by a factor of 4.27) when reacted with fluorescamine, the maximum contribution from hydrolysis of the Arg–Val bond is 8.5% of the total fluorescence, i.e., within the error in the measurement (see Figure 3). The Michaelis–Menten parameters determined above are, therefore, representative of the hydrolysis of the Arg–Gly bond in peptide F-8 by thrombin.

## Discussion

The sequences of the relevant synthetic fibrinogen-like peptides and the kinetic constants for the hydrolysis of their Arg–Gly bonds by thrombin are given in Table I. For comparison, the corresponding data are given for the A$\alpha$ chain in intact (bovine) fibrinogen. Of the synthetic peptides studied thus far, F-8 is clearly the best substrate for thrombin. Peptide F-8 differs from F-5 only in the additional Asp at position $P_{10}$. It can be seen that peptide F-8 has the lowest value of $K_M$, and the highest values of $k_{cat}$ and of $k_{cat}/K_M$, than any other synthetic peptide including F-5. These differences become more significant by recognizing that $K_M$ and $k_{cat}$ for most of the other synthetic peptides (F-1–F-5) are higher and lower, respectively, than shown in Table I (as explained in footnote *b* of the Table). For example, by the earlier method, $K_M$ and $k_{cat}$ for peptide F-6 were found to be $934 \times 10^{-6}$ M and $11 \times 10^{-11}$ M [(NIH unit/L) s]$^{-1}$, respectively (Meinwald et al., 1980), as compared to the more recently determined values of $K_M = 3500 \times 10^{-6}$ M and $k_{cat} = 6.9 \times 10^{-11}$ M [(NIH unit/L) s]$^{-1}$ (Marsh et al. 1982). It thus appears that Asp at position $P_{10}$ makes a significant contribution to the effectiveness of these peptides as substrates for thrombin.

To account for the improved Michaelis–Menten parameters observed for F-8, Asp($P_{10}$) must necessarily interact either

Table I. Comparison of the Kinetic Constants for Hydrolysis of the Arg-Gly Bonds of Various Substrates by Bovine Thrombin at pH 8.0 and 25°C.

| Substrate | $P_{10}$ | $P_9$ | $P_8$ | $P_7$ | $P_6$ | $P_5$ | $P_4$ | $P_3$ | $P_2$ | $P_1$ | $P_1'$ | $P_2'$ | $P_3'$ | $P_4'$ | $P_5'$ | $P_6'$ | $P_7'$ | $K_M \times 10^6$ (M) | $k_{cat} \times 10^{11}$ [M(NIH unit/L)s]$^{-1}$] | $k_{cat}/K_M \times 10^7$ [(NIH unit/L)s]$^{-1}$] | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fibrinogen (bovine) Aα chain | | | | | | | | | | | | | | | | | | 9.2 | 73 | 793 | Martinelli & Scheraga (1980) |
| CNBr Aα | | | | | | | | | | | | | | | | | | 47 | 48 | 100 | Hageman & Scheraga (1974) |
| F-1[a,b] | | | | | | | | | | | Ac-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$ | | | | | | | 630 | 0.3 | 0.05 | van Nispen et al (1977) |
| F-2[a,b] | | | | | | | | | | | Ac-Ala-Glu-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$ | | | | | | | 1560 | 0.3 | 0.02 | van Nispen et al (1977) |
| F-3[a,b] | | | | | | | | | | | Ac-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$ | | | | | | | 680 | 11 | 1.6 | van Nispen et al (1977) |
| F-4[b,c] | | | | | | | | | | | Ac-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-$NHCH_3$ | | | | | | | 789 | 16 | 2.0 | Meinwald et al (1980) |
| F-5[b,c] | | | | | | | | | | | Ac-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$ | | | | | | | 633 | 20 | 3.2 | Meinwald et al (1980) |
| F-8[c] | | | | | | | | | | | Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$ | | | | | | | 310 | 31 | 10 | This work |
| F-6[c] | | | | | | | | | | | Ac-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ | | | | | | | 3500 | 6.9 | 0.2 | Marsh et al (1982) |
| F-7[c] | | | | | | | | | | | Ac-Leu-Ala-Glu-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ | | | | | | | -- | -- | <0.01 | Marsh et al (1982) |
| O[d] | | | | | | | | | | | H-Gly-Val-Arg-Gly-Pro-Arg-Leu-OH | | | | | | | 3700 | 0.5 | 0.01 | Liem & Scheraga (1974) |
| P[d] | | | | | | | | | | | H-Gly-Val-Arg-Gly-Gly-Arg-Leu-OH | | | | | | | 9600 | 0.2 | 0.002 | Liem & Scheraga (1974) |
| Q[d] | | | | | | | | | | | H-Gly-Val-Arg-Gly-Pro-Gly-Leu-OH | | | | | | | 15300 | 0.1 | 0.001 | Liem & Scheraga (1974) |

[a] Peptides F-1, F-2, and F-3 were formerly called A, B, and C, respectively (van Nispen et al., 1977), and are based on the sequence of human fibrinogen.

[b] As shown by Marsh et al (1982), the values of $K_M$ for these peptides should be somewhat higher than those reported here, and the values of $k_{cat}$ (and hence of $k_{cat}/K_M$) should be somewhat lower, because of an improvement in the analysis of the data.

[c] Peptides F-4 to F-8 are also based on the sequence of human fibrinogen.

[d] Peptides O, P, and Q were reported by Liem & Scheraga (1974) and are based on the sequence of bovine fibrinogen, which differs from that of human fibrinogen at position $P_4'$ (Leu for Val) (Martinelli et al, 1979).

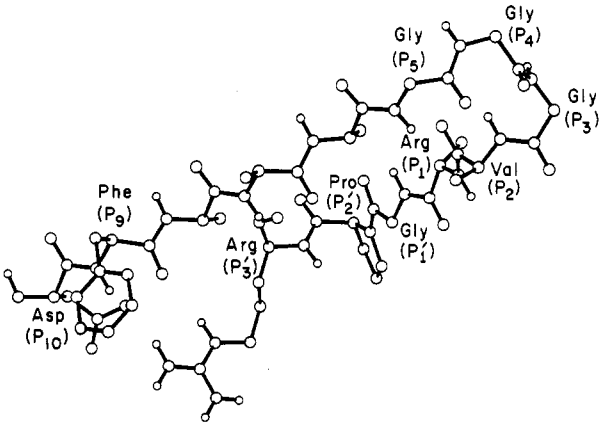


FIGURE 4: Schematic representation of a hairpin-like structure in this portion of the Aα chain of fibrinogen. This structure is only a *hypothetical* one and should not be taken literally. Its purpose is to illustrate the proposed salt link between the side chains of Asp at $P_{10}$ and of Arg at $P_3'$. Additional bends may be present at Pro–Arg ($P_2'$ and $P_3'$) (Von Dreele et al., 1978) and possibly between $P_4$ and $P_8$ to bring Phe at $P_9$ (Rae & Scheraga, 1979) and the phosphorylatable Ser at $P_{14}$ (L. S. Hanna and H. A. Scheraga, unpublished data) close to Val-Arg-Gly at $P_2$, $P_1$, and $P_1'$.

directly with the thrombin molecule itself or with a residue(s) within positions $P_{10}$–$P_4'$ of the fibrinogen Aα chain. The distinction between these two alternatives—or some combination of the two—cannot be made on the basis of the kinetic evidence presented here.

As can be seen in Table I, the specificity constant, $k_{cat}/K_M$, for peptide F-8 is smaller by a factor of 10 than that for CNBr Aα, due almost entirely to differences in substrate affinities as reflected in the values of $K_M$. This difference in $K_M$ corresponds to only 1–2 kcal/mol in free energy of association. Actually, the value of $K_M$ reported here for peptide F-8 is within the range of error (20–500 μM) reported by Hageman & Scheraga (1974) for CNBr Aα. A small difference in binding affinities between these substrates, however, is not unreasonable. Long-range interactions involving a residue (or residues) outside the region of $P_{10}$ through $P_4'$ of CNBr Aα may stabilize the native conformation and thus provide the necessary additional free energy of association. Alternatively, some residue(s) outside the region of $P_{10}$ through $P_4'$ of CNBr Aα may interact directly with thrombin, although this would probably require that the enzyme have an unusually large active site.

Blombäck et al. (1976) reported that peptide fragments consisting of residues 1–23 and 1–33 from the Aα chain of human fibrinogen are cleaved at a rate that is approximately 6% of that for CNBr Aα. Since this figure was close to the 2% figure for peptide F-3, which corresponds to residues 8–23 of the Aα chain, Van Nispen et al. (1977) proposed that residues located between positions 23 and 51, and probably between positions 33 and 51, are important for the interaction of the Aα chain with thrombin. In addition, since the thrombin hydrolysis rates for residues 1–23 of the Aα chain and for residues 1–33 of disulfide-linked Aα chains were much lower than for residues 1–51 and 1–44, Blombäck et al. (1977) concluded that the sequence 33–44 was of considerable importance in thrombin–fibrinogen Aα chain interactions. The data reported here indicate that, if residues between 23 and 51 are involved, they contribute at most only 1–2 kcal/mol in binding affinity.

It has been suggested (Morris et al., 1981; Nagy et al., 1982) that a salt link between Asp at position $P_{10}$ and Arg at position $P_3'$ stabilizes an intervening β-bend, thus providing the proper orientation of Phe($P_9$) in the active site of thrombin. A

schematic representation of such a hairpin-like structure is illustrated in Figure 4. The absence of such long-range interactions would explain the delayed release of fibrinopeptide A from fibrinogen Lille, in which Asn replaces Asp at $P_{10}$ (Morris et al., 1981), and from fibrinogen Munich, in which Asn replaces Arg at $P_3'$ (Henschen et al., 1981). Alternatively, Asp($P_{10}$) may interact directly with the thrombin molecule itself.

These kinetic studies demonstrate the importance of Asp at position $P_{10}$ in thrombin–fibrinogen interactions. An NMR study of peptide F-8 is currently in progress to try to obtain direct evidence for the proposed Asp($P_{10}$)···Arg($P_3'$) salt link and intervening $\beta$-bend in solution.

## Acknowledgments

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## Supplementary Material Available

Detailed experimental procedure and properties of the intermediates (4 pages). Ordering information is given on any current masthead page.

**Registry No.** I, 86177-73-3; II, 86177-74-4; III, 86177-75-5; IV, 86196-54-5; V, 86177-76-6; VI, 86196-44-3; F-8, 86177-72-2; Z-Asp(OBu$^t$)-OSu, 3338-32-7; H-Phe-Leu-Ala-Glu(OBu$^t$)-Gly-OH, 86177-77-7; H-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$, 86177-78-8; Boc-Gly-OSu, 3392-07-2; H-Pro-Arg-Val-$NHCH_3$, 86177-79-9; thrombin, 9002-04-4; Asp, 56-84-8.

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