Mechanism of Action of Thrombin on Fibrinogen. Size of the Aα Fibrinogen-like Peptide That Contacts the Active Site of Thrombin†

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ABSTRACT: The following peptides were synthesized by classical methods in solution: Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-$NHCH_3$ (F-4), Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$ (F-5), and Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ (F-6). The rates of hydrolysis of the Arg–Gly bond in these peptides by thrombin were measured, and the values of the specificity constant, k_{cat}/K_M, were all found to be $\sim 2 \times 10^{-7}$ [(NIH unit/L)s]$^{-1}$, similar to that for a peptide (F-3) having an additional Arg residue between Glu- and -$NHCH_3$ of F-4. The difference between this value and that for the Aα chain of bovine fibrinogen is attributed to slight conformational differences arising from long-range interactions present in fibrinogen but not in the synthetic peptides. In addition to the requirement for the Phe residue, demonstrated earlier, it is shown here that no residues on the C-terminal side of Pro are required for interaction between thrombin and fibrinogen. The active site of thrombin thus appears to interact with a peptide of the size of F-6, with the Phe residue possibly being in close spatial proximity to the Val-Arg-Gly moiety.

The mechanism of action of thrombin on fibrinogen is being studied by an active-site mapping approach: the rates of hydrolysis of specific Arg–Gly bonds in peptide substrates analogous to the relevant portions of the Aα and Bβ chains of fibrinogen are determined and compared with the rates of hydrolysis of the Aα and Bβ chains in fibrinogen (Scheraga, 1977). Most of these experiments have focused on the Aα chain since the rate of hydrolysis of the specific Arg–Gly bond in this chain to release fibrinopeptide A parallels the rate of fibrin formation (Blombäck & Vestermark, 1958). The peptide substrates which have been examined as models of the Aα chain have included synthetic oligopeptides (Andreatta et al., 1971; Liem et al., 1971; Liem & Scheraga, 1973, 1974; van Nispen et al., 1977) and CNBr Aα,[1] the 51-residue N-terminal CNBr fragment of the Aα chain (Hageman & Scheraga, 1974).

Based upon a comparison with the recently determined kinetic parameters for the Aα chain in the intact fibrinogen molecule (Martinelli & Scheraga, 1980), it was concluded that CNBr Aα probably contained all the residues essential for the specific interaction with thrombin. Accepting this as the upper limit to the size of the peptide which interacts with thrombin in the same manner as the Aα chain in intact fibrinogen, smaller synthetic oligopeptides were examined in order to determine the *minimum*-size peptide sufficient for the specific interaction with thrombin and also to identify which residues lead to this specificity (van Nispen et al., 1977). These peptides varied in the length of the amino acid sequence on the N-terminal side of the Arg–Gly bond (see Table I for the amino acid sequences of the synthetic substrates for thrombin and for the definitions of the sites P_i and P_i'). The longest of these peptides, F-3, was a much better substrate than the other peptides that had been synthesized up to that time.

As a result of these experiments, it was concluded that the Phe residue at position P_9 was important in the interaction with thrombin. The essential nature of this Phe residue had been postulated earlier by Blombäck (1967), based upon the observation that the amino acid sequence of this portion of fibrinogen has been strongly conserved in various species of this protein.

Having determined which residues on the N-terminal side of the Arg–Gly bond are involved in the interaction with thrombin, the experiments reported here have been designed to determine the extent of interaction of the residues on the C-terminal side of the Arg–Gly bond. For this purpose peptides F-4, F-5, and F-6, with variable length on the C-terminal side of the Arg–Gly bond, containing 15, 13, and 11 residues, respectively, have been synthesized. These peptides were prepared by classical synthesis in solution because this allows for extensive purification and characterization of all intermediates. The large peptides used in the fragment-condensation approach were chosen in such a way that the carboxy-activated component has a glycine residue at the C terminus, thus eliminating the danger of racemization in the final coupling steps. Since the 16-residue peptide F-3 had been synthesized successfully in this laboratory by the same method (van Nispen et al., 1977), wherever possible the previously characterized intermediates were utilized.

These three peptides do not contain any free primary amines so that the action of thrombin on these substrates could be monitored by measuring the rate of formation of free α-amino groups in the product. Thrombin cleaves the Arg(P_1)–Gly(P_1') bond in each of these peptides to form a product with a free α-amino group of Gly. In addition, in peptides F-4 and F-5, thrombin also cleaves the Arg(P_3')–Val(P_4') bond to yield a product with a free α-amino group of Val. The rate of formation of these products was determined by means of a

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[1] Abbreviations used: CNBr Aα, the N-terminal CNBr fragment of the Aα chain of fibrinogen; [S̄], the average substrate concentration over the time period in which the kinetic experiment was performed; Boc, *tert*-butoxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; DMF, *N,N*-dimethylformamide; HOBt, 1-hydroxybenzotriazole; OBu^t, *tert*-butyloxy; ONp, 4-nitrophenoxy; Fl_3AcOH, trifluoroacetic acid; TLC, thin-layer chromatography; Z, benzyloxycarbonyl; Ac, acetyl; AP-M, aminopeptidase M. 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).

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Table I: Amino Acid Sequences of Synthetic Oligopeptide Substrates

peptide	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'
F-1[a]	Ac-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$
F-2[a]	Ac-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$
F-3[a]	Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$
F-4	Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-$NHCH_3$
F-5	Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$
F-6	Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$
O[b]	Gly-Val-Arg-Gly-Pro-Arg-Leu-OH
P[b]	Gly-Val-Arg-Gly-Gly-Arg-Leu-OH
Q[b]	Gly-Val-Arg-Gly-Pro-Gly-Leu-OH

[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] Peptides O, P, and Q were reported by Leim & 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).

fluorescence assay utilizing the fluorogenic reagent fluorescamine (Weigele et al., 1972; Udenfriend et al., 1972; Böhlen et al., 1973). The cleavage of the Arg(P_3')–Val(P_4') bond complicates the analysis, as it did for peptides F-1, F-2, and F-3 (van Nispen et al., 1977) but, as in the case of these three peptides, the rate of hydrolysis of the Arg–Gly bond could be determined for peptides F-4 and F-5 because of the strictly sequential mechanism with which thrombin cleaves these two bonds in these peptides. Analysis of the products of the thrombin digestion of these peptides at various times by thin-layer chromatography demonstrates that all the Arg–Gly bonds present are cleaved before any Arg–Val bonds are cleaved.

Experimental Section

Materials

Bovine prothrombin (Lot GS-C-8400) was obtained from Sigma Chemical Co., and thrombin was prepared from this material as described earlier (Hageman et al., 1975); it had a specific activity of 2200 NIH units/mg. Trypsin from Worthington Biochemical Corp. and aminopeptidase M from Röhm and Haas were used without further purification. Fluorescamine was obtained from Pierce Chemical Co.

All of the amino acids (except glycine) were of the L configuration. Z-Glu(OBu^t)-OH was purchased from Bachem Inc. HOBt, DCC, and Ac-ONp were purchased from Aldrich Chemical Co. and were purified before use.

Methods

The purity of the amino acid derivatives and peptides was checked routinely by TLC on Merck silica gel plates (F-254; 0.25 mm) using the following solvent systems: (a) chloroform–methanol, 9:1; (b) chloroform–methanol–acetic acid, 95:20:3; (c) 1-butanol–acetic acid–water, 4:1:1; (d) 2-propanol–formic acid–water, 20:1:5; (e) 1-butanol–pyridine–acetic acid–water, 4:1:1:2.

Methods used for the detection of components on TLC plates were ultraviolet light (quenching of fluorescence after exposure of the plates at 254 nm), ninhydrin reagent for free amino groups, chlorine/potassium iodide–starch reagent for NH groups, Sakaguchi reagent for free guanidino groups, and Barton reagent for hydrazides.

Melting points (uncorrected) were determined in a Thomas-Hoover apparatus. Specific rotations were measured with a Perkin-Elmer 141 polarimeter. NMR spectra were obtained for all intermediates with a Varian EM-390 spectrometer and found to be in full agreement with the proposed structures. Amino acid analyses were carried out with a Technicon TSM-1 autoanalyzer on samples that had been hydrolyzed in HCl in evacuated, sealed ampules. To check for racemization, we carried out enzymatic digestions with aminopeptidase M on

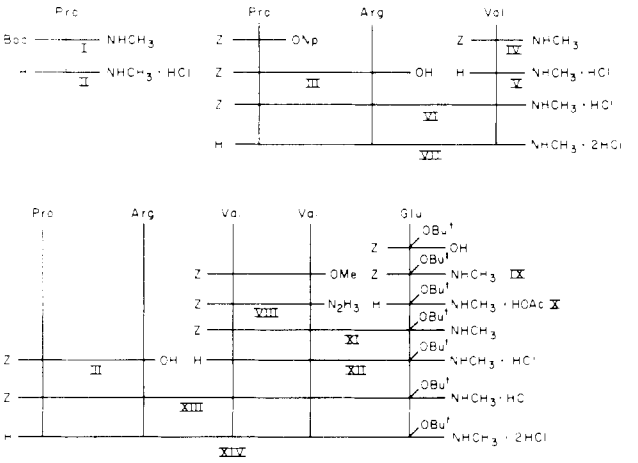

FIGURE 1: Schematic representation of the synthesis of the C-terminal fragments.

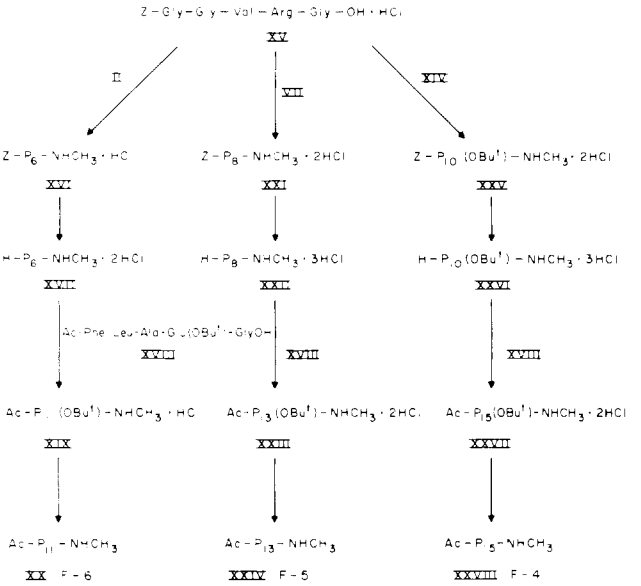

FIGURE 2: Schematic representation of the synthesis of peptides F-4, F-5, and F-6.

the peptides H-Pro-Arg-Val-$NHCH_3$ and H-Pro-Arg-Val-Val-Glu-$NHCH_3$ in 0.1 M Tris-HCl buffer (pH 8.3) at 37 °C until completion. The amino acid composition of these enzymatic hydrolyzates was determined with the TSM-1 autoanalyzer. Microanalysis for C, H, and N composition was carried out by Galbraith Laboratories.

Synthetic Methods. The synthesis of the three peptides, F-4, F-5, and F-6, is outlined in Figures 1 and 2. The details of the syntheses are given in the supplementary material (see paragraph at end of paper regarding supplementary material).

(*1*) *Nitrogen Analysis.* The concentrations of peptide stock solutions from which the solutions used in the kinetic experiments were prepared were determined by micro-Kjeldahl nitrogen analysis (Lang, 1958; Noel & Hambleton, 1976a,b).

(*2*) *Kinetic Experiments.* The procedure used to determine the initial rate of hydrolysis of the Arg–Gly bond in these peptides was essentially the same as described previously (van Nispen et al., 1977).

Peptides F-4, F-5, an F-6 do not contain any primary amines. The rates of hydrolysis of these peptides by thrombin could be monitored by measuring the rate of formation of the primary amine groups [of Gly(P_1') and Val(P_4') for peptides F-4 and F-5 and of only Gly(P_1') for peptide F-6] that were exposed upon cleavage of the respective peptide bonds. A fluorescence assay using the fluorogenic reagent fluorescamine was used to analyze the reaction mixture for the concentration of primary amine present. Fluorescamine was ideal for this purpose since it reacts with primary amines (with an average half-time of tenths of a second) to give a fluorescent product, while the excess reagent is hydrolyzed by water to nonfluorescent products with an average half-life of several seconds (Weigele et al., 1972; Udenfriend et al., 1972; Böhlen et al., 1973). There is no need to quench the thrombin reaction since fluorescamine will react only with the primary amines present at the time it is added to the aqueous solution.

The kinetic experiments were carried out at 25 °C in 0.15 M KCl and 0.05 M sodium borate buffer, pH 8.00. The reaction mixtures were prepared from quantitative dilutions of stock solutions of the peptide substrates. The concentrations of thrombin added to these solutions were determined by clotting assays (Liem et al., 1971). Aliquots of the reaction mixture were removed at timed intervals after the addition of thrombin and assayed with fluorescamine. The fluorescence was measured on a Perkin-Elmer Model MPF-3 fluorescence spectrophotometer. The exciting wavelength was 390 nm, and the emission was measured at 480 nm.

In the case of peptide F-6, only the Arg–Gly bond is hydrolyzed by thrombin, and the fluorescence intensity was directly proportional to the degree of hydrolysis. In the case of peptides F-4 and F-5, as with peptides F-1, F-2, and F-3 (van Nispen et al., 1977), both the Arg–Gly and Arg–Val bonds are hydrolyzed, but in sequential fashion, the Arg–Gly bond being hydrolyzed completely before any hydrolysis of the Arg–Val bond can be detected. This was demonstrated here by thin-layer chromatography of samples of reaction mixtures of peptides F-4 and F-5 and thrombin at various times (using solvent system e); samples of H-Val-$NHCH_3$ and H-Val-Val-Glu-$NHCH_3$ were run as standards to help identify the products. Despite the hydrolysis of the Arg–Val bond in these two peptides, the rate of hydrolysis of the Arg–Gly bond could be determined by the procedure of van Nispen et al. (1977). The fluorescence intensity at any time, $F(t)$, is given by

$$F(t) = F(\infty) - C_1e^{-k_1t} - C_2e^{-k_2t} \quad (1)$$

where $F(\infty)$ is the intensity at infinite time, C_1 and C_2 are the relative contributions to the fluorescence from the N-terminal Gly and N-terminal Val products, respectively, and k_1 and k_2 are equal to $k_{cat}[T]_0/K_M$ for the hydrolysis of the Arg–Gly and Arg–Val bonds, respectively, $[T]_0$ being the initial concentration of thrombin. The total amount of hydrolyzable peptide bonds was determined by using trypsin, instead of thrombin, since the reaction proceeded to completion much faster with the former enzyme; the fluorescence from completely hydrolyzed peptide is designated as F(trypsin).

Plots of ln $[F(\infty) - F(t)]$ against time are linear at large values of t; the linear portion has a slope of $-k_2$ and an intercept of ln C_2. Plots of ln $[F(\infty) - F(t) - C_2]$ or ln $[C_1 - F(t)]$ against time are linear for small values of t with a slope of $-k_1$ and an intercept of ln C_1.

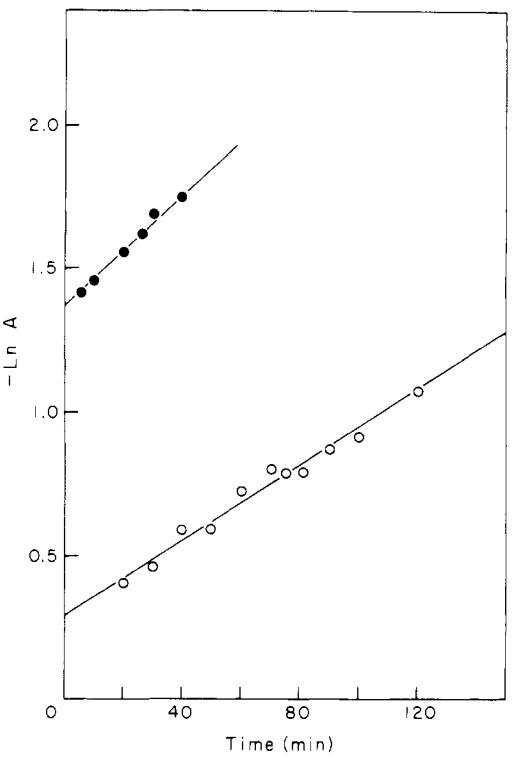

FIGURE 3: Plot of $-\ln A$ vs. time for peptide F-4, where $A = [F(\infty) - F(t)]/F$(trypsin) for the open circles and $[[C_1 - F(t)]/F$(trypsin)] for the solid circles. Peptide concentration was 1.99×10^{-5} M in both experiments. Thrombin concentration was 20 NIH units/mL for the open circles and 0.80 NIH unit/mL for the closed circles. The lines were determined by least-squares analysis of the data.

With C_1/F(trypsin) determined as described previously (van Nispen et al., 1977), the initial rates of hydrolysis of the Arg–Gly bond in peptides F-4 and F-5 could be measured, from which the values of the Michaelis–Menten parameters could be calculated. The hydrolysis of each substrate by thrombin was measured at five different substrate concentrations. The initial slope of the line determined by least-squares analysis of a plot of the fluorescence of each sample, $F(t)$, against time of removal of the sample from the reaction was designated m_R. The extent of reaction in each experiment was always less than 20% of completion. Next, the fluorescence of the infinite-time digestion of each peptide (using trypsin) was plotted against the peptide concentration. These plots were linear, and the slope of the line was determined by least-squares analysis and designated by m_T. Then m_TC_1/F(trypsin) is the amount of fluorescence due to glycine per unit concentration of peptide. Therefore, the initial velocity, v_0, was equal to m_RF(trypsin)$/(m_TC_1)$. The determination of the initial velocity for peptide F-6 was simpler. The initial velocity was equal to m_R/m_T, i.e., essentially C_1/F(trypsin) = 1 and C_2/F(trypsin) = 0 for peptide F-6.

The Michaelis–Menten parameters were then determined from Lineweaver–Burk plots of $1/v_0$ vs. $1/[\bar{S}]$. Triplicate experiments were run for the five different concentrations of each peptide.

Results

Determination of C_1/F(trypsin). Figures 3 and 4 show the results of the experiments designed to measure C_1/F(trypsin) for peptides F-4 and F-5, respectively. The fluorescence of the peptide completely hydrolyzed by thrombin is the same

Table II: Comparison of the Kinetic Constants for the Hydrolysis of Arg-Gly Bonds by Thrombin at pH 8.0 and 25 °C

substrate	$K_M \times 10^6$ (M)	$k_{cat} \times 10^{11}$ [M [(NIH unit/L)s]$^{-1}$]	$k_{cat}/K_M \times 10^7$ [[(NIH unit/L)s]$^{-1}$]	ref
fibrinogen (bovine) Aα chain	9.2 ± 75%	73 ± 50%	793 ± 25%	Martinelli & Scheraga (1980)
CNBr Aα	47 (20-500)[c]	48 (38-330)	100 (60-200)	Hageman & Scheraga (1974)
F-1	630 ± 70%	0.30 ± 90%	0.05 ± 20%	van Nispen et al. (1977)
F-2	1560 ± 66%	0.32 ± 87%	0.02 ± 20%	van Nispen et al. (1977)
F-3[a]	680 ± 18%	11 ± 33%	1.6 ± 20%	van Nispen et al. (1977)
F-4	789 ± 79%	16 ± 60%	2.0 ± 20%	this work
F-5	633 ± 61%	20 ± 40%	3.2 ± 20%	this work
F-6	934 ± 88%	11 ± 85%	1.2 ± 27%	this work
O[b]	3700	0.45	0.01	Liem & Scheraga (1974)
P[b]	9600	0.22	0.002	Liem & Scheraga (1974)
Q[b]	15300	0.09	0.001	Liem & Scheraga (1974)

[a] The kinetic parameters for F-3 were redetermined in this work. The values were $K_M = 840 \times 10^{-6}$ M, $k_{cat} = 12 \times 10^{-11}$ M [(NIH unit/L)s]$^{-1}$, and $k_{cat}/K_M = 1.5 \times 10^{-7}$ [(NIH unit/L)s]$^{-1}$. These values are in good agreement with those reported previously. [b] No error analysis was carried out for these peptides. [c] Range is in parentheses.

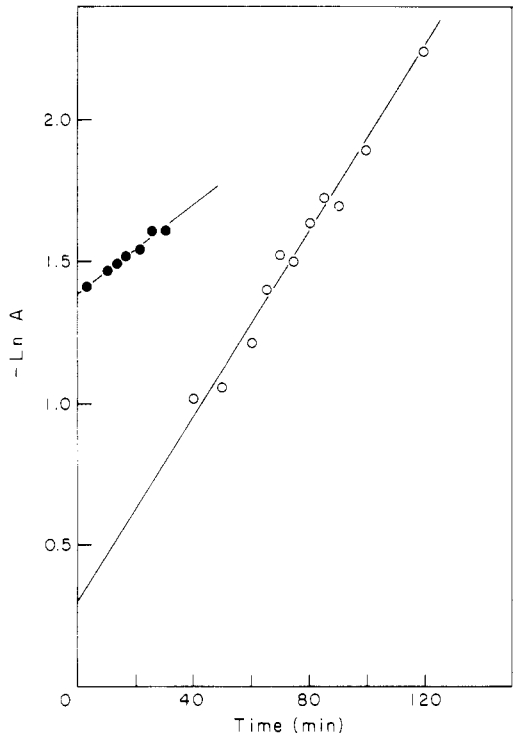

FIGURE 4: Plot of $-\ln A$ vs. time for peptide F-5. A is defined in the same way as for Figure 3. Peptide concentration was 1.77×10^{-5} M in both experiments. Thrombin concentration was 9.1 NIH units/mL for the open circles and 0.36 NIH unit/mL for the solid circles. The lines were determined by least-squares analysis of the data.

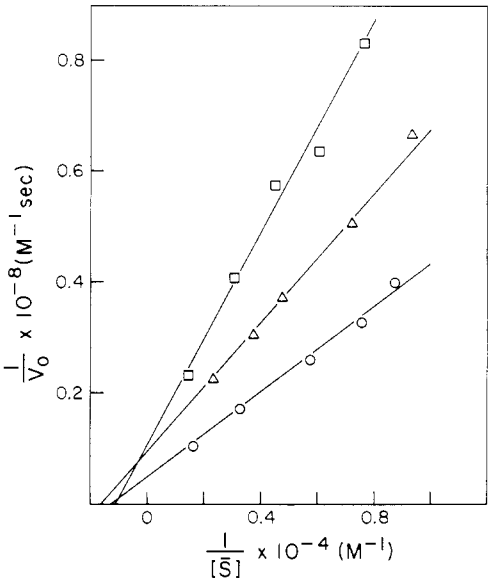

FIGURE 5: Lineweaver–Burk plots of initial rates of hydrolysis of the Arg–Gly bond in peptides F-4, F-5, and F-6. The thrombin concentrations were 1.28, 0.43, and 0.88 NIH units/mL for peptides F-4 (○), F-5 (△), and F-6 (□), respectively. All experiments were performed at pH 8.0 and 25 °C. The lines were determined by least-squares analysis of the data.

as that for complete trypsin hydrolysis; this is consistent with the fact that both enzymes cleave the same bonds in these substrates.

For peptide F-4, the linear portion of the plot of $\ln [F(\infty) - F(t)]/F(\text{trypsin})$ against time (open circles) has a slope of $-0.00652 \pm 27\%$; this gives a value of $5 \times 10^{-9} \pm 27\%$ [(NIH unit/L)s]$^{-1}$ for k_{cat}/K_M for the hydrolysis of the Arg–Val bond by thrombin. The intercept of the line ($t = 0$ axis) is $-0.29 \pm 38\%$. The value of $C_2/F(\text{trypsin})$ calculated from the intercept is $0.75 \pm 12\%$. Then $C_1/F(\text{trypsin}) = [F(\infty)/F(\text{trypsin})] - C_2/F(\text{trypsin}) = 0.25$. The solid circles of Figure 3 are a plot of $\ln [[C_1 - F(t) - F(t)]/F(\text{trypsin})]$ against time for the low thrombin concentration (0.80 NIH unit/mL). The line has a slope of $-0.0101 \pm 12\%$ which gives a value of $2.1 \times 10^{-7} \pm 12\%$ [(NIH unit/L)s]$^{-1}$ for k_{cat}/K_M for the hydrolysis of the Arg–Gly bond in peptide F-4. The value of $C_1/F(\text{trypsin})$ calculated from the intercept is $0.25 \pm 10\%$, in agreement with the above estimate.

The corresponding analysis of the data in Figure 4 for peptide F-5 gives the following results: k_{cat}/K_M for hydrolysis of the Arg–Val bond by thrombin is $3 \times 10^{-8} \pm 16\%$ [(NIH unit/mL)s]$^{-1}$; k_{cat}/K_M for hydrolysis of the Arg–Gly bond by thrombin is $3.7 \times 10^{-7} \pm 20\%$ [(NIH unit/mL)s]$^{-1}$; $C_1/F(\text{trypsin}) = 0.25 \pm 8\%$ and $C_2/F(\text{trypsin}) = 0.75 \pm 8\%$.

The error limits were calculated by using the 95% confidence intervals on the slopes and intercepts of these plots. The above data differ slightly from those in Table II because the latter pertains to results that are averaged over many substrate concentrations. The analysis of Figures 3 and 4 was not required for peptide F-6 since the latter contained only one thrombin-hydrolyzable peptide bond.

Determination of Michaelis–Menten Parameters. Typical plots of $1/v_0$ vs. $1/[\bar{S}]$ are shown in Figure 5 for each of the peptides. Table II summarizes the results of the kinetic experiments for peptides F-4, F-5 and F-6 as well as for other relevant substrates.

The slopes of the Michaelis–Menten plots of Figure 5 give k_{cat}/K_M directly. Hence, the error in k_{cat}/K_M is the error in the slope plus an assumed 10% error in the thrombin concentration. Correspondingly, the error in k_{cat} is the error in the intercept on the $1/v_0$ axis, and the error in K_M is the error

in the intercept on the $1/[\bar{S}]$ axis. The errors represent 95% confidence intervals.

Discussion

Within experimental error, the kinetic parameters for synthetic peptides F-3 to F-6 appear to be the same and differ by orders of magnitude from those for F-1, F-2, O, P, and Q. The importance of Phe at position P_9 was demonstrated earlier (van Nispen et al., 1977), and the present results suggest that residues on the C-terminal side of Pro at P_2' may not play a significant role in the thrombin–fibrinogen interaction. The nonessentiality of Arg at position P_3' is supported by the observation that Fibrinogen Detroit, a congenitally abnormal fibrinogen, has Ser instead of Arg in this position with apparently no effect on the hydrolysis of the Arg–Gly bond by thrombin, although the subsequent clotting of fibrin monomer is impaired (Blombäck et al., 1968, 1978). Thus, the results of these kinetic experiments are able to distinguish between the sites essential for the specificity of the interactions with thrombin and sites essential for the polymerization of fibrin monomer. This conclusion that Arg at position P_3' is not essential for binding to thrombin would appear to disagree with the results for peptides O and Q, where the substitution of Gly for Arg reduces k_{cat}/K_M (with similar results for the role of Pro in a comparison of peptides O and P). Since these peptides lack the required Phe residue and thus are *very* poor substrates, we feel that these variations in such *small* values of k_{cat}/K_M do not argue against the conclusion deduced from comparisons among peptides F-1 to F-6.

Examination of the values of k_{cat}/K_M shows that, although peptides F-3 to F-6 are better substrates of thrombin than the other synthetic peptides, they are somewhat poorer substrates than fibrinogen or the CNBr Aα fragment of fibrinogen. It should be noted, however, that k_{cat} for these peptides differs from that of fibrinogen by less than 1 order of magnitude. The difference between peptides F-3 to F-6 and the Aα chain in intact fibrinogen, as substrates of thrombin, resides primarily in the factors that determine K_M; i.e., these synthetic substrates do not bind to thrombin as well as the Aα chain in fibrinogen does.

It appears then that additional residues not included in P_9–P_7' are required for efficient binding to thrombin. One can envisage several possible alternative roles for these additional residues. First, they may interact directly with thrombin at an independent binding site. Second, these residues might provide long-range interactions to enable P_9–P_7' to adopt an appropriate conformation for optimum binding of Arg–Gly to thrombin; these long-range interactions might involve the Phe residue. Third, the conformation of P_9–P_7', influenced by long-range interactions, could be such that the Phe residue in native fibrinogen is spatially close to the Arg-Gly moiety and helps bind the Arg-Gly segment to thrombin by also interacting with the active site of the enzyme.

The possibility of a specific spatial relationship between Phe(P_9) and the Arg-Gly segment 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) which 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. The proximity of L-Phe (in the case of the natural substrate, fibrinogen) to Val and the Arg-Gly segment may be specially advantageous for binding to thrombin.

The possible involvement of a specific conformational feature in these peptides that would influence their reactivity toward thrombin is suggested by the NMR results of Von Dreele et al. (1978), who found a type II β turn at Pro-Ala in the hexapeptide H-Gly-Val-Arg-Gly-Pro-Ala-NH_2. Further NMR experiments on peptides F-3 to F-6 are in progress. A chain reversal on the N-terminal side of the Arg-Gly bond could bring the essential Phe at P_9 close to Val-Arg-Gly.

In conclusion, peptide F-6 contains all of the residues required for the detemination of k_{cat}. Whether this same peptide contains *all* the required residues for proper binding to thrombin (i.e., for the specificity of the binding step) is uncertain. Long-range interactions, present to some extent in the fibrinogen molecule but less so in CNBr Aα and even less so in peptide F-6, might be required to maintain a specific conformational feature in P_9–P_2', the existence of which is suggested by NMR observations (Rae & Scheraga, 1979; Von Dreele et al., 1978). The absence of these long-range interactions in peptide F-6 is presumably responsible for the larger values of K_M compared to that for the Aα chain of fibrinogen. It would require less than 3 kcal/mol binding energy to reduce K_M by a factor of 100, to the value observed for fibrinogen. It is easily possible to gain 3 kcal of noncovalent interaction energy from a slight conformational change brought about by such long-range interactions. Thus, a slight alteration in conformation could easily bring the value of K_M for peptide F-6 into coincidence with that for fibrinogen.

Further work is concentrating on the effect of amino acid substitutions in F-6 on the values of k_{cat}/K_M and on a conformational analysis of this peptide and its analogues.

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Supplementary Material Available

Experimental details of the synthesis of the three peptides, F-4, F-5, and F-6 (13 pages). Ordering information is given on any current masthead page.

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Formation of 9-*cis*- and 11-*cis*-Retinal Pigments from Bacteriorhodopsin by Irradiating Purple Membrane in Acid†

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ABSTRACT: Both light-adapted and dark-adapted forms of bacteriorhodopsin in purple membrane in 67% glycerol solution were allowed to stand in acidic conditions by the addition of HCl to final concentrations from 4×10^{-4} to 2×10^{-2} M for 24 h at 3 °C. Over this concentration range, the acid-induced products from both species showed a maximum absorbance around 600 nm and high-performance liquid chromatography of extracted retinal isomers revealed that the acid-induced form of bacteriorhodopsin has 13-*cis*- and *all-trans*-retinals in a molar ratio of 4:6, which is intermediate between those of the dark-adapted and the light-adapted forms at neutral pH values. Exposure of the acid-induced form of bacteriorhodopsin to light at wavelengths longer than 670 nm at 3 °C caused a decrease of the absorbance around 600 nm with a concomitant rise of the absorbance around 500 nm. The extract from the irradiated products of bacteriorhodopsin in acid contained 9-*cis*- and 11-*cis*-retinals in addition to 13-*cis*- and *all-trans*-retinals. The absorbance maximum estimated from the analysis of the absorption spectra and the composition of the isomers was found at 495 nm for the 9-*cis*-retinal pigment and around 560 nm for the 11-*cis*-retinal pigment. On irradiation with 438-nm light, the 9-*cis*-retinal pigment disappeared with a concomitant increase of both the 13-*cis*- and *all-trans*-retinal pigments as judged by chromophore analysis and the absorption spectrum. The 9-*cis*-retinal pigment brought to pH 9 exhibited a maximum absorbance at 450 nm; this could be decomposed by the action of hydroxylamine or converted to a form resembling normal bacteriorhodopsin by 438-nm irradiation.

It was previously shown (Maeda et al., 1979) that the chromophore retinal in squid rhodopsin produces all kinds of mono-cis isomers including 7-*cis*- and 13-*cis*-retinals under appropriately selected conditions of irradiation. A further examination of this process revealed that the 7-cis chromophore was produced from lumirhodopsin, one of the light-induced intermediates, but not directly from bathorhodopsin. This implies that some protein factors may determine the direction for the light-induced isomerization of the chromophore retinals. The 7-*cis*-retinal pigment was also produced in cattle rhodopsin (Maeda et al., 1978) under the same experimental conditions as those employed for squid rhodopsin.

Bacteriorhodopsin (bR)[1] is another pigment which has retinal as a chromophore [see a recent review by Stoeckenius et al. (1979)]. It has been well established that the chromophore retinal extracted from the light-adapted form of bR (bR^L) is *all-trans*-retinal and that from the dark-adapted form of bR (bR^D) is an equimolar mixture of both 13-*cis*- and *all-trans*-retinals (Pettei et al., 1977; Maeda et al., 1977). So far, no other isomers have been found from irradiated bR.

It is known that bR undergoes a reversible structural alteration in acidic solution accompanied by a shift of the absorption spectrum to longer wavelength (Oesterhelt & Stoeckenius, 1971). It is expected that the protein environment of bR molecules in acid will affect the chromophore differently on irradiation from that of bR under neutral conditions. From flash photolytic experiments, Lozier et al. (1978) observed that acidic bR undergoes a unique photochemical reaction, forming short-lived species with the wavelength of maximum absorbance (λ_{max}) at shorter wavelengths.

Under continuous irradiation of bR in acid at 3 °C, we observed the formation of 9-*cis*- and 11-*cis*-retinal pigments, which have not been observed by irradiating bR at neutral pH

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[1] Abbreviations used: bR, bacteriorhodopsin; bR^L, light-adapted form of bacteriorhodopsin; bR^D, dark-adapted form of bacteriorhodopsin; λ_{max}, the wavelength of maximum absorbance; CTAB, cetyltrimethylammonium bromide; LC, high-performance liquid chromatography.

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