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Mechanism of Action of Thrombin on Fibrinogen. Direct Evidence for the Involvement of Phenylalanine at Position P_9[†]

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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-$NHCH_3$ (F-6) and Ac-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ (F-7). The rates of hydrolysis of the Arg–Gly bond in these peptides by thrombin were measured, and the rate for the Phe-containing peptide F-6 was found to be much larger than that for F-7. Previous work [van Nispen, J. W., Hageman, T. C., & Scheraga, H. A. (1977) *Arch. Biochem. Biophys. 182*, 227] has demonstrated the importance of Phe-Leu at positions P_9-P_8 of the Aα chain of fibrinogen for the thrombin–fibrinogen interaction. This work demonstrates that the presence of Leu (P_8) alone is insufficient to account for the enhanced hydrolysis rates and that the presence of Phe (P_9) is essential for normal action of thrombin on the Aα chain of fibrinogen.

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 of the Aα chain of fibrinogen[1] is essential for normal thrombin action. This was supported, in part, by van Nispen et al. (1977), who showed that Phe-Leu at positions P_9-P_8 in the peptide Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$ (F-3) greatly increased the rate of

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[1] Abbreviations: AcONP, 4-nitrophenyl acetate; CNBr Aα, the N-terminal CNBr fragment of the Aα chain of fibrinogen; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; Ac, acetyl; HOAc, acetic acid; OBu^t, *tert*-butyloxy; DEAE-Sephadex, diethylaminoethyl-Sephadex. 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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hydrolysis of its Arg–Gly bond compared to the rates for shorter peptides devoid of Phe-Leu. Further, Meinwald et al. (1980) found that peptide F-6 (Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$) contains all of the residues required to obtain a value of k_{cat} close to that observed for the action of thrombin on the Aα chain of fibrinogen (Martinelli & Scheraga, 1980). This paper provides direct evidence for the requirement for Phe at position P_9 by comparing the thrombin-catalyzed rates of hydrolysis of the Arg–Gly bond in peptides F-6 and Ac-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ (F-7). The amino acid sequences of peptides F-6 and F-7 pertain to a portion of the Aα chain of human fibrinogen.

Experimental Procedures

All of the amino acids (except glycine) were of the L configuration. AcONP, HOBT, and DCC were purchased from Aldrich Chemical Co. and were purified before use. Solvents and inorganic salts were reagent grade or better and were used without further purification. DEAE-Sephadex was obtained from Sigma Chemical Co.

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–acetic acid, 95:20:3; (b) 1-butanol–pyridine–acetic acid–water, 4:1:1:2; (c) 2-propanol–formic acid–water, 20:1:5.

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

Melting points (uncorrected) were determined with a Thomas-Hoover apparatus. NMR spectra were obtained for all intermediates with a Varian EM-390 spectrometer. Amino acid analyses were carried out with a Technicon TSM-1 autoanalyzer.

Ac-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ (F-7) was prepared by utilizing two fragments previously synthesized in this laboratory. H-Leu-Ala-Glu(OBu^t)-Gly-OH·HOAc (van Nispen et al., 1977) was acetylated with AcONP to give the protected tetrapeptide Ac-Leu-Ala-Glu-(OBu^t)-Gly-OH: mp 213–215 °C dec; R_f 0.37 (a). Amino acid analysis (6 N HCl, 110 °C, 24 h): Glu, 1.00; Gly, 0.98; Ala, 1.02; Leu, 1.00. The protected tetrapeptide was coupled with H-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$·2HCl (Meinwald et al., 1980) with DCC/HOBT to give the protected decapeptide Ac-Leu-Ala-Glu(OBu^t)-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$·HCl: R_f 0.54 (b) and 0.42 (c). The *tert*-butyl protecting group was removed by treatment with trifluoroacetic acid and the final product (F-7) was purified by chromatography on a DEAE-Sephadex column (OAc^- form). F-7 showed the following properties: R_f 0.35 (b) and 0.41 (c). Amino acid analysis (12 N HCl, 110 °C, 24 h): Glu, 0.99; Pro, 1.00; Gly, 3.93; Ala, 0.99; Val, 1.06; Leu, 1.01; Arg, 1.00.

Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ (F-6) was synthesized essentially as described previously (Meinwald et al., 1980). Since the F-6 peptide actually examined in these experiments was intended for use in related NMR studies, deuterated glycine was incorporated at position P_4. It was assumed that this isotopic substitution would not affect the rates of hydrolysis by thrombin significantly. The synthesis of the isotopically labeled compound will be published in conjunction with the NMR studies.

Bovine prothrombin (lot no. 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 per milliliter) was constructed by using standard NIH thrombin (lot J); bovine fibrinogen (Sigma, type 1, fraction I) was used in all clotting assays.

The concentrations of peptide stock solutions were determined by automated 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 in these peptides was essentially the same as described previously (van Nispen et al., 1977; Meinwald et al., 1980). 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 by using the fluorogenic reagent fluorescamine (Weigele et al., 1972; Udenfriend et al., 1972; Böhlen et al., 1973) obtained from Sigma Chemical Co. Since neither peptide substrate contains 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 by using 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) by using the equation

$$F_c = 10^{Dd} F_o \qquad (1)$$

where F_c and F_o are the corrected and observed fluorescence intensities, respectively, D is the absorbance per centimeter at 390 nm (the wavelength of fluorescence excitation), and d is *half* of the path length in the fluorescence cell. Absorbances were determined on either a Cary 219, a Cary 118, or a Cary 14 (with modified solid-state circuitry) UV–vis spectrophotometer.

Fluorescence intensities were converted to concentrations of product with the aid of a calibration curve constructed by reacting fluorescamine with varying concentrations of H-Gly-Pro-$NHCH_3$ under the same conditions used in the hydrolysis of the substrate peptides. The dipeptide H-Gly-Pro-$NHCH_3$ is the only thrombin-induced hydrolysis product from either of the peptide substrates (F-6 or F-7) that can react with fluorescamine and was obtained as a precursor in the synthesis of the larger peptide substrates. For comparison with previous work from this laboratory, fluorescence values were also converted to concentrations of product by using infinite-time trypsin digests of each peptide as before (van Nispen et al., 1977; Meinwald et al., 1980).

The extent of reaction was always less than 20% 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 for peptide F-6 represent the averages of five

Table I: Amino Acid Sequences of Synthetic Oligopeptide Substrates

	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 [b]	Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-$NHCH_3$
F-5 [b]	Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$
F-6 [b]	Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$
F-7	Ac-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$
O [c]	H-Gly-Val-Arg-Gly-Pro-Arg-Leu-OH
P [c]	H-Gly-Val-Arg-Gly-Gly-Arg-Leu-OH
Q [c]	H-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 F-4, F-5, and F-6, also based on the sequence of human fibrinogen, were examined by Meinwald et al. (1980). [c] 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).

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]$^{-1}$]	reference
fibrinogen (bovine) Aα chain	9.2	73	793	Martinelli & Scheraga (1980)
CNBr Aα	47	48	100	Hageman & Scheraga (1974)
F-1	630	0.30	0.05	van Nispen et al. (1977)
F-2	1560	0.32	0.02	van Nispen et al. (1977)
F-3	680	11	1.6	van Nispen et al. (1977)
F-4	789	16	2.0	Meinwald et al. (1980)
F-5	633	20	3.2	Meinwald et al. (1980)
F-6	934	11	1.2	Meinwald et al. (1980)
F-6 [a]	1100	7.2	0.6	this work
F-6 [b]	3500	6.9	0.2	this work
F-7			<0.01	this work
O	3700	0.45	0.01	Liem & Scheraga (1974)
P	9600	0.22	0.002	Liem & Scheraga (1974)
Q	15300	0.09	0.001	Liem & Scheraga (1974)

[a] Redetermined by analyzing the data by the method of Meinwald et al. (1980). [b] Redetermined by analyzing the data as described in the text.

experiments employing five concentrations of peptide.

Results

The sequences of the relevant fibrinogen-like peptides are given in Table I. A typical Lineweaver–Burk plot for peptide F-6 is shown in Figure 1. The average values obtained from five such determinations are $K_M = 3.5 \times 10^{-3}$ M, $k_{cat} = 6.9 \times 10^{-11}$ M [(NIH unit/L) s]$^{-1}$, and $k_{cat}/K_M = 1.8 \times 10^{-8}$ [(NIH unit/L) s]$^{-1}$. The standard deviations among the five determinations were 50% in K_M, 87% in k_{cat}, and 53% in k_{cat}/K_M.

These values differ somewhat from those reported earlier (Meinwald et al., 1980) and shown in Table II because of an alteration in the procedure for analysis of the data. In this work, fluorescence is converted to concentration of product by using a calibration curve constructed from the reaction of the dipeptide H-Gly-Pro-$NHCH_3$ with fluorescamine rather than by using the fluorescamine fluorescence value from an infinite-time trypsin digest as before (Meinwald et al., 1980). In the new procedure, we have eliminated errors arising from possible incomplete hydrolysis of peptides by trypsin and from background fluorescence due to reactive amino groups of trypsin and its autolysis products. In addition, all fluorescence values were corrected for inner filter effects which were significant at the high peptide concentrations necessary for the determination of the Michaelis–Menten parameters for these relatively poor substrates. For comparison purposes, infinite-time trypsin digests were obtained and the data were analyzed as before (Meinwald et al., 1980), yielding values

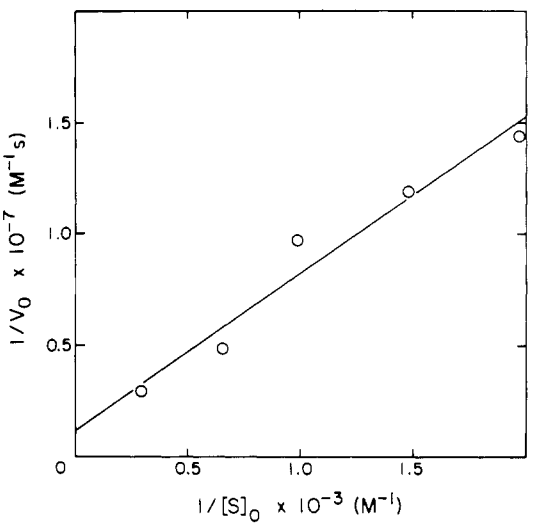

FIGURE 1: Example of a Lineweaver–Burk plot of the initial rates of hydrolysis of the Arg–Gly bond in peptide F-6. The thrombin concentration was 5.2 NIH units/mL.

in good agreement with that previous work (see Table II); i.e., the standard deviations of the redetermined values from those reported previously are 12% in K_M, 30% in k_{cat}, and 41% in k_{cat}/K_M, within the experimental error for these determinations. The use of the improved procedure for analyzing the data suggests that peptides F-1, F-2, F-3, F-4, F-5, and F-6 are slightly poorer substrates than previously reported. The

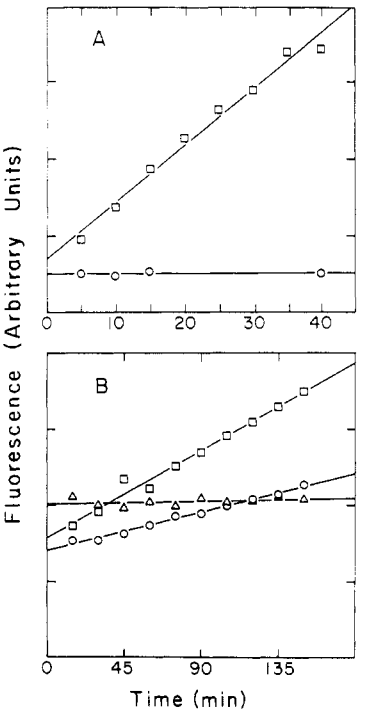

FIGURE 2: (A) Time dependence of fluorescence for peptide F-6 (□) at 0.50 mM and peptide F-7 (○) at 0.55 mM; the thrombin concentration was 9.4 NIH units/mL in both cases. (B) Time dependence of fluorescence for peptide F-7 at concentrations of 2.5 (○), 1.2 (□), and 0.0 mM (△); the thrombin concentration was 737 NIH units/mL in each case.

general trends in the data, however, remain the same as reported previously, and the earlier conclusions remain valid.

Figure 2A shows the time dependence of the fluorescence for peptides F-6 and F-7 at identical thrombin concentrations and similar peptide concentrations. The time course shown depicts the hydrolysis of about 20% of peptide F-6. No significant hydrolysis of peptide F-7 is detected under these conditions. From these data, it is possible to estimate an upper bound for the value of k_{cat}/K_M for peptide F-7. We assume that the maximum change in the observed fluorescence intensity for this peptide (Figure 2A) is 1% during 40 min; this corresponds to an upper bound of V_0 of 4.5×10^{-9} M s^{-1}. We further assume that the value of $[S]_0$ is much less than K_M; this leads to an upper bound of k_{cat}/K_M of 0.01×10^{-7} [(NIH unit/L) s]$^{-1}$. Thus, the Michaelis–Menten parameters for peptide F-7 are similar to those for peptides F-1, F-2, O, P, and Q, all of which lack Phe at position P_9.

In an attempt to increase the rate of hydrolysis of peptide F-7 to an observable level, higher concentrations of thrombin were used. Figure 2B shows the results obtained at an extremely high thrombin concentration (737 NIH units/mL) and at three substrate concentrations, 0, 1.2, and 2.5 mM. The control (0 mM) was identical in all respects to the other two samples except that it contained no peptide. Early time points of samples containing peptide actually gave lower fluorescence values than the control lacking peptide. Intermediate thrombin concentrations of 45, 84, 405, and 541 NIH units/mL all led to the same anomalous behavior that was observed at 737 NIH units/mL. This effect appears more pronounced at higher peptide concentrations (2.5 mM compared to 1.2 mM), suggesting that fluorescamine combined with the active site of thrombin and that the competing peptide inhibits this binding. Such an interaction of fluorescamine with thrombin is not surprising in light of the known affinities of thrombin for the large hydrophobic molecule proflavin (Koehler & Magnusson, 1974) as well as for the hydrophobic groups of a large number of thrombin inhibitors (Geratz & Tidwell, 1977). This interaction is not observed at the very low thrombin concentrations (Figure 2A) normally used in kinetic studies. However, the reaction time (at low thrombin concentration) cannot be extended very much (to hydrolyze peptide F-7) because significant autolysis of thrombin would probably occur under the reaction conditions. In any event, aside from the above estimate of an upper bound, the Michaelis–Menten parameters of peptide F-7 could not be determined by using fluorescamine to measure the extent of hydrolysis.

Discussion

It has been demonstrated that peptide F-7 is an extremely poor substrate for thrombin. Indeed, the peptide is such a poor substrate that Michaelis–Menten parameters are not readily obtainable by this method. This, together with the data for the other peptides in Tables I and II, constitutes direct evidence for the importance of Phe (P_9) in the interaction of thrombin and fibrinogen. The involvement of Phe at P_9 was postulated by van Nispen et al. (1977) to account for the marked differences in Michaelis–Menten parameters of peptides F-2 and F-3; i.e., the presence of Phe-Leu at positions P_9-P_8 in peptide F-3 results in an 80-fold increase in k_{cat}/K_M when compared to peptide F-2. The work presented here demonstrates that the presence of Leu alone at P_8 is insufficient to account for such an increase in k_{cat}/K_M.

While these results are not unexpected in light of earlier work, they do constitute unequivocal evidence for the profound effect of a single amino acid residue distant in the linear sequence from the normal site of enzyme action. Such results might be explained in several ways: (1) the presence of Phe at position P_9 could induce conformational features in the peptide which favor the normal enzyme–substrate interactions in the complex, (2) the site of interaction might span a relatively larger area than normally observed in enzyme–substrate interactions, or (3) the sequence of the peptide might accommodate a feature, such as a β bend, that allows the Phe at position P_9 to be brought into close spatial proximity to the Arg–Gly bond that is hydrolyzed.

The first of these possibilities, that Phe at position P_9 could induce native structure, can be eliminated on the basis of experiments of Nagy et al. (1982). They determined values of K_{conf} for fragment 11–25 of the Aα chain of fibrinogen using antibodies raised against native fibrinogen. The peptide studied was H-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-$NHCH_3$, essentially peptide F-3 of van Nispen et al. (1977), lacking only the N-terminal acetyl group (and the Arg near the C terminus). The value of K_{conf} determined for this peptide, reflecting the equilibrium constant between the "random" and native conformational forms, was $\sim 10^{-6}$. Thus, only $\sim 10^{-4}$% of this peptide adopts a native conformation in aqueous solution.

To assess the second possibility, it would be necessary to know the size of the active site and the conformation of residues in sites P_1–P_9. The size of the active site has been estimated by Berliner et al. (1981) from experiments with nitroxide spin-labels as 15–16 Å. A nine-residue peptide would have a length twice this value if fully extended but could be accommodated in a 15–16-Å site if it were completely α helical. Given its amino acid sequence, especially the three Gly residues at P_3–P_5, it is unlikely that this segment is α helical. Therefore, the second possibility can be eliminated.

Finally, the possible existence of a β bend, to bring Phe at P_9 into close spatial proximity to Arg at P_1 so that both are accommodated in a 15–16-Å active site, seems plausible. This

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.

Examination of Table II reveals that, while peptides F-3, F-4, F-5, and F-6 are substantially better substrates than those lacking Phe (P_9), they are poorer substrates of thrombin than even CNBr Aα, primarily because of differences in binding affinities reflected in the values of K_M. It is possible that these substrates contain all the residues that interact directly at the active site of thrombin yet lack residues which provide the long-range interactions necessary to stabilize the native conformation required for normal binding at the active site. This is the conclusion reached by Nagy et al. (1982) from immunochemical studies.

It has been proposed (Morris et al., 1981; Nagy et al., 1982) that a salt bridge 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. The absence of such long-range interactions would therefore explain the delayed release of fibrinopeptide A observed in fibrinogen Lille in which an Asn replaces Asp at P_{10} (Morris et al., 1981) and in fibrinogen Munich in which Asn replaces Arg at P_3' (Henschen et al., 1981). Kinetic and conformational studies of a synthetic peptide that includes Asp at P_{10} and Arg at P_3' are currently in progress to test this hypothesis.

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