Domingues, D. J. (1987) M.S. Thesis, University of California, Davis.
Giessner-Prettre, C., & Pullman, B. (1971) *J. Theor. Biol. 31*, 287–294.
Harbury, H. A., Cronin, J. R., Fanger, M. W., Hettinger, T. P., Murphy, A. J., Myer, Y. P., & Vinogradov, S. N. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1658–1664.
Hore, P. J. (1983) *J. Magn. Reson. 54*, 539–542.
Kassner, R. J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2263–2267.
Kassner, R. J. (1973) *J. Am. Chem. Soc. 95*, 2674–2677.
Kawano, K., Ozaki, Y., Kyogoku, Y., Ogoshi, H., Sugimoto, H., & Yoshida, Z.-I. (1977) *J. Chem. Soc., Chem. Commun.*, 226–227.
Leitch, F. A., Moore, G. A., & Pettigrew, G. W. (1984) *Biochemistry 23*, 1831–1838.
LeMaster, D. M., & Richards, F. M. (1985) *Biochemistry 24*, 7263–7268.
Leszczynski, J. F., & Rose, G. D. (1986) *Science (Washington, D.C.) 234*, 849–855.
Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70–80.
Mcintosh, L. P., Griffey, R. H., Muchmore, D. C., Nielson, C. P., Redfield, A. G., & Dahlquist, F. W. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1244–1248.
Ozaki, Y., Kyogoku, Y., Ogoshi, H., Sugimoto, H., & Yoshida, Z.-I. (1979) *J. Chem. Soc., Chem. Commun.* 76–77.
Pettigrew, G. W., Meyer, T. E., Bartsch, R. G., & Kamen, M. D. (1976) *Biochim. Biophys. Acta 430*, 197–208.
Pettigrew, G. W., Bartsch, R. G., Meyer, T. E., & Kamen, M. D. (1978) *Biochim. Biophys. Acta 503*, 509–523.
Redfield, A. G., Kunz, S. D., & Ralph, E. K. (1975) *J. Magn. Reson. 19*, 114–117.
Roberts, J. D., Yu, C., Flanagan, C., & Birdseye, T. R. (1982) *J. Am. Chem. Soc. 104*, 3945–3949.
Salemme, F. R., Freer, S. T., Xuong, Ng. H., Alden, R. A., & Kraut, J. (1973) *J. Biol. Chem. 248*, 3910–3921.
Schlauder, G. G., & Kassner, R. J. (1979) *J. Biol. Chem. 254*, 4110–4113.
Smith, G. M. (1979) *Biochemistry 18*, 1628–1634.
Smith, G. M., & Kamen, M. D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4303–4306.
Smith, G. M., Yu, L. P., & Domingues, D. J. (1987) *Biochemistry 26*, 2202–2207.
Takano, T., Trus, B. L., Mandel, N., Mandel, G., Kallai, O. B., Swanson, R., & Dickerson, R. E. (1977) *J. Biol. Chem. 252*, 776–785.
Valentine, J. S., Sheridan, R. P., Allen, L. C., & Kahn, P. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1009–1013.
Weaver, P. F. (1974) Ph.D. Thesis, University of California, San Diego.
Witanowski, M., Stefaniak, L., & Webb, G. A. (1981) *Annu. Rep. NMR Spectrosc. 11B*, 1–502.

Localization of the Binding Site on Fibrin for the Secondary Binding Site of Thrombin†

Zsofia Vali and Harold A. Scheraga*
Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853-1301
Received July 2, 1987; Revised Manuscript Received August 25, 1987

ABSTRACT: Affinity chromatography of active site inhibited thrombin on immobilized fragments derived from the central (desAB-NDSK) and terminal (D_1) globular domains of fibrinogen revealed that the site responsible for the binding of thrombin at its secondary fibrin binding site is located in the central domain. Chromatography of various domains of the central nodule (desAB-NDSK, fibrinogen E, and fibrin E) having nonidentical amino acid sequences showed that all of these fragments are capable of binding to PMSF-thrombin–Sepharose, suggesting that the thrombin binding site resides within the peptide regions common to all of these fragments: $\alpha(Gly_{17}–Met_{51})$, $\beta(Val_{55}–Met_{118})$, and $\gamma(Tyr_1–Lys_{53})$. Competitive affinity chromatography of the same binding domains revealed that there is no detectable difference in their binding constants to PMSF-thrombin–Sepharose, indicating that the $\alpha(Lys_{52}–Lys_{78})$, $\beta(Gly_{15}–Lys_{54})/(Tyr_{119}–Lys_{122})$, and $\gamma(Thr_{54}–Met_{78})$ peptide segments do not contribute significantly to the binding of thrombin. Chromatography of the isolated chains of fibrinogen E showed that the $\alpha(Gly_{17}–Lys_{78})$ peptide region itself contains a strong binding site for PMSF-thrombin–Sepharose. The location of the binding site suggests that the secondary site interaction may play an important role in determining the cleavage specificity of thrombin on fibrinogen and can affect the rate of release of the fibrinopeptides. Affinity chromatography of fragments prepared from polymerized fibrin showed that cross-linked DD (DxD) itself does not bind to thrombin, whereas the DxDE complex remained attached to the column, suggesting that the binding site on fragment E for thrombin is distinct from its binding site for DxD. Thus, the interaction between DxD and E during polymerization is not likely to release thrombin from fibrin. The possible consequences of the fibrin–thrombin interaction on the mechanism of fibrin polymerization and factor XIII activation are discussed.

Thrombin performs a regulatory function in thrombosis and hemostasis. In addition to its proteolytic action on fibrinogen, thrombin also activates factor V, factor VIII, factor XIII, and, in the presence of thrombomodulin, protein C. In each of these cases, activation involves limited proteolysis of the polypeptide chain by thrombin.

The major event in a series of controlled reactions is the activation of fibrinogen to fibrin monomers, which then polymerize spontaneously to form the fibrin network. During the conversion of fibrinogen to fibrin, thrombin is removed

† This work was supported by a research grant (HL-30616) from the National Heart, Lung, and Blood Institute of the National Institutes of Health.

0006-2960/88/0427-1956$01.50/0

from the solution (Eagle, 1935; Seegers et al., 1945; Liu et al., 1979a, 1980; Wilner et al., 1981). Many different aspects of the interaction between α-thrombin and *fibrin* have been characterized. Liu et al. (1979a) studied the diffusion of ^{125}I-thrombin into fibrin clots and found two classes of binding sites with association constants K_a of 5.8×10^5 M^{-1} and 6.8×10^4 M^{-1}, respectively; from an analysis of the binding of thrombin to fibrin monomer–Sepharose, Kaminski and McDonagh (1983) found a similar value of K_a of 4.9×10^5 M^{-1} but observed only one class of sites. Plasmin dissolution of fibrin clots leads to the recovery of active thrombin (Bloom, 1962; Liu et al., 1979a; Francis et al., 1983). It has been shown that the catalytic site of thrombin is not involved in the binding to fibrin (Liu et al., 1979a; Fenton et al., 1981; Wilner et al., 1981; Berliner et al., 1985; Kaminski & McDonagh, 1983, 1987). The mechanism by which bound thrombin is released from fibrin is not known. Kaminski and McDonagh (1983) suggested that thrombin is released during the polymerization process, whereas other workers found evidence that thrombin binds to the fibrin clot and is released during plasmic dissolution of the clot (Bloom, 1962; Liu et al., 1979a; Francis et al., 1983). The contradictory results reported for the number of binding sites present on fibrin, and the effect of Ca^{2+} ions on the fibrin–thrombin interaction, are probably due to the different experimental conditions and assay systems used by the investigators. However, all studies lead to the conclusions that α-thrombin binds to fibrin at a secondary site distinct from its catalytic center, that the interaction is ionic in nature, and that it is abolished at increased ionic strength.

The thrombin–fibrin interaction has many important physiological consequences. It has been noted that, by binding active thrombin, fibrin acts as an anti-thrombin, thereby limiting thrombosis (Liu et al., 1979a). Lack of such binding constitutes a physiological disorder. About 20 cases of dysfibrinogenaemia associated with arterial or venous thrombosis have been reported (Henschen et al., 1983a,b). In some of these cases, the structural defect in the fibrinogen molecule has been characterized. In the cases of fibrinogen New York I (Liu et al., 1979b) and fibrinogen Milano II (Haverkate et al., 1986), the underlying cause of thrombosis was associated with defective binding of thrombin to the fibrin formed from the abnormal fibrinogen. Although binding of thrombin to fibrin may serve as a trap for active thrombin during clot formation, interaction of a secondary site with the substrate can affect the specificity and kinetic properties of the enzyme considerably.

Thrombin is a serine protease that is homologous to trypsin and chymotrypsin. Its proteolytic action is strictly restricted to a very limited number of peptide bonds in its protein substrates. In fibrinogen, thrombin specifically cleaves FpA[1] and FpB from the amino terminus of the Aα and Bβ chains. Restriction of the action of thrombin to these sites has been thought to be due to a unique active site geometry on thrombin and to a specific favorable conformation of the Aα and Bβ chains for binding to this active site. In this respect, immunochemical studies showed that long-range interactions play an important role in defining the conformation of the N-terminal part of the Aα and Bβ chains (Nagy et al., 1982, 1985). Kinetic and NMR studies on synthetic peptides suggested that catalysis might be facilitated by a β-bend conformation in the region of the Aα chain near the hydrolyzable Arg–Gly bond (Marsh et al., 1982, 1983, 1985).

An additional mechanism that might contribute to the specific cleavages of the fibrinopeptides and account for the increased K_M for the binding of the synthetic peptides as compared to the native chains is the interaction of thrombin with fibrinogen at this secondary binding site. There have been no reports as to which region(s) of the fibrin(ogen) molecule is (are) responsible for the binding of thrombin at its secondary binding site. The location of this site may indicate whether such an interaction could play a role in determining the specificity of thrombin for fibrinogen or whether it serves only as a trap for active thrombin during clot formation.

In the present work, we localized the thrombin binding site to the central domain of fibrin(ogen). We obtained different fragments of this domain by proteolysis and compared their affinities to Sepharose-bound PMSF-thrombin in order to establish the structural requirements for thrombin binding. We also showed that the $\alpha(Gly_{17}–Lys_{78})$ segment isolated from fibrinogen E contains a strong thrombin binding site. Our results suggest that, in addition to binding active thrombin during clot formation, the interaction between fibrin(ogen) and thrombin might be an important determinant in thrombin specificity and in the rate of release of the fibrinopeptides. It may also affect the mechanism of fibrin polymerization and factor XIII activation.

Experimental Procedures

Preparation of Bovine Thrombin and PMSF-Inactivated Thrombin. Prothrombin from barium citrate eluate from bovine plasma (Sigma) was activated with *Echis carinatus* venom (Sigma) as described by Ghosh and Seegers (1980). The activation mixture was then gel filtered into 0.025 M sodium phosphate–0.1% PEG, pH 6.5, buffer. Purification of thrombin was carried out by FPLC (Spectra Physics Model 8700) on a Mono S HR 10/10 (Pharmacia) cation-exchange column equilibrated with the above buffer. The separation was carried out with a linear gradient of 0–0.5 M NaCl in 60 min at a flow rate of 2 mL/min. The pool containing bovine thrombin was shown to be homogeneous by SDS–PAGE. Specific activity of the preparations was determined by measuring the rate of release of FpA from fibrinogen with RP-HPLC detection (Martinelli & Scheraga, 1979). Specific activity of the preparations was between 2800 and 3100 NIH units. The concentration of the thrombin solutions was determined by its absorbance with the value of $E^{1\%}_{280} = 19.5$ (Winzor & Scheraga, 1964).

The thrombin preparation was inactivated with PMSF (final concentration 0.2 mM). PMSF-thrombin proved to be inactive in two different assays: release of FpA and clotting time of fibrinogen.

Preparation of Fibrinogen Fragments. Plasmic Digestion of Fibrinogen. Human fibrinogen (grade L, Kabi) was digested in 0.05 M Tris-HCl/0.15 M NaCl, pH 7.4, buffer at a final concentration of 15 mg/mL and dialyzed exhaustively against the same buffer. The concentration of the solution was adjusted to 10 mg/mL, and $CaCl_2$ was added to a final concentration of 5 mM. The fibrinogen solution was digested with 0.1 mg/mL plasminogen (a gift from Laszlo Patthy, Institute of Enzymology, Hungarian Academy of Sciences) and 100 CTA units/mL urokinase (Abbokinase, Abbot) at

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; PEG, poly(ethylene glycol) 6000; PMSF, phenylmethanesulfonyl fluoride; PMSF-thrombin, thrombin inactivated by PMSF; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; FpA and FpB, fibrinopeptides A and B, respectively; NDSK, amino-terminal cyanogen bromide fragment of fibrinogen; desAB-NDSK, amino-terminal cyanogen bromide fragment of fibrinogen lacking both fibrinopeptides; CTA, Committee on Thrombolytic Agents; KIU, kallikrein inhibitor unit(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography; FPLC, fast protein liquid chromatography.

37 °C for 180 min. Proteolysis was arrested by the addition of 200 KIU of Trasylol (Bayer), and the solution was dialyzed against 0.02 M Tris-HCl/0.05 M NaCl, pH 7.4, buffer.

Preparation and Characterization of Fragments D_1 and E. Fragment D_1 was separated from small amounts of contaminating D_2 and D_3 and from fibrinogen E by FPLC on a Mono Q HR 10/10 (Pharmacia) anion-exchange column equilibrated with 0.02 M Tris-HCl/0.05 M NaCl, pH 7.4, buffer with a linear gradient of 0.05–0.5 M NaCl in 60 min at a flow rate of 2 mL/min. Fractions collected from the mono Q column were analyzed by SDS–7% PAGE under both reducing and nonreducing conditions. The sample containing fragment D_1 (α M_r 12000; β M_r 42000; γ M_r 39000) was further analyzed to prove the intactness of the C-terminal part of the γ-chain, which is susceptible to plasmic hydrolysis. An aliquot of the protein (5 mg/mL) was further digested with 0.02 mg/mL plasminogen and 50 CTA units/mL urokinase in the presence of 10 mM EDTA at 37 °C for 60 min. Digestion was terminated by keeping the sample at 100 °C for 5 min. The peptides remaining in solution were separated by RP-HPLC (Spectra Physics 8000 instrument equipped with a Model 770 UV–visible variable-wavelength detector) on a Radial-PAK C_{18} (Waters) cartridge. An eluent system of 0.09% TFA/water (A) and 0.09% TFA/acetonitrile (B) was used, and a linear gradient of 2–60% B in 60 min was applied. The separated peptides were identified quantitatively by amino acid analysis. The practically identical molar recovery of the three C-terminal peptides, γ(303–356), γ(357–373), and γ(374–406), proved that the D_1 preparation contained intact γ-chains.

The protein pool containing fibrinogen E was characterized by its chain composition. An aliquot of the fragment was reduced and sulfonated according to the method of Thannhauser and Scheraga (1985). S-Sulfonated chains were separated by RP-HPLC in an eluent system of 0.09% TFA/water (A) and 0.09% TFA/acetonitrile (B) with a linear gradient of 2–60% B in 60 min on a Radial-PAK C_{18} (Waters) cartridge. The peptides were identified by amino acid analysis. Thus, the fibrinogen E preparation can be characterized by the following chain composition: α(17–78), β(55–122), γ(1–53)/(1–58).

Preparation of DesAB-NDSK. NDSK was prepared by CNBr cleavage of fibrinogen according to the method of Blomback et al. (1968) as modified by Olexa and Budzynski (1979b). FpA and FpB were cleaved off by treatment with thrombin (10 NIH units/mL, 60 min). Complete removal of the fibrinopeptides was detected by following the course of peptide release by RP-HPLC. Active thrombin was inactivated with PMSF (0.2 mM final concentration), and desAB-NDSK was separated from inactivated thrombin and from the fibrinopeptides by gel filtration on a Superose 12 (Pharmacia) FPLC medium in 0.05 M Tris-HCl/1 M NaCl, pH 7.4, buffer. The protein was dialyzed against 0.05 M NH_4HCO_3 buffer and lyophilized.

Preparation of Plasmic Fragments of Cross-Linked Fibrin. Fibrinogen used for these preparations was kindly supplied by Laszlo Patthy (Institute of Enzymology, Hungarian Academy of Sciences) and was obtained from plasminogen-depleted human plasma by the procedure of Chen and Mosesson (1977). This preparation contained a sufficient amount of factor XIII to form cross-linked fibrin when treated with thrombin. This fibrinogen preparation was clotted and freeze-dried as described by Olexa and Budzynski (1979a).

Preparation of DxDE Complex. Homogeneous DxDE complex was obtained by following a procedure described by Olexa and Budzynski (1979a). Samples were analyzed by SDS–PAGE and Tris–glycine gel electrophoresis, and the pool containing the DxDE complex was dialyzed against 0.05 M Tris-HCl/0.05 M NaCl, pH 7.4, buffer and kept frozen at −70 °C.

Preparation of Cross-Linked DD and Fibrin E from DxDE Complex. The complex was dissociated by incubation at 37 °C for 120 min in 0.05 M Tris-HCl/6 M urea, pH 7.4, buffer. DxD was separated from fibrin E by FPLC on a Mono Q HR 10/10 anion-exchange column equilibrated with the above buffer and developed with a linear salt gradient from 0 to 0.5 M NaCl in 60 min at a flow rate of 2 mL/min. Samples were analyzed by SDS–PAGE, and the pooled fragments were dialyzed against 0.05 M NH_4HCO_3 and lyophilized.

Cross-linked DD was also prepared from a late digest of cross-linked fibrin (Olexa & Budzynski, 1979a) and was isolated as described above.

Detection of Complex Formation between Cross-Linked DD and Fibrin E. The ability of our DxD and E preparations to form a strong noncovalent complex was demonstrated by the procedure described by Olexa and Budzynski (1979a) using the nondissociating Tris–glycine gel electrophoresis system (Davis, 1964).

Isolation of the Polypeptide Chains of Fibrinogen E. Fibrinogen E (5 mg/mL) was reduced and sulfonated (Thannhauser & Scheraga, 1985). The peptides were separated by RP-HPLC as described earlier under the characterization of fibrinogen E. All peptides were identified by amino acid analysis.

Preparation of Immobilized Proteins. Fragment D_1, desAB-NDSK, PMSF-thrombin, and bovine serum albumin were each coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the instructions of the manufacturer. Fibrin monomer–Sepharose was prepared by the method of Heene and Matthias (1973).

Affinity Chromatography. Interactions between PMSF-thrombin and fibrin(ogen) fragments were studied by affinity chromatography. In these experiments, the amount of protein applied onto the column did not exceed 50% of the column capacity for that particular fragment. Chromatography was performed in 0.05 M Tris-HCl/0.05 M NaCl–0.1% PEG, pH 7.4, buffer, and the bound protein was eluted with the same buffer containing 0.3 M NaCl. (Preliminary studies showed that the ionic strength of this buffer was adequate to eliminate interactions between the proteins studied.) Washing the columns with 1 M NaCl, 6 M urea, or 6 M guanidine hydrochloride did not result in the elution of additional protein material. Concentrations of the proteins in the unbound and bound fractions were determined by their absorbance at 280 nm, with the following values for $E^{1\%}_{280}$: 20.8 for fragment D and DxD (Marder et al., 1969); 12.0 for fragment E (Davis, 1964); 12.0 for NDSK (Marder et al., 1972). Recovery of all proteins from the affinity columns was between 85 and 95% for both bovine α-thrombin and the fibrinogen fragments.

Competitive Affinity Chromatography. The relative binding constants of fragments derived from the central domain of fibrin(ogen) were determined by competitive affinity chromatography on PMSF-thrombin–Sepharose. In this method, all sites on the immobile phase are saturated by applying a high concentration of an equimolar mixture of the two ligand components being compared. After removal of excess solution from the column, the bound protein is eluted. The ratio of the concentrations of the two eluted ligands is equal to the ratio of their binding constants.

In our experiments, the PMSF-thrombin–Sepharose column was overloaded with an equimolar mixture of fibrinogen E and

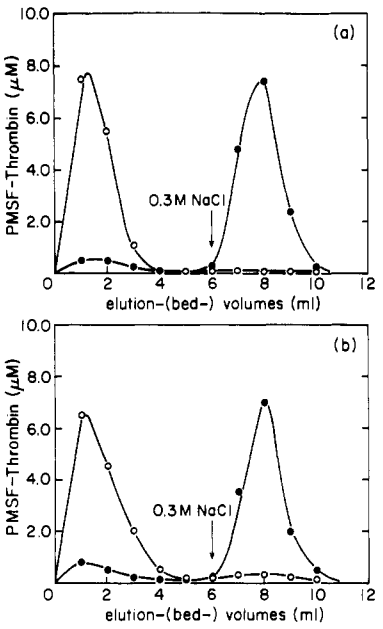

FIGURE 1: Elution profiles of PMSF-thrombin from fibrin–, desAB-NDSK–, D_1–, and BSA–Sepharose. Columns (1-mL bed volumes) were equilibrated with 0.05 M Tris-HCl/0.05 M NaCl, pH 7.4, buffer. After the sample was passed through the column, it was washed with 6 bed volumes of buffer, and the bound protein was eluted with buffer containing 0.3 M NaCl. (a) Binding of PMSF-thrombin to BSA–Sepharose (○) and to fibrin monomer–Sepharose (●). (b) Binding of PMSF-thrombin to desAB-NDSK–Sepharose (●) and to D_1–Sepharose (○).

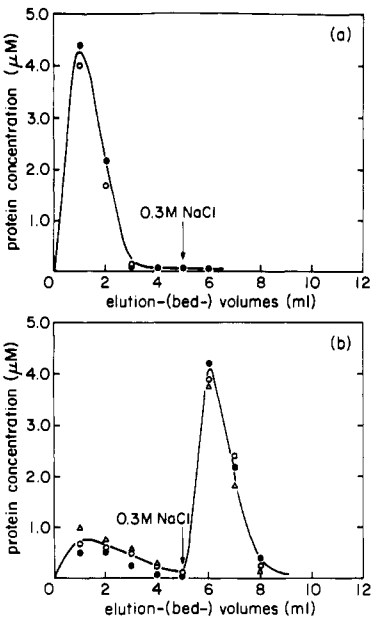

FIGURE 2: Elution profiles of fibrin(ogen) fragments from PMSF-thrombin–Sepharose. The columns (1-mL bed volumes) were equilibrated with 0.05 M Tris-HCl/0.05 M NaCl, pH 7.4, buffer. After application of the samples (7 μmol of protein), the columns were washed free of unbound protein with 5 bed volumes of buffer and the bound fractions were eluted with the same buffer containing 0.3 M NaCl. (a) Binding of bovine serum albumin (●) and fragment D_1 (○). (b) Binding of fragments fibrinogen E (○), fibrin E (●), and desAB-NDSK (△).

fibrin E, or fibrinogen E and desAB-NDSK. The column was washed free of unbound proteins, and the bound fraction was eluted with buffer containing 0.3 M NaCl. The compositions of the applied sample, the effluent, and the bound fraction were determined by SDS–PAGE.

Gel Electrophoresis. Protein samples were analyzed by SDS–PAGE on 7% and 10% gels under reducing or nonreducing conditions (Laemmli, 1970). The DxDE complex was detected in a nondissociating medium with 9% Tris–glycine–PAGE gels prepared by the method of Davis (1964).

Amino Acid Analysis. Amino acid analysis was performed on a Technicon TSM autoanalyzer. The samples were hydrolyzed according to the method of Swadesh et al. (1984).

RESULTS

Assignment of the Thrombin Binding Site to the N-Terminal Domain of Fibrin. It is known that active site inhibited thrombin binds to fibrin monomer–Sepharose and is eluted at increased ionic strength (Berliner et al., 1985). We used this property of the thrombin–fibrin interaction to specify the conditions for the affinity chromatographic experiments (see Experimental Procedures). We showed that PMSF-thrombin binds to fibrin monomer–Sepharose and can be eluted with the same buffer at higher ionic strength (Figure 1). Further washing of the column with 1 M NaCl, 6 M urea, and 6 M guanidine hydrochloride did not release additional protein material. The amount of protein bound to the column under these conditions was independent of the extent of washing. In contrast to its binding to fibrin monomer–Sepharose, PMSF-thrombin did not bind to bovine serum albumin immobilized on Sepharose or to the CNBr-activated Sepharose matrix that had been treated by the same procedure used for protein immobilization.

To determine which region of the fibrin monomer molecule is responsible for the binding of thrombin, we attached the two nonoverlapping fragments (NDSK and D_1) of fibrin to the Sepharose matrix (the D_1 domain represents the C-terminal and NDSK the N-terminal globular domains of the molecule). To rule out a possible interaction between active site inhibited thrombin and the sites that are cleaved by active thrombin at the N-terminus of the Aα and Bβ chains, both fibrinopeptides (FpA and FpB) were removed from NDSK prior to immobilization. As shown in Figure 1, PMSF-thrombin binds to desAB-NDSK-Sepharose whereas no binding to immobilized D_1 could be detected.

Thus, we can conclude that the structures involved in the binding of active site inhibited thrombin reside in the N-terminal domain of fibrin.

Comparison of the Affinity of the Various N-Terminal Domains of Fibrin(ogen) to PMSF-Thrombin–Sepharose. Two subspecies of fragment E can be derived from the central nodule of fibrinogen by limited plasmic digestion, fibrinogen E from fibrinogen and fibrin E from cross-linked fibrin. Fibrin E shows affinity for fragment DD whereas fibrinogen E prepared from a terminal digest of fibrinogen has lost the peptide regions essential for interaction with DD (Olexa & Budzynski, 1979a,b). We found that both purified fibrinogen E and fibrin E bind to PMSF-thrombin immobilized on the Sepharose matrix (Figure 2). As expected from the results presented in Figure 1, desAB-NDSK also bound to the column, whereas D_1 did not show any interaction with immobilized thrombin and passed through the column unretarded, similar to the control protein (bovine serum albumin) (Figure 2). Table I summarizes the amino acid sequences of fibrinogen E, fibrin E, and desAB-NDSK. The $\alpha(Gly_{17}–Met_{51})$, $\beta(Val_{55}–Met_{118})$, and $\gamma(Tyr_1–Lys_{53})$ peptide chain segments are present in all three fragments. This leads to the conclusion that the regions essential for the binding of PMSF-thrombin lie within these boundaries.

If the additional peptide segments that distinguish between these fragments are involved in thrombin binding, these fragments would bind to PMSF-thrombin–Sepharose with

Table I: Comparison of the Peptide Segments of Thrombin–Sepharose-Bound Fragments Derived from the Central Domain of Fibrin(ogen)

fragment	α-chain	β-chain	γ-chain
desAB-NDSK[a]	Gly_{17}–Met_{51}	Gly_{15}–Met_{118}	Tyr_1–Met_{78}
fibrinogen E[b,c]	Gly_{17}–Lys_{78}	Val_{55}–Lys_{122}	Tyr_1–Lys_{53} Tyr_1–Lys_{58}
fibrin E[d]	Gly_{17}–Lys_{78}	Gly_{15}–Lys_{122}	Tyr_1–Lys_{62}
sequence common to all fragments	Gly_{17}–Met_{51}	Val_{55}–Met_{118}	Tyr_1–Lys_{53}
sequence differences between the fragments	Lys_{52}–Lys_{78}	Gly_{15}–Lys_{54} Tyr_{119}–Lys_{122}	Thr_{54}–Met_{78}

[a] Kowalska-Loth et al. (1973). [b] Takagi and Doolittle (1975a,b). [c] Sequence confirmed in the present work. [d] Olexa et al. (1981).

different affinities. Therefore, we compared the relative binding constants of the various fragments derived from the central nodule of fibrinogen to PMSF-thrombin–Sepharose by competitive affinity chromatography. PMSF-thrombin–Sepharose was overloaded with a 1:1 mixture of fibrinogen E and fibrin E. The column was washed free of unbound protein, the bound fraction was eluted, and its composition was determined by gel electrophoresis (see Experimental Procedures). Comparison of the protein composition of the applied sample with that of the bound fraction showed no shift in the relative amount of the two fragments. The same experiment carried out with a 1:1 mixture of fibrinogen E and desAB-NDSK revealed no detectable difference in their binding to the resin (data not shown). This indicates that there is no significant difference in their binding constants to PMSF-thrombin–Sepharose. These data suggest that the α(Lys_{52}–Lys_{78}), β(Gly_{15}–Lys_{54})/(Tyr_{119}–Lys_{122}), and γ(Thr_{54}–Met_{78}) peptide segments do not contribute significantly to the binding of the central domain to thrombin (Table I).

Binding of the Isolated Chains of Fibrinogen E to PMSF-Thrombin–Sepharose. We have addressed the question as to whether the individual chains of the N-terminal domain themselves can bind to PMSF-thrombin–Sepharose. Reduced, S-sulfonated chains of fibrinogen E were chromatographed on the thrombin–Sepharose column, and the peptides present in the unbound and bound fractions were separated by RP-HPLC (Experimental Procedures). Three peptides were isolated from the unbound fraction, and one peptide was isolated from the bound fraction. Identification of these peptides on the basis of amino acid analysis is presented in Table II. The α(Gly_{17}–Lys_{78}) peptide chain was recovered from the bound fraction, showing that this chain itself can bind to thrombin–Sepharose, whereas neither the β(Val_{55}–Lys_{122}) nor the γ-(Tyr_1–Lys_{53}) or the γ(Tyr_1–Lys_{58}) peptide bound to PMSF-thrombin–Sepharose under the given conditions.

Binding of Fragments Derived from Polymerized Cross-Linked Fibrin to PMSF-Thrombin–Sepharose. It has been suggested that thrombin bound to fibrin monomer is released during the polymerization process (Kaminski & McDonagh, 1983). The major structural event of polymerization is the interaction of fibrin E with properly aligned D domains, leading to the formation of the strong noncovalent complex DDE. Subsequently, the D domains are cross-linked by factor XIII to form the DxDE complex.

We tested the hypothesis that the binding of cross-linked DD (DxD) to fibrin E would abolish the interaction between thrombin and fibrin E. DxD and fibrin E fragments were prepared from the DxDE complex obtained from cross-linked fibrin. Chromatography of these fragments on PMSF-thrombin–Sepharose showed that, although fibrin E was bound to the column, the DxD fragment passed through the matrix unretarded (Table III). Then we prepared the DxDE complex

Table II: Amino Acid Composition of Peptides Isolated from the Thrombin–Sepharose-Unbound and -Bound Samples by RP-HPLC[a]

	peptides recovered from the unbound fraction			recovered from the bound fraction
	P-1, β(55–122)	P-3, γ(1–53)	P-4, γ(1–58)	P-2, α(17–78)
Asx[b]	8.2 (8)[d]	8.3 (8)	8.2 (8)	11.9 (11)
Glx[c]	11.3 (11)	6.4 (6)	7.5 (7)	7.7 (7)
Ser	6.0 (6)	3.5 (3)	4.0 (4)	5.1 (5)
Gly	4.0 (4)	2.3 (2)	2.1 (2)	2.8 (3)
His	1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)
Arg	3.4 (3)	2.5 (2)	2.3 (2)	4.2 (4)
Thr	2.1 (2)	4.2 (5)	5.2 (6)	1.1 (1)
Ala	4.9 (5)	2.1 (2)	2.1 (2)	1.4 (1)
Pro	4.0 (4)	1.2 (1)	1.1 (1)	2.8 (3)
Tyr	2.5 (2)	2.3 (3)	2.8 (3)	2.5 (2)
Val	4.7 (5)	2.8 (3)	3.5 (4)	2.4 (3)
Met	1.0 (1)	0.2 (0)	0.2 (0)	0.9 (1)
Cys	3.5 (3)	3.1 (4)	3.2 (4)	4.5 (4)
Ile	0.9 (1)	2.6 (3)	3.3 (3)	1.7 (2)
Leu	8.5 (9)	4.5 (5)	5.3 (5)	3.0 (3)
Phe	1.3 (1)	2.0 (2)	1.9 (2)	2.8 (3)
Lys	1.9 (2)	3.4 (3)	4.1 (4)	6.1 (6)
Trp	nd[e] (0)	nd (0)	nd (0)	nd (2)

[a] The composition is expressed as number of moles per mole of peptide. [b] Asp + Asn. [c] Glu + Gln. [d] Theoretical values (Henschen et al., 1983) are given in parentheses. [e] nd, not determined.

Table III: Binding of Fibrin(ogen) Fragments to Thrombin–Sepharose

fragment	detected in unbound fraction	detected in bound fraction
desAB-NDSK	–	+
fibrinogen E	–	+
fibrin E	–	+
D_1	+	–
DxD	+	–
DxDE	–	+

from these same components by incubating an equimolar mixture of DxD and fibrin E at room temperature for 60 min. Formation of the complex was followed by analyzing aliquots of the sample in the Tris–glycine–polyacrylamide gel electrophoretic system (Experimental Procedures). The DxDE complex formed was chromatographed on PMSF-thrombin–Sepharose under the same conditions as the separated and purified DxD and fibrin E. The DxDE complex was recovered from the bound fraction (Table III).

These results indicate that the interaction of fibrin E with PMSF-thrombin–Sepharose can lead to the binding of DxD to immobilized thrombin. This suggests that fibrin E has a separate binding site for DxD and thrombin.

Discussion

Specificity of Affinity Chromatography To Detect Interactions between Fibrin(ogen) Fragments and Thrombin. In the present work, we used affinity chromatography to detect interactions between active site inhibited thrombin and fibrin(ogen) fragments. We showed that PMSF-thrombin binds to fibrin monomer–Sepharose and is eluted at increased ionic strength. In control experiments, we found that the same thrombin preparation did not show any binding to CNBr-activated Sepharose or immobilized bovine serum albumin. Chromatography of PMSF-thrombin on fragments of fibrin(ogen) was carried out under the same conditions (Figure 1). We used the same conditions in the reverse experiments, when PMSF-thrombin was immobilized on the Sepharose matrix. Bovine serum albumin did not bind to this matrix (Figure 2).

Although we cannot exclude the possibility that the proteins undergo conformational changes during immobilization, the characteristic sensitivity of the interactions to high ionic strength that had been reported for both the in vivo and in vitro systems (Liu et al., 1979a; Berliner et al., 1985) and the compatible results obtained for the different types of immobilized ligands suggest that a specific ionic interaction between the secondary binding site of thrombin and fibrin fragments is operative in all cases (compare Figures 1 and 2).

Assignment of the Thrombin Binding Site to the Central Domain of Fibrin. We studied the binding of active site inhibited thrombin (PMSF-thrombin) to the largest fragment derived from the terminal globular domain of fibrinogen, D_1, and to desAB-NDSK, which corresponds to the central nodule of the molecule. We found that PMSF-thrombin bound stoichiometrically to immobilized desAB-NDSK whereas no interaction with D_1-Sepharose was detected. Thus, the site that is responsible for binding to the secondary binding site in active site inhibited thrombin is located in the N-terminal (or central) domain of fibrinogen.

We used competitive affinity chromatography to detect differences in binding strength between the three bound species (desAB-NDSK, fibrinogen E, and fibrin E). This method is very sensitive to small differences in the relative binding constants of the bound proteins (Varadi & Patthy, 1983). We did not find any difference in binding affinity of fibrin E, fibrinogen E, or desAB-NDSK to thrombin–Sepharose. Thus, we conclude that the $\alpha(Lys_{52}–Lys_{78})$, $\beta(Gly_{15}–Lys_{54})/(Tyr_{119}–Lys_{122})$, and $\gamma(Thr_{54}–Met_{78})$ peptide segments do not alter the secondary thrombin binding site on the central domain of fibrinogen.

On the basis of their finding that the thrombin–fibrin interaction is sensitive to the presence of Ca^{2+} ions and that thrombin is released at some stage of fibrin polymerization, Kaminski and McDonagh (1983) speculated that it might be the D domain of fibrinogen that carries an extended thrombin binding site, since this domain contains both polymerization sites (Olexa & Budzynski, 1981; Varadi & Scheraga, 1986) and Ca^{2+} binding sites (Nieuwenhuizen et al., 1979; Varadi & Scheraga, 1986). However, at present it is not known when thrombin is released from fibrin (see later section). Since the central domain is also involved in polymerization (Olexa & Budzynski, 1981) and contains a Ca^{2+} binding site (Nieuwenhuizen et al., 1983), our finding that the thrombin binding site is on the central domain does not contradict their experimental results.

Affinity of the Isolated Chains of the Central Domain for PMSF-Thrombin–Sepharose. Affinity chromatography of reduced and S-sulfonated chains of fibrinogen E showed that the isolated α-chain itself is capable of binding to PMSF-thrombin–Sepharose (Table II). Since weak binding would go undetected during affinity chromatography, our results do not exclude a possible interaction between the PMSF-thrombin–Sepharose and the β- and/or the γ-chains nor rule out the contribution of these chains to thrombin binding but show that the $\alpha(Gly_{17}–Lys_{78})$ peptide segment itself carries a strong thrombin binding site.

The importance of the $\alpha(Gly_{17}–Lys_{78})$ peptide sequence in binding thrombin is in accordance with earlier results reported from this and other laboratories on the kinetic constants for the hydrolysis of the thrombin-sensitive Arg–Gly bond in the Aα chain. Active site mapping experiments suggested that the Aα chain derived from NDSK is as good a substrate for thrombin as the intact Aα chain of fibrinogen (Hagemann & Scheraga, 1974). Martinelli and Scheraga (1980) reached the same conclusion after determining K_M and k_{cat} for the cleavage of FpA from intact fibrinogen. They pointed out that NDSK Aα probably contains all the amino acids required for interaction with thrombin and that the small difference in the specificity constant (k_{cat}/K_M) between the two structures might be caused by slight perturbation of the three-dimensional structure in the NDSK Aα chain due to the harsh conditions used during CNBr cleavage.

On the other hand, numerous studies on the kinetic constants of the thrombin-catalyzed hydrolysis of synthetic peptides with sequences identical with that of Aα around the cleaved Arg–Gly bond revealed that K_M for the hydrolysis of these peptides is considerably higher than that measured for fibrinogen Aα or NDSK Aα [Marsh et al. (1983) and references cited therein].

Two different mechanisms can account for the higher K_M measured for the small synthetic peptides. One is that a favorable conformation of the Aα chain is needed for the interaction with the active site of thrombin. Immunochemical studies indicated that long-range interactions in fibrinogen stabilize the domain around the scissile bond in a conformation that is different from what is observed for the synthetic peptides (Nagy et al., 1982). NMR studies suggested that catalysis may be facilitated by a β-bend conformation of the N-terminus of the Aα chain that places Phe_8 in close proximity to the cleaved $Arg_{16}–Gly_{17}$ bond (Marsh et al., 1985; Ni et al., 1988). This β-bend has been proposed to be stabilized by a salt bridge between Asp_7 and Arg_{19} to account for the smaller K_M measured for the peptide containing Asp_7 as the HN_2-terminal residue (Marsh et al., 1983).

A second mechanism that could account for the larger K_M measured for the synthetic peptide substrates is that the fibrinogen Aα chain may contain an additional binding site that interacts with thrombin at a secondary site that is distinct from its active center whereas this region is missing from the synthetic peptides. Blomback et al. (1976) reported that the peptides $A\alpha(Ala_1–Arg_{23})$ and $(Ala_1–Trp_{33})_2$ are cleaved at a rate of only about 6% of the rate measured for NDSK Aα. Moreover, the hydrolysis rate for these peptides was found to be much smaller than for residues $Ala_1–Lys_{44}$, and this was still lower than the value obtained for $Ala_1–Met_{51}$ (Hogg & Blomback, 1978). The authors suggested that the sequences between residues Trp_{33} and Lys_{44} and probably between Cys_{45} and Met_{51} are important for thrombin–fibrinogen interaction. However, since they were studying the rate of release of FpA from these peptides, their data do not provide information about the mechanism of the observed enhancement of the hydrolysis rate. The effect of the additional peptide segments can be due to their ability to stabilize a favorable conformation of the Aα chain for thrombin cleavage, and/or they can constitute an independent site that can interact with thrombin at a site other than its active center.

From our results, we can conclude that the $\alpha(Gly_{17}–Lys_{78})$ and probably the $\alpha(Gly_{17}–Met_{51})$ sequences play an important role in the interaction with the secondary binding site of thrombin and that this binding does not require the presence of FpA.

The interaction of thrombin with fibrin(ogen) at this secondary site may also affect the release of FpB. In contrast to the NDSK Aα chain, NDSK Bβ is a poor substrate for thrombin as compared to intact fibrinogen (Hagemann & Scheraga, 1977), and the difference can be attributed to the larger value of K_M obtained for NDSK Bβ (Hagemann & Scheraga, 1977; Martinelli & Scheraga, 1980; Hanna et al., 1984). These authors speculated that the NDSK Bβ may

contain all the residues that interact directly with thrombin but, once removed from the intact molecule, it may no longer retain the proper conformation required for hydrolysis by thrombin.

Our results show that, in contrast to the NDSK Aα chain, NDSK Bβ does not contain all sequence information required for thrombin recognition. Binding of thrombin at the secondary site can considerably alter the kinetics of release of FpB. It would be of interest to determine the rate of release of FpA and FpB from fibrinogen under conditions where the secondary site interaction is inhibited and characterize the effect of polymerization on the release of FpB under such conditions.

Effect of Polymerization on Thrombin Binding. The fate of fibrin-bound thrombin has been the subject of some controversy. Liu et al. (1979) presented evidence that thrombin is specifically adsorbed to the fibrin clot. Francis et al. (1981) reported that active enzyme is recovered from fibrin-bound thrombin after plasmic dissolution of the clot. In contrast to these findings, others presented evidence that the thrombin–fibrin interaction occurs mainly with fibrin monomers (Carney et al., 1979; Wilner et al., 1981; Liu et al., 1985). Kaminski and McDonagh (1983) speculated that bound thrombin might be released during polymerization.

The major structural event in the formation of fibrin polymer from monomers is the binding of the N-terminal E domain to the C-terminal domain (D) of fibrinogen, leading to the assembly into two-stranded protofibrils, followed by a lateral aggregation of the protofibrils to fibers. Two properly aligned D domains are then cross-linked by factor XIII to produce covalently linked DxD dimers.

We tested the hypothesis that these structural events could lead to the release of thrombin from fibrin because of a competition of binding the E domain by thrombin and DxD. We studied the binding of fibrin-E, DxD, and the DxDE complex to PMSF-thrombin–Sepharose. Fibrin E prepared from the DxDE complex was found to bind to thrombin, whereas cross-linked DD did not show any interaction with the immobilized protein (Table III). On the other hand, fibrin E could mediate the binding of DxD to PMSF-thrombin–Sepharose, indicating that the interaction between E and DxD does not inhibit the binding of E to thrombin. This suggests that the binding site in E for thrombin is distinct from its binding site for DxD. The consequence of the existence of a separate binding site is discussed in the next section. Our result does not rule out the possibility that other structural events (e.g., conformational changes) that probably occur during polymerization can lead to the release of fibrin-bound thrombin.

Physiological Importance of the Secondary Site Interaction between Thrombin and Fibrin. Our findings that the thrombin binding site in fibrin is present on the N-terminal domain of the molecule and that the $\alpha(Gly_{17}–Lys_{78})$ peptide segment itself carries a strong thrombin binding site suggest that the binding of thrombin to this site may play an important role in determining the specificity of thrombin action on fibrinogen (of the 14 Arg–Gly bonds present in fibrinogen, only 4 are cleaved by thrombin). This interaction can also affect the kinetic parameters for the release of FpA and FpB significantly.

In addition to clotting fibrinogen, thrombin plays a central role in the regulation of haemostasis by acting on a large number of protein substrates and zymogens, leading to their activation. Fibrinogen and fibrin are not passive protease substrates in the coagulation cascade either. They regulate the enzymes that act upon them (thrombin, factor XIII, plasminogen, plasmingen activator), and they interact with a large number of proteins to modulate the lifetime, strength, adhesiveness, and location of the fibrin clot. Some of the proteins that bind to fibrin(ogen) are also substrates for thrombin (e.g., fibrinogen and factor XIII). Since fibrin-bound thrombin has a free active site (Liu et al., 1979a; Fenton et al., 1981; Wilner et al., 1981; Berliner et al., 1985; Kaminski & McDonagh, 1983, 1987), it may display distinct catalytic properties toward some of its substrates. Such binding can affect the mechanism of fibrin polymerization. After the release of the fibrinopeptides, the enzyme remains bound to fibrin monomer, forming fibrin monomer–thrombin complex (FMxThr), in which the polymerization sites in the E domain are available for interaction with the complementary sites on the D fragment. (Our results show that the E fragment has separate binding sites for DxD and thrombin.) This leads to the formation of fibrinogen–FMxThr- and fibrinogen–FMxThr–fibrinogen-type dimers and trimers. The weak binding of thrombin to fibrin monomer allows its dissociation and binding to a nearby fibrinogen molecule without being released into the blood stream. This mechanism can considerably increase the speed of fibrin polymerization by localizing the enzyme to its site of action with the formation of fibrinogen–FM–FMxThr–fibrinogen-type polymers.

Activation of factor XIII is increased dramatically in the presence of fibrin (Janus et al., 1983; Lewis et al., 1985). Kinetic analysis suggested that both polymeric fibrin I and fibrin II (but not fibrinogen or fibrin monomer) are promoters for factor XIII activation (Lewis et al., 1985). It is not known where the binding site for factor XIII is localized in fibrin, but it is tempting to speculate that thrombin bound to fragment E at its secondary binding site could be the catalytically active form for this activation and the mechanism of promotion of the release of the activation peptide is the formation of a ternary complex between the E domain of fibrin, thrombin, and factor XIII.

ACKNOWLEDGMENTS

We thank Drs. L. Patthy and A. Varadi for their helpful comments on the manuscript. We gratefully acknowledge the preparative work of F. J. Moy.

REFERENCES

Berliner, L. J., Sugawara, Y., & Fenton, J. W., II (1985) *Biochemistry 24*, 7005.

Blomback, B., Blomback, M., Henschen, A., Hessel, B., Iwanaga, S., & Woods, K. R. (1968) *Nature (London) 218*, 130.

Blomback, B., Hogg, D., Gardlund, B., Hessel, B., & Kudryk, B. (1976) *Thromb. Res. 8 (Suppl. 2)*, 329.

Bloom, A. L. (1962) *Brit. J. Haematol. 8*, 129.

Carney, D. H., Glenn, K. C., Cunningham, D. D., Das, M., Fox, C. F., & Fenton, J. W., II (1979) *J. Biol. Chem. 254*, 6244.

Chen, A. B., & Mosesson, M. W. (1977) *Anal. Biochem. 79*, 144.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.

Eagle, H. J. (1935) *J. Gen. Phys. 18*, 547.

Fenton, J. W., Zabinski, M. P., Hsieh, K., & Wilner, G. D. (1981) *Thromb. Haemostasis 46*, 177.

Francis, C. W., Markham, R. E., Barlow, G. H., Florack, T. M., Dobrzynski, D. M., & Marder, V. J. (1983) *J. Lab. Clin. Med. 102*, 220.

Ghosh, A., & Seegers, W. H. (1980) *Thromb. Res. 20*, 281.

Hageman, T. C., & Scheraga, H. A. (1974) *Arch. Biochem. Biophys. 164*, 707.

Hageman, T. C., & Scheraga, H. A. (1977) *Arch. Biochem. Biophys. 179*, 506.
Hanna, L. S., Scheraga, H. A., Francis, C. W., & Marder, V. J. (1984) *Biochemistry 23*, 4681.
Haverkate, F., Koopman, J., Kluft, C., D'Angelo, A., Cattaneo, M., & Manucci, P. M. (1986) *Thromb. Haemostasis 55*, 131.
Heene, D. L., & Matthias, F. R. (1973) *Thromb. Res. 2*, 137.
Henschen, A., Lottspeich, F., Kehl, M., & Southan, C. (1983a) *Ann. N.Y. Acad. Sci. 408*, 28.
Henschen, A., Kehl, M., Southan, C., Lottspeich, F., & Geogopoulos, D. (1983b) in *Fibrinogen, Structure, Functional Aspects, Methabolism* (Haverkate, F., Henschen, A., Nieuwenhuizen, W., & Straub, P. W., Eds.) p 125, de Gruyter, Berlin.
Hogg, D. H., & Blomback, B. (1978) *Thromb. Res. 12*, 953.
Janus, T. J., Lewis, S. D., Lorand, L., & Shafer, J. A. (1983) *Biochemistry 22*, 6269.
Kaminski, M., & McDonagh, J. (1983) *J. Biol. Chem. 258*, 10530.
Kaminski, M., & McDonagh, J. (1987) *Biochem. J. 242*, 881.
Kowalska-Loth, B., Gardlund, B., Egberg, N., & Blomback, B. (1973) *Thromb. Res. 2*, 423.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lewis, S. D., Janus, T. J., Lorand, L., & Shafer, J. A. (1985) *Biochemistry 24*, 6772.
Liu, C. Y., Nossel, H. L., & Kaplan, K. L. (1979a) *J. Biol. Chem. 254*, 10421.
Liu, C. Y., Nossel, H. L., & Kaplan, K. L. (1979b) *Thromb. Haemostasis 42*, 79.
Liu, C. Y., Kaplan, K. L., Markowitz, A. H., & Nossel, H. L. (1980) *J. Biol. Chem. 255*, 7627.
Liu, C. Y., Handley, D. A., & Chien, S. (1985) *Anal. Biochem. 147*, 49.
Marder, V. J., Shulman, N. R., & Carroll, W. R. (1969) *J. Biol. Chem. 244*, 2111.
Marder, V. J., Budzynski, A. Z., & James, H. L. (1972) *J. Biol. Chem. 247*, 4775.
Marsh, H. C., Jr., Meinwald, Y. C., Lee, S., & Scheraga, H. A. (1982) *Biochemistry 21*, 6167.
Marsh, H. C., Jr., Meinwald, Y. C., Thannhauser, T. W., & Scheraga, H. A. (1983) *Biochemistry 22*, 4170.
Marsh, H. C., Jr., Meinwald, Y. C., Lee, S., Martinelli, R. A., & Scheraga, H. A. (1985) *Biochemistry 24*, 2806.
Martinelli, R. A., & Scheraga, H. A. (1979) *Anal. Biochem. 96*, 246.
Martinelli, R. A., & Scheraga, H. A. (1980) *Biochemistry 19*, 2343.
Nagy, J. A., Meinwald, Y. C., & Scheraga, H. A. (1982) *Biochemistry 21*, 1794.
Nagy, J. A., Meinwald, Y. C., & Scheraga, H. A. (1985) *Biochemistry 24*, 882.
Ni, F., Scheraga, H. A., & Lord, S. T. (1988) *Biochemistry* (submitted for publication).
Nieuwenhuizen, W., Vermond, A., Nooijen, W. J., & Haverkate, F. (1979) *FEBS Lett. 98*, 257.
Nieuwenhuizen, W., Vermond, A., & Hermans, J. (1983) *Thromb. Res. 31*, 81.
Olexa, S. A., & Budzynski, A. Z. (1979a) *Biochemistry 18*, 991.
Olexa, S. A., & Budzynski, A. Z. (1979b) *J. Biol. Chem. 254*, 4925.
Olexa, S. A., & Budzynski, A. Z. (1981) *J. Biol. Chem. 256*, 3544.
Olexa, S. A., Budzynski, A. Z., Doolittle, R. F., Cottrell, B. A., & Greene, T. C. (1981) *Biochemistry, 20*, 6139.
Seegers, W. H., Nieft, M., & Loomis, E. C. (1945) *Science (Washington, D.C.) 101*, 520.
Swadesh, J. K., Thannhauser, T. W., & Scheraga, H. A. (1984) *Anal. Biochem. 141*, 397.
Takagi, T., & Doolittle, R. F. (1975a) *Biochemistry, 14*, 940.
Takagi, T., & Doolittle, R. F. (1975b) *Thromb. Res. 7*, 813.
Thannhauser, T. W., & Scheraga, H. A. (1985) *Biochemistry 24*, 7681.
Varadi, A., & Patthy, L. (1983) *Biochemistry 22*, 2440.
Varadi, A., & Scheraga, H. A. (1986) *Biochemistry 25*, 519.
Wilner, G. D., Danitz, M. P., Mudd, M. S., Hsieh, K. H., & Fenton, J. W., II (1981) *J. Lab. Clin. Med. 97*, 403.
Winzor, D. J., & Scheraga, H. A. (1964) *Arch. Biochem. Biophys. 104*, 202.