Immunochemical Determination of Conformational Equilibria for Fragments of the Aα Chain of Fibrinogen†

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ABSTRACT: A study of the conformation of the Aα chain of the intact fibrinogen molecule and of fibrinogen derivatives in the region that is hydrolyzed by thrombin has been carried out by using a highly sensitive immunochemical method [Sachs, D. H., Schechter, A. N., Eastlake, A., & Anfinsen, C. B. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3790]. Anti-fibrinogen antibodies were induced in rabbits by immunization with native bovine fibrinogen. Two separate populations of antibodies, specific for the (same) native antigenic determinant within fragment 16–25 and fragment 11–25, respectively, of the Aα chain, were isolated by immunoadsorption. These preparations were used to determine the value of K_{conf}, the equilibrium constant for the interconversion of the nonnative and native conformations of this determinant. Values of K_{conf} were measured for this determinant within native fibrinogen, the disulfide knot (DSK), CNBrAα, fragment 11–25, fragment 16–25, and fibrinopeptide A. ^{125}I-Labeled fibrinogen ([^{125}I]F) was used in the determination of K_{conf} by measuring the competition between [^{125}I]F and the fibrinogen derivatives under study for binding to the purified antibody. For the antigenic region in F, the DSK, and CNBrAα, the values of K_{conf} at 4 °C were infinity, $(4.9 \pm 2.9) \times 10^{-3}$, and $(5.2 \pm 4.8) \times 10^{-5}$, respectively. The values of K_{conf} for fragments 11–25 and 16–25 and fibrinopeptide A at 4 °C were less than $(2.0 \pm 1.8) \times 10^{-6}$. These measurements indicate that the antigenic region in F is unperturbed by iodination and that 0.5% of the DSK molecules, 0.005% of the CNBrAα molecules, and less than 0.0002% of the smaller fragments adopt the native conformation within this antigenic determinant in aqueous solution. The antigenic determinant involved in this study is localized among residues 16–25 of the Aα chain (the numbering referring to the sequence of *bovine* fibrinogen). The values of K_{conf} are correlated with the values obtained previously for k_{cat} and K_M [for the thrombin-induced hydrolysis of the Arg_{19}–Gly_{20} bond within these peptides [Meinwald, Y. C., Martinelli, R. A., van Nispen, J. W., & Scheraga, H. A. (1980) *Biochemistry 19*, 3820]]. The less efficient kinetics of hydrolysis of these fragments (as compared to that of native fibrinogen) is attributed to incomplete formation of the native conformation around residues 16–25 of the Aα chain. Both the kinetic and immunochemical results suggest that long-range interactions are necessary for the stabilization of the native structure of that (those) portion(s) of fibrinogen that interacts with thrombin and the antibody, respectively.

Thrombin releases fibrinopeptides A and B from the N-terminal portions of the Aα and Bβ chains, respectively, of the soluble plasma protein fibrinogen (see Figure 1),[1] an event which initiates the spontaneous polymerization of the resultant fibrin monomer into the insoluble fibrin clot (Scheraga & Laskowski, 1957; Blombäck et al., 1966; Blombäck, 1979; Doolittle et al., 1979a, and references therein). The proteolytic action of thrombin appears limited in fibrinogen to those four Arg–Gly bonds that link the fibrinopeptides to the fibrinogen molecule (Blombäck et al., 1966; Fenton et al., 1979; Bing et al., 1981, and references therein). Six other pairs of Arg–Gly bonds occur in F, yet none of these are cleaved by thrombin, even though some are in exposed regions that are vulnerable to attack by plasmin and other proteases (Doolittle et al., 1978).

An active-site mapping approach (Scheraga, 1977) has been used to investigate the nature of the interactions of the amino acids in fibrinogen that are responsible for this unique thrombin specificity. The rates of hydrolysis of the specific Arg–Gly bonds in various peptide substrates (Andreatta et al., 1971; Liem et al., 1971; Liem & Scheraga, 1973, 1974; Hageman & Scheraga, 1974, 1977; van Nispen et al., 1977; Meinwald et al., 1980) have been compared with the rates of hydrolysis of these same bonds in the Aα and Bβ chains in fibrinogen (Martinelli & Scheraga, 1980). On the basis of a comparison with the kinetic parameters, k_{cat} and K_M, of the complete Aα chain, CNBrAα has been found to contain all of the residues essential for the specific interaction with thrombin (Hageman & Scheraga, 1974; Martinelli & Scheraga, 1980; Meinwald et al., 1980).

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[1] Abbreviations: F, fibrinogen; DSK, disulfide knot, the large disulfide-linked CNBr-cleavage product of F, containing the N-terminal portions of the Aα, Bβ, and γ chains; Aα and Bβ, the peptide chains of fibrinogen that release fibrinopeptides A and B, FpA and FpB, upon hydrolysis by thrombin; CNBrAα, the N-terminal peptide of the Aα chain produced by CNBr cleavage of the Aα chain of F; [^{125}I]F, fibrinogen iodinated with ^{125}I; $NaDodSO_4$, sodium dodecyl sulfate; *S. aureus*, *Staphylococcus aureus*, Cowen strain I; IgG-SORB, trademark for *S. aureus*, Enzyme Center, Boston, MA; Ab, antibody; Ag, antigen; K_{assoc}, the equilibrium constant for the reaction Ab + Ag ⇌ (Ab·Ag); K_{conf}, the equilibrium constant between the unfolded and native conformation of a protein fragment; (X–Y), a polypeptide whose N- and C-terminal residues are X and Y, respectively; Cl_3CCOOH, trichloroacetic acid; Tween 20, poly(oxyethylene) sorbital monolaurate; BBS, borate–saline buffer (0.075 M NaCl, 0.1 M boric acid, and 0.025 M sodium tetraborate, pH 8.5); PBS, phosphate–saline buffer (0.15 M NaCl, 0.012 M Na_2HPO_4, and 0.008 M KH_2PO_4, pH 7.4); PBS/oval/azide, PBS containing 0.1% ovalbumin and 0.1% sodium azide; BBS/Tween, BBS containing Tween 20 (0.1%) and additional NaCl (1 M); BS, borate–saline buffer (0.05 M boric acid, 0.0012 M sodium tetraborate, and 0.15 M NaCl); Tris, tris(hydroxymethyl)aminomethane; RIA, radioimmunoassay; anti-X, antibody against X, where X is F or a fragment thereof; DCC, *N,N'*-dicyclohexylcarbodiimide; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; Ac, acetyl; OBu^t, *tert*-butyloxy; F_3CCOOH, trifluoroacetic acid; TLC, thin-layer chromatography; Z, benzyloxycarbonyl. 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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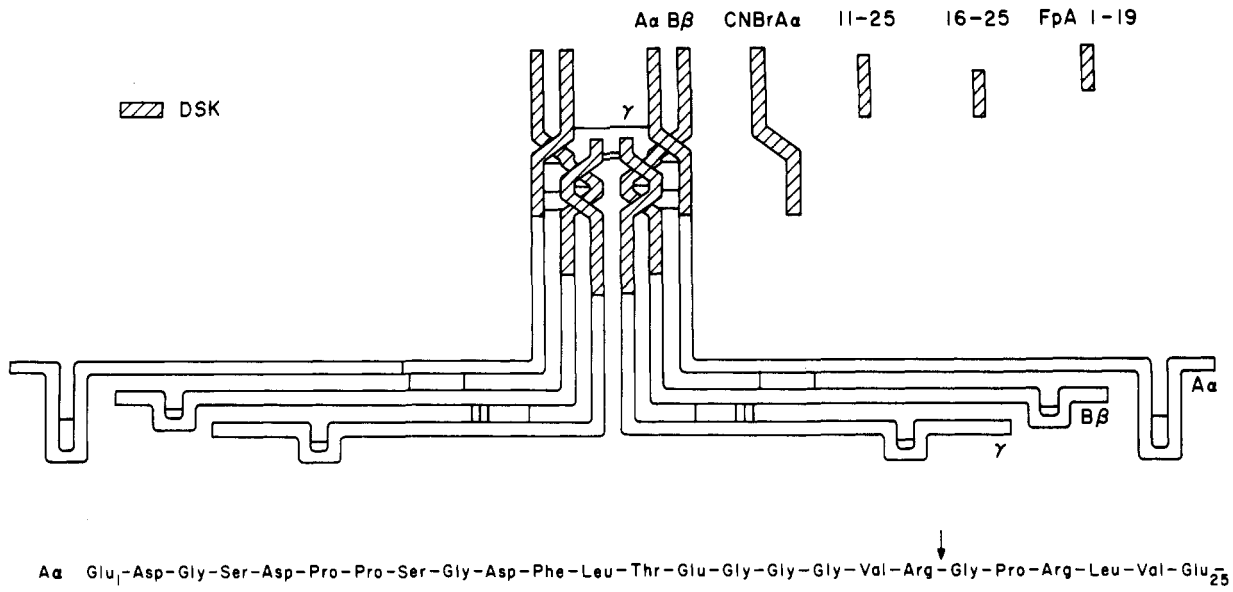

FIGURE 1: Schematic diagram of fibrinogen and its fragments used in this study. [Adapted with permission of Blombäck (1979) (Figure 91)]. The hatched portion of the whole F molecule represents the DSK; smaller fragments thereof are also shown. The amino acid sequence of residues 1–25 of the Aα chain of bovine fibrinogen is listed below the diagram. The arrow marks the location of the bond hydrolyzed by thrombin.

While the peptide[2] Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ [F6 in the nomenclature of Meinwald et al. (1980)] (see Figure 1) has been reported to contain all of the residues necessary for the determination of k_{cat} (Meinwald et al., 1980), examination of the values of the specificity constant, k_{cat}/K_M, reported by Meinwald et al. (1980) shows that this and other synthetic oligopeptides [F1–F6 of Meinwald et al. (1980)] are somewhat poorer substrates for thrombin than are fibrinogen or the CNBrAα fragment of fibrinogen. Since k_{cat} for the synthetic peptides (F3–F6) differs from that of the fibrinogen Aα chain by less than an order of magnitude, the difference in the specificity constants resides primarily in the factors that determine K_M. Additional residues, not included in these peptides, may be required for efficient binding by direct interaction with thrombin. Alternatively, all of the residues necessary for direct interaction with thrombin may be present within those peptides studied, but additional residues are required to provide long-range interactions that enable these peptides to adopt an appropriate conformation for optimum binding to thrombin. Specific conformational features in the residues near the bond hydrolyzed by thrombin, as suggested by NMR observations (Von Dreele et al., 1978; Rae & Scheraga, 1979), may be required to direct thrombin specificity.

The purpose of this work, therefore, is to examine the conformation of the Aα chain in the region of hydrolysis by thrombin. We have chosen an immunochemical method to assess the role of conformation in the specificity of thrombin action. This immunochemical method provides a quantitative set of parameters with which to describe the conformational equilibrium of a peptide fragment in a range of low concentrations where it is difficult to apply other physicochemical techniques.

The primary assumption of this immunochemical method is that a given polypeptide derived from a protein can exist in two forms, P_N and P_R, where P_N is the conformation that the polypeptide would have in the intact native protein and P_R is the ensemble of unordered or denatured forms. The degree to which an excised peptide has the conformation that it had in the intact protein may be expressed in terms of the equilibrium constant, K_{conf}, for the equilibrium (Sachs et al., 1972)

$$P_R \overset{K_{conf}}{\rightleftharpoons} P_N \qquad (1)$$

When antibodies that bind only with P_N, but not with P_R, are used, it is possible to determine K_{conf}.

Additional assumptions inherent in the application of this technique [summarized by Anfinsen & Scheraga (1975)] include the following: (a) Antibodies produced against the native protein will contain subpopulations that recognize only that fraction of excised peptide that contains an antigenic determinant, which has the native-like conformation. (b) The antibody binds with the same affinity to its corresponding *native* determinant regardless of whether the determinant is in the intact protein or in a fragment thereof. (c) The *simultaneous* equilibria involving eq 1 (and eq 2 and 3) remain unperturbed during the analytical procedures used to carry out the experiment.

The conformational equilibrium constant, K_{conf}, has been determined for various peptide derivatives of the Aα chain of bovine F which contain the segment hydrolyzed by thrombin. Anti-F antibodies have been induced in rabbits by immunization with native bovine F. These antibodies have been fractionated on a series of affinity columns to yield antibodies against F and against the DSK, CNBrAα, fragment 11–25 and fragment 16–25 of the Aα chain. Those purified antibodies, which are specific for the native antigenic determinant[3] within fragment 16–25 of the Aα chain [viz., anti-(16–25) and anti-(11–25)], have been used to determine the values of K_{conf} for this determinant within F, the DSK, CNBrAα, fibrinopeptide A, and fragments 11–25 and 16–25.

These values of K_{conf} provide a measure of the degree to which these F derivatives, containing the site of thrombin cleavage, retain the conformation that they had in the intact native F molecule. The values of K_{conf} have been correlated with the values of K_M for the thrombin-induced hydrolysis of the Arg–Gly bonds in these peptides, thereby providing an

[2] There are only two substitutions in amino acid sequence within residues 11–25 of the Aα chain of bovine and human fibrinogen: at position 13 Thr (bovine) is replaced by Ala (human), and at position 23 Leu (bovine) is replaced by Val (human).

[3] An antigenic determinant is defined as a region of the native protein containing residues that participate directly in binding to the antibody by means of interactions with the antibody combining site.

assessment of the role of both amino acid sequence and conformation in the overall specificity of thrombin for F.

Materials and Methods

Preparation of Fibrinogen and Its Derivatives. Bovine F was prepared from Sigma fraction I (lot 117C-0140, 82% clottable) as described by Hageman & Scheraga (1977). Preparation of the DSK followed the procedure outlined by Telford et al. (1980). The purification of CNBrAα was that of Martinelli et al. (1979). Fibrinopeptides A and B were prepared from bovine F (Blombäck & Vestermark, 1958). The purity of these peptides was assessed by amino acid analysis and by gel electrophoresis. Figure 1 summarizes the derivatives used in this study.

Synthesis, Purification, and Analysis of Peptides. Two peptides[2] corresponding to residues 16–25, viz., H-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-$NHCH_3$·3HCl, and residues 11–25, viz., H-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-$NHCH_3$·3HCl, of the Aα chain of F have been synthesized. These will be referred to as I and II, respectively. The two peptides were prepared by classical solution methods utilizing intermediates previously synthesized in this laboratory (van Nispen et al., 1977; Meinwald et al., 1980). Because of the availability of synthetic fragment precursors of these peptides which corresponded to the sequence of *human* F, fragments 11–25 and 16–25 were synthesized following the human rather than the bovine sequence.[2]

All of the amino acids (except glycine) were of the L configuration. HOBt, DCC, and *N*-ethylmorpholine 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) by using the following solvent systems: (a) 1-butanol–acetic acid–water, 4:1:1; (b) 1-butanol–pyridine–acetic acid–water, 4:1:1:2; (c) 2-propanol–formic acid–water, 20:1:5; (d) 1-butanol–acetic acid–water, 4:3:3. Methods used for the detection of components on TLC plates were ultraviolet light, ninhydrin reagent for free amino groups, chlorine/potassium iodide–starch reagent for NH groups, and Sakaguchi reagent for free guanidino groups.

Melting points (uncorrected) were determined with a Thomas Hoover apparatus. Specific rotations were measured with a Perkin-Elmer 141 polarimeter. Amino acid analysis was carried out as described below (see Amino Acid Analysis).

H-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu(OBu^t)-$NHCH_3$·3HCl (III) (Meinwald et al., 1980) was treated with F_3CCOOH to give directly the free decapeptide I. Purification was achieved by chromatography on a DEAE-Sephadex column (OAc^- form). I showed a single spot upon TLC analysis: R_f 0.24 in (b).

Coupling of Z-Phe-Leu-Ala-Glu(OBu^t)-Gly-OH (van Nispen et al., 1977) with III using DCC/HOBt gave the fully protected pentadecapeptide. The *tert*-butyl protecting group was removed by treatment with F_3CCOOH and the Z group by hydrogenation, yielding II. After chromatography on a DEAE-Sephadex column (OAc^- form), II showed the following properties: R_f 0.28 in (b) and 0.50 in (d); $[\alpha]_D$ −106° (*c* 0.3, 1% HOAc).

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out on samples of reduced bovine F (Weber & Osborn, 1969). The gels were 7.5% acrylamide and 0.25% bis(acrylamide); they were run at 8 mA/gel and were stained with Coomassie Brilliant Blue. $NaDodSO_4$–polyacrylamide disc gel electrophoresis (Laemmli, 1970) was carried out on samples of DSK and CNBrAα. The running gel was 15% acrylamide and 0.4% bis(acrylamide), and the stacking gel was 3% acrylamide and 0.08% bis(acrylamide).

Table I: Amino Acid Composition of Derivatives of Fibrinogen[a]

amino acid	DSK	CNBrAα	FpA	I (16–25)	II (11–25)
Asp	63 (62)[b]	5.1 (6)[c]	3.0 (3)		
Thr	37 (36)	2.8 (3)	0.9 (1)		
Ser	41 (40)	3.4 (5)	1.7 (2)		
Glu	59 (56)	6.6 (7)	1.9 (2)	1.04 (1)	2.02 (2)
Pro	35 (44)	5.1 (5)	2.0 (2)	1.01 (1)	1.00 (1)
Gly	51 (46)	6.6 (8)	4.8 (5)	2.97 (3)	4.06 (4)
Ala	23 (26)	1.1 (1)			0.82 (1)
Val	27 (26)	2.0 (2)	1.0 (1)	2.85 (3)	2.93 (3)
Ile	22 (18)				
Leu	35 (34)	2.0 (2)	1.0 (1)		0.91 (1)
Tyr	14 (16)				
Phe	11 (12)	1.9 (2)	1.0 (1)		0.99 (1)
Lys	35 (34)	2.0 (2)			
His	5 (4)				
Arg	37 (36)	3.5 (4)	1.0 (1)	2.00 (2)	2.17 (2)

[a] Hydrolysis was performed in 6 N HCl for 24 h under vacuum. Values have not been corrected for hydrolysis losses. Experimental values are given as moles of amino acid per mole of peptide followed in parentheses by the theoretical values. Since hydrolysis was carried out with HCl, the Trp content of the derivatives was not determined. All other values not recorded were less than 0.2 mol of amino acid per peptide. A 48-h hydrolysis of peptide II gave values indistinguishable from those reported here. [b] Calculated from the data of Timpl et al. (1977), Hageman & Scheraga (1977), and Martinelli et al. (1979), assuming for the composition 2(CNBrAα + CNBrBβ + CNBrγ). [c] Calculated from the sequence of CNBrAα (Timpl et al., 1977; Martinelli et al., 1979).

Amino Acid Analysis. All samples were hydrolyzed for 24 h at 105 °C in 6 N HCl (prepared from constant-boiling HCl) (Spackman et al., 1958) and then chromatographed with a Technicon TSM automated amino acid analyzer. The results are shown in Table I.

Determination of Peptide and Protein Concentrations. The concentrations of F solutions were determined spectrophotometrically as described previously (Telford et al., 1980). The concentrations of all peptide and protein derivatives of F used in the measurements of K_{conf} were determined by micro-Kjeldahl nitrogen analysis carried out with the procedures of Lang (1958) and Noel & Hambleton (1976a,b). Concentrations of antibody solutions were estimated by the absorbance at 280 nm assuming that $E^{1\%}_{1cm}$ = 13.5 (Stevenson & Dorrington, 1970).

Antibody Preparation. Antiserum specific to native bovine F was raised in four rabbits by intramuscular injection according to the method of Telford et al. (1980). Rabbits were bled 10 days after each injection. The drawn blood was left at ambient temperature for 1 h and then at 4 °C overnight to allow the blood to clot. Antiserum was isolated by centrifugation of the clotted blood (2000 rpm, 4 °C, 30 min) and stored at −20 °C until used. Antisera drawn on separate days and from different rabbits were not pooled. Antibody titers were determined for each bleeding for each rabbit, and the highest titer antisera from one rabbit were selected. Antisera obtained from the monthly bleedings following the 7th through the 13th booster injections of this rabbit were pooled. This animal was sacrificed, and the serum from the cardiac puncture bleeding was also pooled. The experiments described below were performed with the hyperimmune antiserum from this rabbit.

Preparation of Sepharose Immunoadsorbents for Affinity Chromatography. Bovine F, the DSK, CNBrAα, and syn-

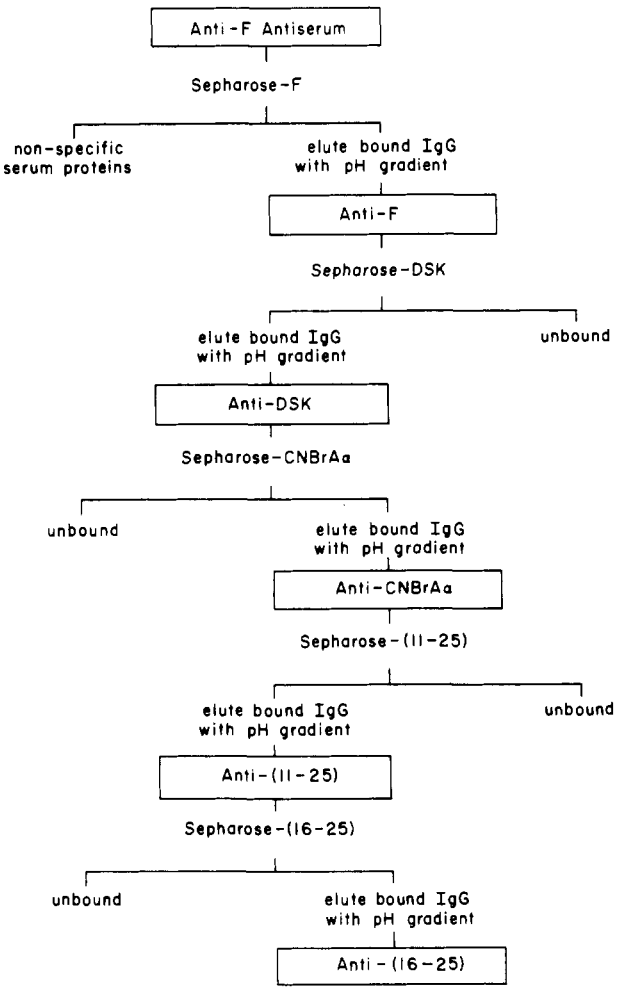

FIGURE 2: Affinity chromatography scheme.

thetic peptides 11–25 and 16–25 were coupled to activated-CH Sepharose 4B (Pharmacia Fine Chemicals) in 0.1 M $NaHCO_3$ and 0.50 M NaCl buffer, pH 8.0, following the instructions of the manufacturer. Steric hindrance of antibody approach to the linked peptide was reduced by attaching the peptides to this Sepharose support which contained a six-carbon spacer arm between the resin support and the active ester group. The amount of protein derivative bound to the dry resin was 40 mg of F to 4 g of resin, 32 mg of DSK to 4 g, 30 mg of CNBrAα to 2 g, 25 mg of fragment 11–25 to 1.25 g, and 20 mg of fragment 16–25 to 1.33 g. Excess active sites on the resin were removed by suspending the linked resin in 0.05 M Tris-HCl buffer, pH 8.0, for 1 h. The rinsed gel was stored in BS at 4 °C until used. The coupling efficiency, as judged by absorbance of the peptide or protein solution, before linking, and the supernatant, after linking, was greater than 95%.

Fractionation of Antibody Subpopulations. Antibodies were fractionated by affinity chromatography by using columns of Sepharose to which F or F fragments had been covalently coupled.[4] The rationale behind the affinity columns is shown in Figure 2.

[4] Immunoadsorption of antibodies to columns of polypeptide antigens covalently bound to Sepharose can be used to select between conformationally specific antibodies to the native or disordered antigen (Ab_N or Ab_R) since it is the material used as immunogen which determines the reactive specificity of the antibody, not the ligand on the Sepharose matrix. Of the large excess of antigen bound to Sepharose, which is in a conformational equilibrium between native and nonnative states, only those antigenic determinants in the native conformation are recognized by the antibody that had been elicited to the native protein (Furie et al., 1975).

Upon completion of the entire series of chromatographic operations with the affinity columns depicted in Figure 2, the yields of both anti-$(11–25)_N$ and anti-$(16–25)_N$ were found to be too low to obtain sufficient amounts of antibodies to perform the complete series of K_{conf} experiments. An alternative route of purification of these particular populations of antibodies was, therefore, followed. The Sepharose–F, fragment 11–25, and fragment 16–25 columns were enlarged 3-fold to increase their capacity, and anti-$(11–25)_N$ and anti-$(16–25)_N$ were prepared directly from anti-F_N.

For the preparation of anti-F_N, whole hyperimmune antisera (25 mL) were applied at room temperature to a column containing approximately 35 mL of Sepharose–F (packed volume) equilibrated with BBS/Tween buffer. For elution of nonspecifically bound proteins, the column was first washed with BBS/Tween (Smith et al., 1978) at a flow rate of 20 mL/h. Fractions of 3.5 mL were collected. The eluate was monitored, and elution was continued until the eluate had an absorbance at 280 nm of less than 0.05*A* (Zeiss Model PMQ II spectrophotometer).

Antibodies specifically bound to the column were then eluted with 0.5 M HOAc (Smith et al., 1978), and the pH of the eluate was adjusted immediately to pH 8.0 with NaOH. Specific anti-F_N antibodies were detected by determining the ability of a 50-μL aliquot of each fraction to bind [^{125}I]F; the procedure used was the same as that described below (Binding Assays). The fractions that contained anti-F_N were pooled, dialyzed overnight against BS, concentrated on an Amicon ultrafiltration apparatus using an Amicon XM 50 membrane, and stored at 4 °C for subsequent use. Sodium azide (0.1%) was added to all samples as a bacteriostat.

Anti-F_N was then applied directly to either Sepharose–fragment 11–25 or Sepharose–fragment 16–25 columns. Typically, 10–15 mL of anti-F_N (1.0 mg/mL) was loaded onto a Sepharose–(11–25) or –(16–25) column (14-mL bed volume). Anti-$(11–25)_N$ and anti-$(16–26)_N$ were purified from the other anti-F_N antibodies by using the same elution procedure as described above. One complication introduced by this second procedure was that the resulting antibodies were found to be heterogeneous in their affinities, as indicated by biphasic Scatchard plots (data not shown). Reapplication of these heterogeneous antibody populations to the same column 3 times (Sepharose–fragment 11–25 or –fragment 16–25), however, yielded populations of antibodies, [anti-$(11–25)_N$ or anti-$(16–25)_N$], which gave Scatchard plots in [^{125}I]F binding experiments indicative of populations of antibodies homogeneous with respect to their binding affinities and their specificity (Calvert et al., 1979) (see Results). These isolated "homogeneous" populations were subsequently used in the K_{conf} experiments.

Consistent with the previously employed nomenclature (Sachs et al., 1972; Furie et al., 1975; Hurrell et al., 1977a; Chavez & Scheraga, 1977, 1979, 1980a,b), we designate the antibody populations purified with Sepharose–fragment 11–25 and Sepharose–fragment 16–25 as anti-$(11–25)_N$ and anti-$(16–25)_N$, respectively, where the subscript N indicates that the antibodies were produced by using native F as the immunogen.

Preparation of [^{125}*I*]*F.* Purified bovine F was trace labeled with ^{125}I by using the solid phase lactoperoxidase method (Marchalonis, 1969; Thorell & Johansson, 1971; David, 1972; David & Reisfeld, 1974; Krohn & Welch, 1974) incorporating the glucose oxidase procedure (Hubbard & Cohn, 1972). The iodination was performed on approximately 1 mg of F contained in 100 μL of sodium phosphate buffer, pH 7.5, using

a New England Nuclear radioiodination kit following the instructions of the supplier. When the reaction was quenched, 0.5 mL of PBS/oval/azide was added to the reaction mixture to act as carrier and to avoid losses of F in transfer. The [^{125}I]F was separated from excess Na^{125}I on a Sephadex G-25 (Pharmacia Fine Chemicals) column (1.0 × 18 cm) previously equilibrated with PBS/oval/azide. The ovalbumin was purchased from Sigma Chemical Co. (grade V).

Binding Assays. The binding of unfractionated antiserum to [^{125}I]F was carried out to determine which serum samples had the highest titer. Use of the method of Farr (1958) to separate the antibody-bound [^{125}I]F from the free [^{125}I]F by precipitation of the antibody–antigen complexes with 50% saturated ammonium sulfate was not possible since F alone precipitates at only 33% saturation of ammonium sulfate. Hence, separations were carried out with heat-inactivated *S. aureus* cells (IgG-SORB, Enzyme Center, Boston, MA) (Jonsson & Kronvall, 1974a,b; Madar et al., 1980).

Serial dilutions of antiserum were made by using either PBS/oval/azide or BBS/Tween as the diluent. Assays were performed in triplicate in microfuge tubes (Beckman) containing 100 μL of each dilution of antiserum, 50 μL of [^{125}I]F, and 250 μL of either buffer. Radiolabeled F had been diluted to give approximately 50 000 cpm/50 μL, corresponding to 15 ng of F. The reaction mixture was incubated overnight at 4 °C and allowed to come to equilibrium after which 50 μL of 10% (w/v) suspension of IgG-SORB was added to each tube. The IgG-SORB had been prepared according to the instructions of the supplier. The tubes were incubated for 10 min and then centrifuged (10 000 rpm, 1.5 min) on a Beckman Microfuge B, and the supernatant was removed by aspiration (or an aliquot was removed and the radioactivity in the supernatant was counted, and the remaining supernatant was aspirated). The pellet was rinsed with 0.4 mL of PBS/oval/azide or BBS/Tween buffer and the tube vortexed to resuspend the pellet and recentrifuged. The supernatant was again aspirated off, and the radioactivity in the pellet was counted. The addition of Tween 20 at a final concentration of 0.1% (v/v) to the BBS was found here to reduce the nonspecific uptake of radioactivity to less than 5%. It is unlikely that any dissociation of bound [^{125}I]F occurred during the washing process, since the sum of the concentrations of free [^{125}I]F in the supernatant and bound [^{125}I]F in the washed precipitate closely approximated the concentration of [^{125}I]F added to the system, indicating that there was no perturbation of the equilibrium concentrations by the separation procedure. Therefore, in subsequent experiments, only the bound fraction was counted.

Antibody titers (Hunter, 1978) were determined for each serum sample to assess its potential use in the experiments designed to measure K_{conf}. A correction was made for nonspecific binding by the method of Farr (1958) and Minden & Farr (1978) with data provided by a preimmune serum control included in each series of assays.

Determination of K_{assoc} for Anti-$(11–25)_N$ and Anti-$(16–25)_N$ to Native Fibrinogen. The association constants for the binding of anti-$(11–25)_N$ and anti-$(16–25)_N$ to native bovine F were measured by using the assay procedure described for analysis of the antiserum (see Binding Assays, above). [^{125}I]F (50 μL), purified antibody (50 μL), and buffer (100 μL) were mixed and equilibrated. The concentration of anti-$(16–25)_N$ was 3.5×10^{-11} M and that of anti-$(11–25)_N$ was 5.2×10^{-11} M throughout, and a range of concentrations of [^{125}I]F from 2.5×10^{-8} to 2.5×10^{-12} M was used in the binding experiments for both antibody populations. For the control assay to analyze for nonspecific binding of [^{125}I]F, antibody was replaced with 50 μL of buffer. The binding data for both anti-$(16–25)_N$ and anti-$(11–25)_N$ were analyzed by the method of Scatchard (1949), after the antibody concentration was determined by the method of Steward & Petty (1972) and Steward (1978). The data were plotted as r/c vs. r, where r = [bound antigen]/[total antibody], c = [free antigen], and the brackets designate molar concentrations. The values of K_{assoc} for the binding of anti-$(16–25)_N$ and anti-$(11–25)_N$ to F were determined from the binding data by the method of Calvert et al. (1979).

Competition Studies. The ability of various derivatives of F to lead to competitive inhibition of the binding of specific antibodies [either anti-$(11–25)_N$ or anti-$(16–25)_N$] to [^{125}I]F was examined by means of a competitive radioimmunoassay (Yalow & Berson, 1970). Assays were performed in triplicate for each concentration of competing antigen by adding to each microfuge tube [^{125}I]F (50 μL), buffer (PBS/oval/azide or BBS/Tween, 50 μL), and competing antigen (50 μL). The final concentrations of competing antigen were varied from 0 to 6.0×10^{-7} M for unlabeled bovine F, to 1.0×10^{-5} M for DSK, to 1.5×10^{-4} M for CNBrAα, to 1.5×10^{-4} M for fragment 11–25, to 5.0×10^{-4} M for fragment 16–25, and to 3.0×10^{-3} M for FpA. A 50-μL sample of the specific antibody was then added, bringing the total final volume to 200 μL. For anti-$(16–25)_N$ the competitive RIA's were performed by using three concentrations of F, viz., 1.2×10^{-9}, 6.6×10^{-10}, and 1.3×10^{-10} M. The concentration of anti-$(16–25)_N$ was 3.5×10^{-11} M throughout. For anti-$(11–25)_N$, the concentrations of F and specific antibody were 2.7×10^{-9} and 5.2×10^{-11} M, respectively. The tubes were incubated overnight at 4 °C to achieve equilibrium; free [^{125}I]F was separated from antibody-bound [^{125}I]F by the IgG-SORB method described above (Binding Assays). After centrifugation, the supernatant was discarded, the precipitate was washed, and the radioactivity in the precipitate was then counted.

The analysis of competitive RIA data becomes complicated if the antigen under study is multivalent (as is the dimeric F molecule). Anomalous effects of multiple binding of an antibody molecule to a multivalent antigen are substantially reduced when the concentration of labeled antigen is sufficiently high, such that the concentration of antibody combining sites is limiting; hence, the antibodies are saturated (Creighton, 1980). This requires that the concentration of antigen be greater than the value of the largest dissociation constant of the antigen–antibody complex (Creighton et al., 1978; Creighton, 1980). Under these conditions, each antigen molecule is likely to have at most a single antibody molecule bound to it, and consequently multiple binding will be negligible.

Therefore, the concentration of [^{125}I]F was chosen to be in large (20-fold molar) excess over the concentration of the antibody in the competitive RIA studies, and the antibody concentration was adjusted in the competitive RIA so that 5–33% of the [^{125}I]F was bound in the absence of any inhibitor, thereby saturating the antibody combining sites. Dimeric F was therefore operationally "univalent". Finally, the fractionation of the antibody populations by means of immunoadsorption provided operationally "homogeneous" populations for these experiments.

The molecular weights for the fragments used in this study are the following: F, 340 000; DSK, 60 000; CNBrAα, 5700; fragment 11–25, 1555; fragment 16–25, 1081; FpA, 1889. Molar ratios of peptide and unlabeled F to [^{125}I]F ranged from 1:1000 to 100 000:1.

The competition between unlabeled F and [^{125}I]F was carried out as a control to determine whether iodination of F altered its response to antibody. A counting standard containing only labeled F was included in each experiment to correct the specific activity for radioactive decay. The control assay to analyze for nonspecific binding of [^{125}I]F contained no antibody in the assay tube. The control assay to determine the maximum amount of precipitable [^{125}I]F (i.e., antibody-bound [^{125}I]F) contained no inhibitor in the assay tube.

Data are presented graphically as the ratio of B_i, the percent of [^{125}I]F bound in the presence of a particular concentration of a competitive inhibitor, to B_0, the percent of [^{125}I]F bound in the absence of any inhibitor, vs. log of the concentration of inhibitor. In the absence of any inhibitor, the value of bound [^{125}I]F is normalized to 100%. The inhibition data for each inhibitor were linearized as a plot of ln $[Y/(100 - Y)]$ vs. log of the concentration of competing antigen, where $Y = (B_i/B_0) \times 100$. [This corresponds to the "logit" transformation of Feldman & Rodbard (1971); Rodbard & Hutt, 1974.] By use of this logit analysis, linear least-squares parameters were obtained and then applied in the calculation of a best-fit sigmoidal curve to the experimental data for each inhibitor.

For determination of K_{conf}, the competitive radioimmunoassay data were analyzed by the method of Furie et al. (1975) as described by Chavez & Scheraga (1980b) with further modifications as outlined below.

Definition and Derivation of K_{conf}. The effectiveness with which a polypeptide derivative of F inhibits the binding of one of the purified antibodies, Ab_N, to [^{125}I]F depends on the magnitude of the equilibrium constant of eq 1. The two competing reactions are

$$Ab_N + P_N \overset{K_1}{\rightleftharpoons} Ab_N{\cdot}P_N \quad (2)$$

$$Ab_N + F_N \overset{K_2}{\rightleftharpoons} Ab_N{\cdot}F_N \quad (3)$$

where Ab_N represents an available antibody combining site, P_N refers to a free polypeptide derivative with its antigenic determinant in the native conformation, and F_N represents a free (iodinated) F molecule. Equations 1–3 represent simultaneous equilibria which can be analyzed by the procedure described below.

For the case of a monovalent antigen, we may assume[5] that $K_2 = K_1$ (Sachs et al., 1972; Furie et al., 1975). For the case of F, however, there are two potential antigenic binding sites per molecule, $-F_N-$; therefore $K_2 = 2K_1$, because of the statistical factor for multiple binding of an antibody combining site to a divalent antigen. Making use of this relation, eq 2 and 3 may be combined to give

$$[P_N] = \frac{2[Ab_N{\cdot}P_N][F_N]}{[Ab_N{\cdot}F_N]} \quad (4)$$

If the concentration of F_R is assumed to be negligible, $[F_N] = [F_{tot}] - [Ab_N{\cdot}F_N]$. Therefore

$$K_{conf} = \frac{[P_N]}{[P_R]} = \frac{2[Ab_N{\cdot}P_N]([F_{tot}] - [Ab_N{\cdot}F_N])}{[Ab_N{\cdot}F_N][P_R]} \quad (5)$$

Using the substitution $[P_R] = [P_{tot}] - [Ab_N{\cdot}P_N] - [P_N]$ and recombining terms, we obtain

$$K_{conf} = \frac{2[Ab_N{\cdot}P_N]([F_{tot}] - [Ab_N{\cdot}F_N])}{[Ab_N{\cdot}F_N][P_{tot}] - [Ab_N{\cdot}P_N](2[F_{tot}] - [Ab_N{\cdot}F_N])} \quad (6)$$

The mole ratio of [^{125}I]F to Ab_N in the systems was adjusted to be approximately 20:1. The quantities $[F_{tot}]$ and $[P_{tot}]$ were the total concentrations of [^{125}I]F and inhibitor added, respectively, as determined by nitrogen analyses. By use of iodinated F, the concentration of $Ab_N{\cdot}F_N$ at equilibrium is determined from the radioactivity present in the precipitate in the assay. The concentration of $Ab_N{\cdot}P_N$ is calculated by subtracting the amount of [^{125}I]F in the precipitate in the presence of competing inhibitor from the amount of [^{125}I]F in the precipitate in the absence of competing inhibitor.

Results

Purity of Fibrinogen and Peptide Derivatives. NaDodSO$_4$–polyacrylamide gel electrophoresis of samples of reduced F gave only three bands corresponding to the Aα, Bβ, and γ chains of F (McDonagh et al., 1972). The purity of the peptide fragments, DSK, and CNBrAα was established by NaDodSO$_4$–polyacrylamide disc slab gel electrophoresis. The fragments exhibited single bands, and the amino acid compositions of Table I determined from amino acid analysis were consistent with the known reported compositions (Timpl et al., 1977; Martinelli et al., 1979). The results of amino acid analysis of fibrinopeptide A and the synthetic fragments 11–25 and 16–25 were in agreement with the data of Blombäck & Vestermark (1958) and Doolittle et al. (1979b).

Antibody Preparation. Rabbits immunized with bovine F produced antisera which contained precipitating antibodies directed against F. The concentration of anti-F_N in the antisera, estimated by affinity chromatography on an anti-F_N column, was 1 mg/mL of serum.

Previous studies have indicated that antibodies raised against proteins in their native form are specific for the native conformation of these proteins (Sachs et al., 1972; Furie et al., 1975; Anfinsen & Scheraga, 1975; Berzofsky et al., 1976b; Smith et al., 1977). Therefore, immunization with the native protein would not be expected to yield significant amounts of antibody to the unfolded form, as discussed by Sachs et al. (1972), Hurrell et al. (1977a), and Chavez & Scheraga (1980b).

Preparation of [^{125}I]F. Bovine F was trace labeled with ^{125}I to high specific activity by means of the lactoperoxidase/glucose oxidase method of iodination. Greater than 95% of the radioactivity, contained in an aliquot of the first radioactive peak isolated after passage of the iodination reaction mixture through a Sephadex G-25 column, could be precipitated with 10% Cl_3CCOOH or with excess anti-F_N antibody. The average percent recovery of F from the iodination procedure was 55%. The percent incorporation of ^{125}I into F ranged from 55% to 72%, yielding an average of 0.3 mol of ^{125}I/mol of F. The average specific activity of the radiolabeled F was 3.2×10^6 cpm/μg.

The Aα chain of *bovine* DSK does not contain any tyrosyl residues (Martinelli et al., 1979). Presumably, within the DSK and near the antigenic determinant under investigation, tyrosyl residues Bβ-48, Bβ-117, and γ-1 of bovine F (Chung et al., 1981) have been iodinated, as suggested by the work of York & Blombäck (1979) with *human* F. Additional tyrosyl residues in bovine F (not present in human F) may be found at Bβ-6 and Bβ-26 (Chung et al., 1981), and these residues may also have been iodinated. Major changes in the conformation of [^{125}I]F as a result of iodination by the lactoperoxidase method have been ruled out for the following reasons: (1) the

[5] Since the antigenic determinant within P_N is, by definition, in all respects identical with the corresponding antigenic determinant within the native protein (see footnote 3), the association constants for the binding of antibody to P_N and to F_N are therefore assumed to be equal. The value is determined experimentally by the reaction of the antibody with the native protein.

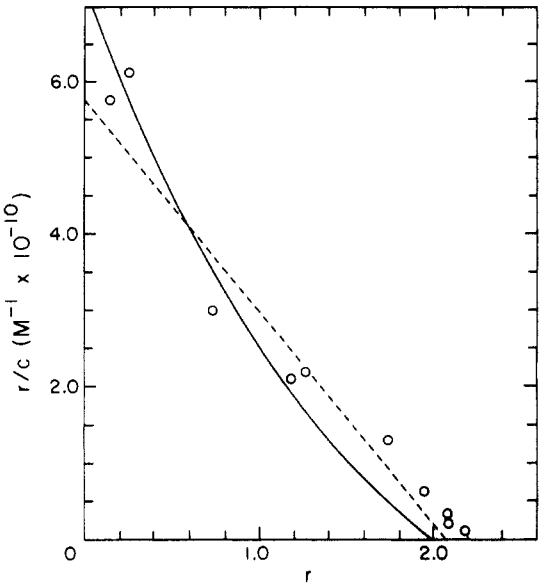

FIGURE 3: Scatchard plot for the binding of anti-$(16–25)_N$ to [^{125}I]F at 4 °C. (O) Experimental data; (---) least-squares line; (—) curve calculated from the value of K_{assoc} determined by using the equation of Calvert et al. (1979).

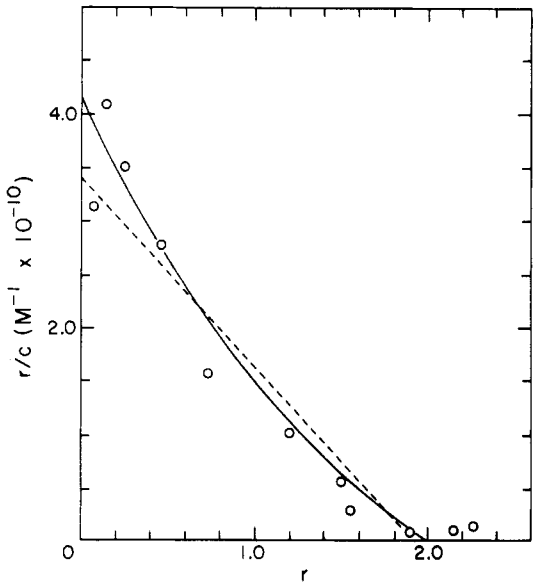

FIGURE 4: Scatchard plot for the binding of anti-$(11–25)_N$ to [^{125}I]F at 4 °C. See the legend for Figure 3.

level of incorporation of I atoms per F molecule has been kept low (York & Blombäck, 1979), (2) [^{125}I]F was 92% clottable as compared to 95% for the unlabeled material, and (3) an equivalent concentration of F gave 50% inhibition of precipitation of [^{125}I]F by both anti-$(11–25)_N$ and anti-$(16–25)_N$, indicating that there was no inactivation of the antigenic activity of [^{125}I]F by iodination.

Determination of K_{assoc}. The Scatchard plots for the binding of anti-$(16–25)_N$ and anti-$(11–25)_N$ antibodies to [^{125}I]F at 4 °C are shown in Figures 3 and 4, respectively. Since F is a dimeric protein, it has two potential (equivalent) binding sites for each antibody molecule isolated to the particular antigenic determinant within fragments 11–25 and 16–25. The effect of multideterminant antigens on the conventional Scatchard plot analysis for the binding of antibody to antigen has been discussed by Berzofsky et al. (1976a), Calvert et al. (1979), and Steensgaard et al. (1980). A general binding equation has been derived for the case of a bivalent ligand and a multivalent acceptor (Calvert et al., 1979).

For the present case of a bivalent ligand (F) and a bivalent acceptor (Ab), the general binding equation [eq 9a,b of Calvert et al. (1979)] has been used to determine the best values of K_{assoc} for anti-$(16–25)_N$ and anti-$(11–25)_N$ from the experimental data presented in Figures 3 and 4. Linear least-squares regression analyses of the binding data for anti-$(16–25)_N$ and anti-$(11–25)_N$ (dashed lines in Figures 3 and 4, respectively) were used to determine initial estimates of K_{assoc} for each antibody. With these estimates, the best value of K_{assoc} for each antibody was found by using a nonlinear least-squares method (Bevington, 1969) which used the free ligand concentration c as the independent variable and r [as given by eq 9a,b of Calvert et al. (1979)] as the dependent variable (Rodbard & Tacey, 1978; Munson & Rodbard, 1980; Faure et al., 1980). The values of K_{assoc} for anti-$(16–25)_N$ and anti-$(11–25)_N$ have thus been determined as $(1.2 \pm 0.2) \times 10^{10}$ and $(7.5 \pm 0.5) \times 10^{9}$, respectively. The solid curves in Figures 3 and 4 have been calculated by using these best values of K_{assoc} for each antibody population.

The limiting value of r for each antibody was found to be equal to 2 (see Figures 3 and 4), in agreement with simulated Scatchard plots for the case of a bivalent ligand and a bivalent acceptor (Nichol & Winzor, 1976). This supports the fact that there was no steric hindrance of antigen binding to antibody, i.e., one antibody molecule may indeed bind to two F molecules, as already demonstrated by the immunoelectron microscopy studies of Telford et al. (1980).

The simulation studies (Nichol & Winzor, 1976) also reveal the fact that, for a bivalent ligand and a bivalent acceptor, the binding curves exhibit a dependence on acceptor concentration that is not found for a univalent antigen. Depending on the concentration of the acceptor, the Scatchard plot for a bivalent ligand may be linear, concave, or convex. Therefore, in contrast to the case of a monovalent antigen,[6] deviations from linearity cannot be taken as an indication of cooperativity of antibody combining sites or the presence of heterogeneous populations of antibody. The fact that the experimental binding data may be fit by using the model of Calvert et al. (1979), derived under the assumption of homogeneous reactants, so that one binding constant governs all equilibria, supports the conclusion that these affinity-purified antibody populations are homogeneous with respect to their binding constants and their specificity.

Determination of K_{conf}. Inhibition of binding of [^{125}I]F to anti-$(16–25)_N$ by unlabeled F, the DSK, CNBrAα, fragment 11–25, fragment 16–25, and FpA at 4 °C is shown in Figure 5. The error symbols represent 95% confidence limits for the experimental points (±2 standard deviations). The experimental points have been treated by using a logit analysis (Rodbard & Hutt, 1974), and the curve drawn is derived from the results of this analysis. The values of K_{conf} determined from the data of Figure 5 and those calculated from the best-fit curves, respectively, are given in Table II. Values of K_{conf} have also been determined by using anti-$(11–25)_N$ purified from anti-F_N. The competitive RIA data are presented in Figure 6, and the values of K_{conf} determined from these data are given in Table III. The values of K_{conf} for the monovalent competitive inhibitors CNBrAα, fragment 11–25, and frag-

[6] When r is defined as [bound antigen]/[total antibody] and c as [free antigen], and the binding data are plotted as r/c vs. r, the resulting Scatchard plot should be linear for a *monovalent* antigen–homogeneous antibody population; the slope would then represent the negative of the association constant, K_{assoc}. For a monovalent antigen, nonlinearity in the Scatchard plot would then be attributed either to cooperativity of the two combining sites on the same antibody molecule or to the presence of multiple populations of antibody each with its own value of K_{assoc} (Rodbard & Bertino, 1973).

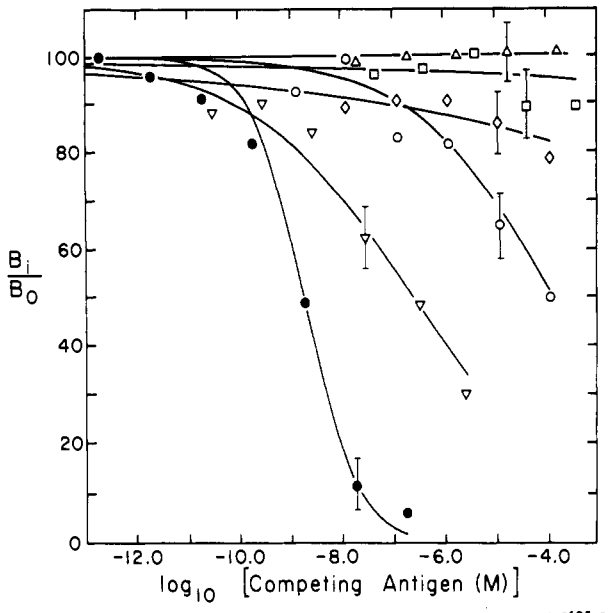

FIGURE 5: Competitive binding curves for anti-$(16–25)_N$ and [^{125}I]F in the presence of unlabeled F (●), DSK (▽), CNBrAα (○), fragment 11–25 (◇), fragment 16–25 (□), and fibrinopeptide A (△). All samples were incubated for 24 h at 4 °C. Binding in the absence of competitive inhibitor corresponds to 100% [^{125}I]F bound. The curves were drawn by analyzing the data using a logit analysis (see Materials and Methods).

Table II: Values of K_{conf} for Derivatives of Fibrinogen Using Anti-$(16–25)_N$ at 4 °C, pH 8.3

inhibitor	concn (M)	$K_{conf,exptl}$ [a]	$K_{conf,calcd}$
F	0 to 2.0×10^{-7}	*b*	*b*
DSK	0 to 3.1×10^{-5}	$(4.9 \pm 2.9) \times 10^{-3}$	$(3.2 \pm 1.8) \times 10^{-3}$
CNBrAα	0 to 1.4×10^{-4}	$(5.2 \pm 4.8) \times 10^{-5}$	$(3.6 \pm 1.6) \times 10^{-5}$
11–25	0 to 1.4×10^{-4}	$(2.0 \pm 1.8) \times 10^{-6}$	$(8.8 \pm 4.4) \times 10^{-7}$
16–25	0 to 4.8×10^{-4}	$(8.8 \pm 7.3) \times 10^{-7}$	$(2.8 \pm 1.8) \times 10^{-7}$
FpA	0 to 2.1×10^{-3}	*c*	*c*

[a] The values of K_{conf} represent the average of three separate determinations using [^{125}I]F concentrations of 1.2×10^{-9}, 6.6×10^{-10}, and 1.3×10^{-10} M. The Ab concentration was 3.5×10^{-11} M throughout. The mixture of all species was incubated for 24 h at 4 °C. The values of K_{conf} for F and the DSK were determined by using eq 6 of Chavez & Scheraga (1980b), while the values of K_{conf} for CNBrAα, fragment 11–25, and fragment 16–25 were determined by using eq 6 of this paper (see Results). [b] Equation 6 of Chavez & Scheraga (1980b) leads to infinite theoretical values, because of the neglect of $[F_R]$. If this term were included, it would make a very small contribution in both the numerator and denominator of eq 6. Thus, the actual values are really finite and very large, but inaccurate, because of the small differences between two large numbers in the denominator of eq 6. [c] Below the limit of detection.

ment 16–25 were determined by using eq 6. The values of K_{conf} for the divalent competitive inhibitors F and the DSK were determined by using eq 6 of Chavez & Scheraga (1980b) since $K_1 = K_2$ for these dimeric competitive inhibitors.

When unlabeled F_N is used as the inhibitor (i.e., if $[Ab_N{\cdot}F_N]$ is substituted for $[Ab_N{\cdot}P_N]$ and $[F_N] + [Ab_N{\cdot}F_N]$ is substituted for $[P_{tot}]$ in eq 6), then the value of K_{conf} should become infinite since $[F_R]$ is taken as zero. The experimentally determined value of K_{conf} for F_N is a very large finite number because of a nonzero $[F_R]$. The fact that the experimental values of K_{conf} are very large for unlabeled F for both antibodies studied

Table III: Values of K_{conf} for Derivatives of Fibrinogen Using Anti-$(11–25)_N$ at 4 °C, pH 8.3

inhibitor	concn (M)	$K_{conf,exptl}$ [a]	$K_{conf,calcd}$
F	0 to 6.0×10^{-7}	*b*	*b*
DSK	0 to 1.4×10^{-5}	$(3.4 \pm 2.2) \times 10^{-3}$	$(3.9 \pm 1.1) \times 10^{-3}$
CNBrAα	0 to 6.6×10^{-4}	$(5.4 \pm 4.2) \times 10^{-5}$	$(4.8 \pm 2.9) \times 10^{-5}$
11–25	0 to 1.3×10^{-4}	$(3.4 \pm 2.7) \times 10^{-7}$	$(3.2 \pm 1.9) \times 10^{-7}$
16–25	0 to 4.4×10^{-4}	$(2.9 \pm 2.4) \times 10^{-7}$	$(1.6 \pm 1.2) \times 10^{-7}$
FpA	0 to 3.0×10^{-3}	*c*	*c*

[a] The Ab concentration and the [^{125}I]F concentration were 5.2×10^{-11} and 2.8×10^{-9} M throughout. The mixture of all species was incubated for 24 h at 4 °C. [b] See footnote *b* in Table II. [c] Below the limit of detection.

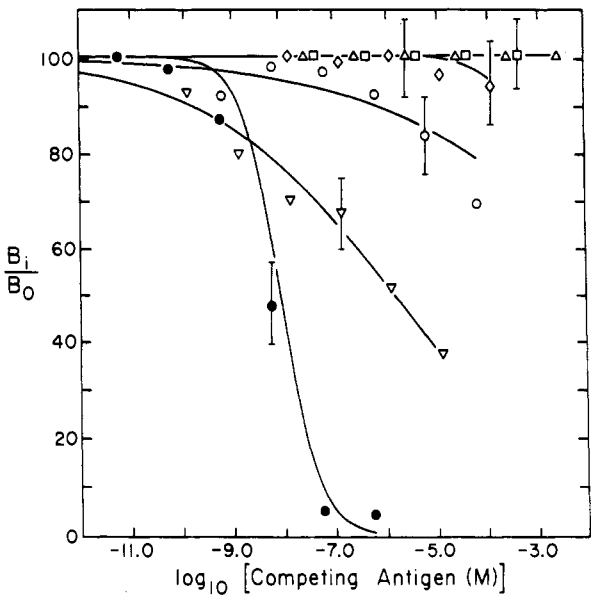

FIGURE 6: Competitive binding curves for anti-$(11–25)_N$ and [^{125}I]F in the presence of unlabeled F (●), DSK (▽), CNBrAα (○), fragment 11–25 (◇), fragment 16–25 (□), and fibrinopeptide A (△). All samples were incubated for 24 h at 4 °C. Binding in the absence of competitive inhibitor corresponds to 100% [^{125}I]F bound. The curves were drawn by analyzing the data using a logit analysis (see Materials and Methods).

confirms that trace-labeled [^{125}I]F exists in the native conformation and is not detectably denatured in the regions of the antigenic determinants.

Inhibition by the DSK and CNBrAα was less efficient in the displacement of [^{125}I]F than that by unlabeled F by nearly 3 and 5 orders of magnitude, respectively, in the concentration of inhibitor (see Figures 3 and 4). Approximately a 1000-fold increase in DSK concentration relative to that of F_N is required to displace 50% of the [^{125}I]F, while a 100 000-fold excess of CNBrAα is required to achieve 50% displacement of [^{125}I]F. Fragment 11–25 and fragment 16–25 show only slight inhibition at a 10^6-fold excess in concentration. No inhibition of the binding of [^{125}I]F to anti-$(16–25)_N$ or anti-$(11–25)_N$ by FpA could be detected even at 10^7-fold excess in concentration. The minimum inhibition that can be determined experimentally is ±200 cpm in 50 000 total cpm when 10% is bound (Rodbard, 1978). This limit of detection established the value of K_{conf} for FpA as $<10^{-7}$.

As F is degraded progressively to the DSK, to CNBrAα, and finally to the synthetic peptides, there is a decrease in the values of K_{conf} for the antigenic determinant within this region

Table IV: Comparison of Kinetic Constants for the Hydrolysis of Arg-Gly Bonds by Thrombin (at pH 8.0 and 25 °C) with K_{conf} (at pH 8.3 and 4 °C)

substrate	$k_{cat} \times 10^{11}$ [M (NIH units/L)$^{-1}$ s^{-1}]	$K_M \times 10^6$ (M)	K_{conf}[a]
fibrinogen[b]	73 ± 50%	9.2 ± 75%	c
DSK	d	d	$(4.2 \pm 2.6) \times 10^{-3}$
CNBrAα[e]	48 (range 38-330)	47 (range 20-500)	$(5.3 \pm 4.5) \times 10^{-5}$
11-25[e]	11 ± 33%	680 ± 18%	$(11.0 \pm 10.0) \times 10^{-7}$
16-25[e]	0.30 ± 90%	630 ± 70%	$(5.9 \pm 4.9) \times 10^{-7}$

[a] The values in this column represent the averages of the values of K_{conf} determined by using both anti-$(11-25)_N$ and anti-$(16-25)_N$. [b] These values pertain to the Aα chain of the intact fibrinogen molecule (Martinelli & Scheraga, 1980). [c] See footnote *b* in Table II. [d] No value of k_{cat} or of K_M has been reported for the DSK, although it is known that thrombin will release FpA from the DSK at nearly the same rate as it releases this peptide from the F molecule (Hogg & Blombäck, 1974). [e] The kinetic parameters for CNBrAα were determined by Hageman & Scheraga (1974) and those for 11-25 and 16-25 by van Nispen et al. (1977).

of the Aα chain, as can be seen from the data of Tables II and III. Within experimental error, the values of K_{conf} showed no systematic variation as the concentrations of peptide fragments were varied. This indicates that the equilibria of eq 1-3 are not perturbed by the assay procedure. Purification of anti-F_N on a Sepharose–fragment 11-25 column did not result in the isolation of an antibody population that recognized any additional antigenic activity within fragment 11-15, since fragment 11-25 did not inhibit the binding of [^{125}I]F to anti-$(11-25)_N$ more effectively than fragment 16-25. The similarity of the data of these two tables, for a given inhibitor, provides further support for the suggestion that this antigenic determinant most likely lies within residues 16-25.

Table IV contains a summary of the values of K_{conf} determined in this work as well as the values of k_{cat} and K_M previously determined for the fragments under study (Hageman & Scheraga, 1974; van Nispen et al., 1977; Martinelli & Scheraga, 1980).

Discussion

Antigenic Region on Fibrinogen. The values of K_{conf} for unlabeled F, the DSK, CNBrAα, fragment 11-25, fragment 16-25, and FpA lead to several conclusions about the structure of the antigenic determinant in the region of residues 16-25, both in the Aα chain of native F and within this region in all of the fragments studied. The DSK, CNBrAα, fragment 11-25, and fragment 16-25 do possess antigenic activity, although the values of K_{conf} for these fragments are several orders of magnitude lower than the value of K_{conf} for native F. These lower values of K_{conf} indicate that the derivatives of F_N possess fewer conformation-stabilizing interactions in the region of the antigenic determinant than native F_N. The fact that FpA shows no inhibition indicates that the native antigenic determinant within fragment 16-25 either (1) spans the Arg_{19}–Gly_{20} bond or (2) requires a specific conformation within residues Gly-Gly-Val-Arg, residues 16-19, that is stabilized by long-range interactions not present in FpA.

The value of K_{conf} for CNBrAα is an order of magnitude greater than the corresponding values of K_{conf} for fragments 11-25 and 16-25, which do not differ significantly from each other (see Tables II and III). This increased inhibitory capability of CNBrAα supports the hypothesis that residues outside of residues 11-25 are necessary for the stabilization of the native conformation in the region containing the site of hydrolysis by thrombin (Scheraga, 1977; Blombäck et al., 1977; Hogg & Blombäck, 1974, 1978). Since the synthetic fragments with the *human* F sequence served satisfactorily here to fractionate antibodies to *bovine* F by affinity chromatography, it appears that differences in sequence between human and bovine F in these limited regions[2] have little effect on the antigenicity of this region, in agreement with the observations of Tanswell et al. (1978). Further, since the values of K_{conf} for *bovine* F (and, likewise, the values of K_{assoc}) are similar no matter whether anti-$(16-25)_N$ or anti-$(11-25)_N$ is used, it may be concluded that the antigenic region may consist of residues 16-25. This conclusion is supported by the similarity of the values of K_{conf} in Tables II and III for the derivatives of F. Further, the similarity of the values of K_{conf} for fragments 11-25 and 16-25, despite the presence of a free α-amino group on Gly_{16} in the latter fragment, suggests that Gly_{16} may not be part of the antigenic determinant, which is therefore localized at residues 17-25. This would account for the absence of binding of FpA to these antibodies since FpA contains only three residues of this determinant (and its α-carboxyl group is free).

In summary, one of the antigenic regions in the Aα chain has been localized among residues 16-25, which spans the Arg_{19}–Gly_{20} thrombin-cleavable bond. Since this segment lies on the surface of the molecule, with a tendency of residues 21-22 to lie in the central portion of a β bend (Von Dreele et al., 1978), and antigenic determinants identified in proteins often include β bends (Rae et al., 1981), we speculate that the Arg_{19}–Gly_{20} bond is in a structure near a β bend and that the conformational integrity of this structure is essential for the binding of the antibodies isolated here and also for the binding of thrombin.

Before discussing the numerical values of K_{conf}, we must consider the nature of the antigenic determinant. Antibodies are formed against antigenic determinants which constitute parts of the surface of the protein. Each local region of the protein surface has its own unique chemical and physical properties. The antigenic surface must be complementary to the molecular surface of the antibody combining site; however, the exact nature of an antigenic determinant, i.e., the exact molecular structure of the surface in contact with the antibody, is still not well understood. A "primary antigenic determinant" has been defined (Hurrell et al., 1977b) as "any single linear sequence of amino acids which, in isolation, exhibits antigenic activity". It has been suggested that two or more such sequences, spatially close to each other but not necessarily linear in sequence, may be required to provide a complete description of the spatial antigenic domain in the native protein (East et al., 1980, 1981).

The exact way in which certain residues external to the linear "primary" determinant influence the binding of antibody to this determinant is still an unsettled matter. In the absence of a crystal structure of an antibody–protein antigen complex, the role of these external amino acid residues may be attributed to a number of possibilities: (1) The native conformation of a linear sequence is required for efficient binding to antibody (Atassi, 1975, 1978; Ibrahimi et al., 1979), and the interaction of these external residues with this linear sequence helps preserve the native conformation. (2) In addition to the linear sequence, these external residues may participate by *direct* involvement in binding between antibody and antigen (Hurrell et al., 1977b; East et al., 1980, 1981). (3) These external residues may be determinants for *other* antibodies, and the formation of these *other* antigen–antibody complexes may induce a conformational change in the antigenic region of

interest so that it can then bind to its own specific antibody (Celada & Strom, 1972). The use of monoclonal antibodies rather than polyclonal antisera would enable possibility 3 to be assessed; however, possibilities 1 and 2 cannot be distinguished at present.

It, therefore, must be considered that poor inhibition of antibody binding to a native protein by an excised peptide may be due to the absence of these surrounding residues and not entirely to incomplete formation of the native conformation within these peptides. There remain two possible explanations for the low values of K_{conf} for the synthetic peptides: (1) Residues near the spatial region of 16–25 may be required to fold residues 16–25 into the native (antigenic) conformation, by long-range interactions; however, only residues 16–25 interact directly with the antibody combining site. (2) Residues near the spatial region of 16–25, in addition to residues 16–25, constitute the antigenic determinant, the entirety of which interacts with the antibody combining site.

The values of K_{conf} determined for CNBrAα indicate that this fragment possesses conformation-stabilizing interactions not present in the synthetic fragments. Considering the amino acid sequences of both human and bovine fibrinogen, many amino acid residues on both sides of the site of thrombin cleavage of CNBrAα, extending from Phe in FpA to residue 32 in CNBrAα, are conserved in this peptide (Timpl et al., 1977; Martinelli et al., 1979). These residues may be necessary for the stabilization of the native conformation within this region. These residues alone, however, are not sufficient for the stabilization of the intact native conformation within this region. The difference in the values of K_{conf} between CNBrAα and F might also be attributed, however, to a perturbation of the native three-dimensional structure in CNBrAα brought about by CNBr cleavage and by carboxymethylation of its Cys residues.

For the case of the DSK, however, all of the residues surrounding the region under study are present, since the DSK has been shown to occupy the central nodule of F (Telford et al., 1980). Therefore, the decreased inhibitory activity of this fragment must be due to conformational differences between it and the corresponding portion of the native F molecule. Presumably, this antigenic determinant within the DSK has undergone an alteration from its native conformation when the DSK was cleaved from the native F molecule.

Significance of K_{conf}. The values of K_{conf} measured for a fragment pertain to that portion of the amino acid sequence which forms the antigenic determinant, not to the entire fragment. Following the arguments of Anfinsen & Scheraga (1975), we may evaluate the significance of the magnitudes of the values of K_{conf} in Tables II and III as follows. As an approximate calculation, if we assume that each amino acid residue of the fragment can exist in five discrete backbone conformational states, each of which is accessible with equal probability, then there are 5^{10} or 1×10^7 possible conformations of a fragment containing 10 residues, such as fragment 16–25, of which the native conformation is only one. Thus, if the native conformation of the antigenic determinant within these 10 residues were attained as a random event, then K_{conf} for this antigenic determinant would be approximately 1×10^{-7}. Values of K_{conf} which are greater than 1×10^{-7} indicate the presence of additional conformation-stabilizing interactions that increase the probability that the fragment will attain the native conformation.

When viewed in this perspective, it can be concluded that the synthetic peptides are in essentially a random or unordered conformation. A value of K_{conf} of $\sim 5 \times 10^{-3}$ for the antigenic determinant within the DSK indicates that the percentage of DSK molecules with the native conformation is greater by 4 orders of magnitude than the percentage of the synthetic fragments having the native conformation; many of the DSK molecules, however, are still in an ensemble of nonnative forms. A value of K_{conf} of 5×10^{-3} corresponds to a standard free energy change of approximately 3 kcal at 300 K, for the nonnative-to-native equilibrium. In order to increase K_{conf} to 10^3 (i.e., to make the fragment become essentially completely native), an additional 7 kcal must be supplied from long-range stabilizing interactions or ~ 0.5 kcal per residue, for an antigenic determinant of 15 residues. It is reasonable to expect that an additional 0.5 kcal per residue can be obtained from long-range interactions, when the fragment interacts with the rest of the protein molecule to form the native structure.

As discussed by Anfinsen & Scheraga (1975), this immunochemical approach almost certainly does not measure the presence of fully folded peptide fragments because the antibody might also recognize many almost-native conformations. A tendency to form the native and near-native structure exists, because of short- and medium-range interactions (Scheraga, 1973, 1978); however, long-range interactions are necessary for folding to the *stable* native conformation. Since the precise nature of the conformation recognized by the antibodies and the fidelity of that recognition are not known, the precise values measured in the conformational equilibrium experiments appear to be less meaningful than the *relative* values for the same determinant within a series of fragments of a protein, using the same antibody population (Creighton et al., 1978).

Immunochemistry of Fibrinogen. The values of K_{conf} determined in this work correlate well with the results of other immunological studies of F. Previous analyses have shown that some CNBr fragments of bovine F do not react strongly with antibodies to native bovine F (Timpl et al., 1975). When antibodies directed against native F are used, it has been reported that only very weak precipitin lines are formed with the DSK (Blombäck & Blombäck, 1972; Timpl et al., 1975; Telford et al., 1980). A previous interpretation of these observations was that the possible antigenic structures on the DSK were buried in the native molecule (Blombäck et al., 1973). An alternative hypothesis (based on the results of our present work) would be that recognition of native antigenic determinants within these fragments by antibodies to native F requires that these fragments adopt conformations similar to those of the native protein. This implies that the conformation of the DSK in the intact F molecule may not be that of the excised DSK fragment. Our examination of a particular antigenic determinant within the DSK does indeed suggest that conformational changes accompany CNBr cleavage, at least in this one region, as reflected by the value of K_{conf} for the DSK.

Other studies also suggest that stabilization of the native conformation within the *entire* N-terminal region of F requires the presence of the *intact* F molecule. When anti-F and [^{125}I]F were employed in a competitive RIA, a sequential loss of native antigenicity was found as F was degraded with plasmin from F to Fg-X, Fg-Y, Fg-D:E, Fg-D, and finally Fg-E[7] (Plow & Edgington, 1973). Fg-E, even at a 3 orders of magnitude increase in concentration relative to F, was found to be unable to compete against F for anti-F antibodies (in agreement with the results of the present work for DSK). Therefore, the conformation of the N-terminal region of F appears to be stabilized by interaction with the rest of the F molecule.

[7] This nomenclature refers to the plasmin degradation products of F (Marder et al., 1969).

The immunochemistry of CNBrAα has also been studied (Tanswell et al., 1978, 1979). The region which spans the thrombin cleavage site in the Aα chain of F has been found to be antigenic in CNBrAα (Tanswell et al., 1978); our assignment of the antigenic region to residues 16–25 of the Aα chain is in agreement with this result. From the fact that antisera to the isolated Aα chain showed stronger binding with CNBrAα than antisera to native F or to the DSK (Tanswell et al., 1978), it was concluded that a closer similarity exists between the conformation of the antigenic determinants within CNBrAα and the corresponding determinants in the unfolded Aα chain, compared to the conformation of these determinants in native F or in the DSK (Tanswell et al., 1978). The results of our present study support the conclusion that CNBrAα does not retain a large proportion of its native conformation when it is excised from the parent F molecule.

Both FpA and FpB are located within the central nodule of F (Telford et al., 1980), but their precise spatial location relative to the exposed surface and to other parts of the F molecule is not known. Structural information concerning FpA and the region of its attachment to F may be inferred from the results of the present and other immunochemical work. In the development of a RIA specific for free FpA (Nossel et al., 1971, 1974), two different rabbit antisera (R2 and R33), prepared against human FpA conjugates, were found to possess different degrees of cross-reactivity with F. A comparison of these two antisera (Canfield et al., 1976) showed that one of them (R2) exhibited high specificity for FpA and less than 2% cross-reactivity with F or FpA-containing fragments such as the DSK, CNBrAα, and fragment E (a plasmin degradation product of F similar to the DSK). This antiserum (R2) was found to recognize an antigenic determinant within residues 7–16 of FpA (Wilner et al., 1976). The other antiserum (R33) possessed a much higher degree of cross-reactivity with FpA-containing fragments (DSK, CNBrAα, and E) and was found to react with residues on the amino-terminal side of Asp_7 in FpA (Wilner et al., 1976).

The relative selectivity of R2 antiserum for free FpA was explained by the inability of R2 antigenic determinants to achieve the particular conformation specified by the antibody combining site when these determinants are attached to the parent Aα chain (Wilner et al., 1979). On the other hand, R33, which reacts with antigenic determinants within the N-terminal portion of FpA, may be able to recognize these determinants in all of the F derivatives studied, thereby accounting for the cross-reactivity of this antiserum (Canfield et al., 1976). The N-terminal region of FpA may be freely accessible and in a random conformation even in the parent F molecule, while the C-terminal region may be in a fixed and specific conformation as part of the point of attachment of FpA to the rest of the F molecule.

The results of our experiments complement and support these studies of Nossel et al. (1974) and Canfield et al. (1976). Antisera induced against native F and then fractionated against a specific determinant, within residues 16–25 of the Aα chain, are able to recognize the native conformation of this limited portion of the Aα chain and distinguish those F derivatives which retain this conformation. Our results show that the native conformation within this particular antigenic determinant is stabilized by interactions present within an *intact* native molecule.

Correlation between K_{conf} and K_M. The values of k_{cat} and K_M previously determined for the fragments under study (Hageman & Scheraga, 1974; van Nispen et al., 1977; Martinelli & Scheraga, 1980) as well as the values of K_{conf} determined in this work are summarized in Table IV. The values of K_{conf} for the DSK, CNBrAα, fragment 11–25, fragment 16–25, and FpA indicate that (among other possibilities) these fragments do not have a completely native conformation around residues 16–25 of the Aα chain. The values of k_{cat} and K_M indicate that the kinetics of the thrombin-induced hydrolysis of the Arg–Gly bond within these fragments is less efficient as compared to that of native fibrinogen. The ability of thrombin to bind to these fragments, as indicated by the values of K_M, decreases as the size of the fragment decreases and, as suggested by the values of K_{conf}, as the fraction of native conformation decreases. The less efficient kinetics of hydrolysis may then be attributed to the incomplete formation of the native conformation around residues 16–25 of the Aα chain. This implies that thrombin requires the native conformation of the Aα chain at or near residues 16–25 for proper binding and efficient hydrolysis. The proper interactions between both specific antibody and thrombin with these fragments evidently can occur only when the Aα chain in this region is held in the proper three-dimensional conformation by interactions with the rest of the molecule. It is probable that thrombin interacts with slightly different residues than the antibody within the region surrounding the cleavage site; e.g., thrombin interacts with Phe_{11}. It may be concluded, however, that the native conformation that is recognized by thrombin within F is not present to a large extent within the fragments studied in this work. On the other hand, the parallelism between K_M and K_{conf} suggests that fragment 11–25 does have all of the amino acid residues required for binding to thrombin; fragment 11–25 requires only the long-range interactions with the rest of the fibrinogen molecule in order to put it in the correct conformation for optimal binding to thrombin.

Mechanistic Implications. The implications of the results of this work for the mechanism of action of thrombin on fibrinogen may be summarized as follows. The immunochemical (as well as kinetic) results suggest that long-range interactions are necessary for the stabilization of the native structure of that (those) portion(s) of fibrinogen which interacts with thrombin. These long-range interactions may possibly involve an electrostatic interaction between Asp_{10} and Arg_{22} which might stabilize a β-bend structure near Arg_{19}–Gly_{20} and orient the Phe_{11} residue that is critical for effective thrombin action. The absence of these long-range interactions might be responsible for the delayed release of FpA seen in the case of fibrinogen Lille (Morris et al., 1981), in which Asp has been replaced by Asn, and in the case of fibrinogen Munich (Henschen et al., 1981), in which Arg has been replaced by Asn, resulting in a disruption of the proposed electrostatic stabilization.

Conclusion

The immunochemical results presented in this work suggest that long-range interactions are necessary for the stabilization of the native structure of F in the region of the antigenic determinant contained within residues 16–25 of the Aα chain. The less efficient kinetics of thrombin-induced hydrolysis previously observed for fragments of F containing these residues may be attributed to incomplete formation of the native conformation around residues 16–25 of the Aα chain. Presumably, fragment 11–25 contains all of the residues that bind to thrombin but lacks the long-range interactions to put it into the correct conformation for optimal binding to this enzyme.

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