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

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ABSTRACT: The conformations of the Bβ chain of the intact fibrinogen molecule and of various fragments of the Bβ chain of fibrinogen that contain the region that is hydrolyzed by thrombin have been compared by an 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. An antibody population specific for the native antigenic determinant within the Bβ fragment 20–28 was isolated by immunoadsorption. This preparation was 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), CNBrBβ, Bβ fragment 16–28, Bβ fragment 20–28, and fibrinopeptide B (FpB). ^{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 CNBrBβ, the values of K_{conf} at 4 °C were infinity, $(5.9 \pm 3.5) \times 10^{-3}$, and $(1.2 \pm 0.7) \times 10^{-3}$, respectively. The values of K_{conf} for Bβ fragment 16–28, Bβ fragment 20–28, and FpB at 4 °C were less than $(6.0 \pm 3.9) \times 10^{-7}$. These measurements indicate that the antigenic region in F is unperturbed by iodination and that 0.6% of the DSK molecules, 0.1% of the CNBrBβ molecules, and less than 0.00006% of the smaller fragments adopt the native conformation within the antigenic determinant in aqueous solution. The value for CNBrBβ is 2 orders of magnitude greater than that obtained earlier for CNBrAα [Nagy, J. A., Meinwald, Y. C., & Scheraga, H. A. (1982) *Biochemistry 21*, 1794]. The antigenic determinant involved in this study is localized among residues 20–28 of the Bβ chain of bovine fibrinogen (corresponding to residues 13–21 of the human Bβ chain). The immunochemical results, and kinetic data reported earlier [Hageman, T. C., & Scheraga, H. A. (1977) *Arch. Biochem. Biophys. 179*, 506], suggest that long-range interactions are necessary for the stabilization of the native structure in the region of fibrinogen that interacts with the antibody and with thrombin, respectively. In addition, the observed ability of the anti-$B\beta(20–28)_N$ antibodies to bind to intact native bovine F molecules supports the conclusion that the Arg–Gly region is accessible to thrombin-catalyzed hydrolysis in both the Aα and Bβ chains of native bovine and human fibrinogen [Martinelli, R. A., & Scheraga, H. A. (1980) *Biochemistry 19*, 2343; Hanna, L. S., Scheraga, H. A., Francis, C. W., & Marder, V. J. (1984) *Biochemistry 23*, 4681].

The rates of the thrombin-catalyzed release of fibrinopeptides[1] A and B from bovine and human fibrinogens have been measured in vitro prior to the gel point (Martinelli & Scheraga, 1980; Hanna et al., 1984) by using a high-performance liquid chromatography assay (Martinelli & Scheraga, 1979). The results of these studies have confirmed previous observations (Blombäck & Yamashina, 1957; Bilzekian et al., 1975; Higgins et al., 1983) that thrombin releases FpA more rapidly than FpB from fibrinogen. In addition, these experiments (Martinelli & Scheraga, 1980; Hanna et al., 1984) have shown that FpB is released from the start of the reaction, demonstrating that, initially, the Aα and Bβ chains of fibrinogen can compete with each other as substrates of thrombin and, in addition, that FpA cleavage is not obligatory for release of FpB, a finding confirmed by Hurlet-Jensen et al. (1982).

To determine the minimum-size peptide that possesses all of the residues essential for proper interaction of thrombin with the Bβ chain of fibrinogen and thereby elucidate the inherent structural properties of fibrinogen responsible for the observed differences in the initial rates of release of FpB compared to FpA, k_{cat} and K_M were determined for various peptide fragments of the Bβ chain of fibrinogen. The N-terminal cyanogen bromide cleavage fragment from the Bβ chain of bovine fibrinogen, CNBrBβ, was found to be a poor substrate for thrombin, as compared to the intact fibrinogen molecule (Hageman & Scheraga, 1977). Although the values of k_{cat} for the hydrolysis of CNBrBβ and the Bβ chain of fibrinogen are approximately equal, the value of K_M for CNBrBβ is 10–20

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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 upon hydrolysis by thrombin; FpA and FpB, fibrinopeptides A and B; CNBrAα, the N-terminal peptide of the Aα chain produced by CNBr cleavage of the Aα chain of F; CNBrBβ, the N-terminal peptide of the Bβ chain produced by CNBr cleavage of the Bβ chain of F; ^{125}I-F, fibrinogen iodinated with ^{125}I; 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; anti-X, antibody against X, where X is F or a fragment thereof; RIA, radioimmunoassay; ACC, *Ancistrodon contortrix contortrix*, the venom of which is capable of releasing FpB from F.

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Table I: Partial Amino Acid Sequences of Bovine and Human Bβ Chains

Bovine[a]

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	c 22	23	24	25	26	27	28
p-Glu-	Phe-	Pro-	Thr-	Asp-	Tyr(OSO_3H)-	Asp-	Glu-	Gly	-Gln-	Asp-	Asp	-Arg-	Pro-	Lys-	Val-	Gly-	Leu-	Gly-	Ala-	Arg-	Gly	-His-	Arg-	Pro-	Tyr-	Asp-	Lys

Human[b]

1	2	3	4	5	6	7	8	9	10	11	12	13	14	c 15	16	17	18	19	20	21
p-Glu-	Gly	-Val-	Asn-	Asp	-Asn-	Glu-	Glu-	Gly-	Phe-	Phe-	Ser-	Ala-	Arg-	Gly	-His-	Arg-	Pro-	Leu-	Asp-	Lys

[a] Doolittle & Blombäck (1964); Timpl et al. (1977); Martinelli et al. (1979). [b] Iwanaga et al. (1969); Watt et al. (1978); Hessel et al. (1979).. [c] This is the Arg–Gly bond that is hydrolyzed by thrombin to liberate FpB.

times larger than the value of K_M for the Bβ chain of fibrinogen (Hageman & Scheraga, 1977; Martinelli & Scheraga, 1980; Hanna et al., 1984). Thus, the overall rate of release of FpB from CNBrBβ is much less than that from the intact fibrinogen molecule. It has been suggested that the CNBrBβ fragment may contain all of the residues that interact directly with thrombin but, once removed from the fibrinogen molecule, it may no longer retain the proper conformation required for hydrolysis by thrombin (Hageman & Scheraga, 1977; Martinelli & Scheraga, 1980; Hanna et al., 1984).

Therefore, the purpose of the present work is to examine the conformation of the Bβ chain within fibrinogen and several of its fragments in the region of the thrombin-catalyzed hydrolysis. The equilibrium constant, K_{conf}, between the unfolded and native conformation of a protein fragment (Sachs et al., 1972), has been determined for bovine fibrinogen and for various peptide fragments containing the hydrolysis site in the Bβ chain, using the approach outlined by Nagy et al. (1982) for corresponding fragments of the Aα chain of fibrinogen. Anti-F_N antibodies,[2] induced in rabbits by immunization with native bovine F, have been fractionated to yield antibodies against fragment 20–28 of the Bβ chain. These antibodies have been used to determine the values of K_{conf} for this determinant within F, the DSK, CNBrBβ, Bβ fragment 16–28, Bβ fragment 20–28, and FpB. For reference, we list the relevant portions of the amino acid sequences of the bovine and human Bβ chains in Table I.

The results of the current work support the hypothesis that the conformation of CNBrBβ in solution is unlike the conformation that this fragment assumes within the native F molecule. In addition, the observed ability of anti-Bβ(20–28)$_N$ antibodies to bind to the intact native bovine F molecule supports the conclusion (Martinelli & Scheraga, 1980; Hanna et al., 1984) that the Arg–Gly region is accessible to thrombin in the native F molecule in both the Aα and the Bβ chains. Finally, the immunochemical data presented in this paper suggest that long-range interactions are necessary for the stabilization of the native fibrinogen-like conformation within fragments of the N-terminal portion of the Bβ chain. Similar findings have been reported for fragments of the N-terminal region of the Aα chain (Nagy et al., 1982).

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 and purification of the DSK and of CNBrBβ was carried out with the procedures outlined by Hageman & Scheraga (1977). Fibrinopeptide B was prepared from bovine F (Blombäck & Vestermark, 1958).

The synthesis, purification, and analyses of two peptides (H-Ala-Arg-Gly-His-Arg-Pro-Tyr-Asp-Lys-$NHCH_3$ and H-Val-Gly-Leu-Gly-Ala-Arg-Gly-His-Arg-Pro-Tyr-Asp-Lys-$NHCH_3$, corresponding to residues 20–28 and 16–28, respectively, of the Bβ chain of bovine F), the preparation and fractionation of anti-Bβ(20–28)$_N$ antibodies,[2] and the radioimmunoassays with ^{125}I-F are described in detail in the supplementary material (see paragraph at end of paper regarding supplementary material) and in Nagy et al. (1982).

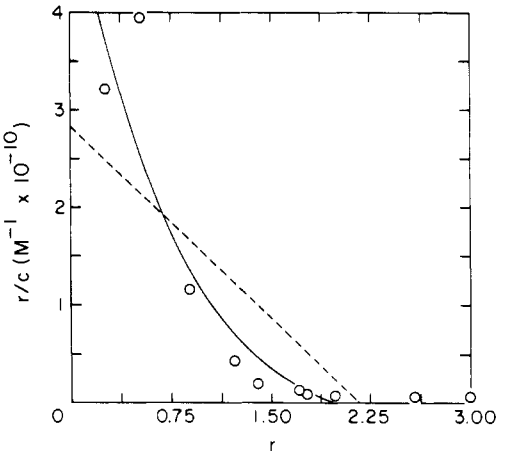

FIGURE 1: Scatchard plot for the binding of anti-Bβ(20–28)$_N$ to ^{125}I-F at 4 °C, where r = [bound antigen]/[total antibody], c = [free antigen], and the brackets designate molar concentration: (O) experimental data; (--) least-squares line; (—) curve calculated from the value of K_{assoc} determined by using the equation of Calvert et al. (1979).

Results

Purity of Peptide Derivatives. The results of the amino acid analyses of FpB and the synthetic fragments 20–28 and 16–28 were in agreement with the data of Timpl et al. (1977) and Martinelli et al. (1979). The results of the amino acid analysis of CNBrBβ are in agreement with the sequence data of Chung et al. (1981).

Determination of K_{assoc}. A respresentative Scatchard (1949) plot for the binding of anti-Bβ(20–28)$_N$ antibodies to ^{125}I-F at 4 °C is shown in Figure 1. The concentration of anti-Bβ(20–28)$_N$ in this experiment was 2.5×10^{-10} M, and that of ^{125}I-F was varied from 5.0×10^{-8} to 2.5×10^{-12} M. The binding data have been treated with the general binding equation of Calvert et al. (1979) as described by Nagy et al. (1982). Linear least-squares regression analysis of the binding

[2] In accordance with the nomenclature used previously (Sachs et al., 1972; Nagy et al., 1982), we designate the antibody populations purified by affinity chromatography with Sepharose–Bβ fragment 20–28 as anti-Bβ(20–28)$_N$, where the subscript N indicates that the antibodies were produced by using native F as the immunogen.

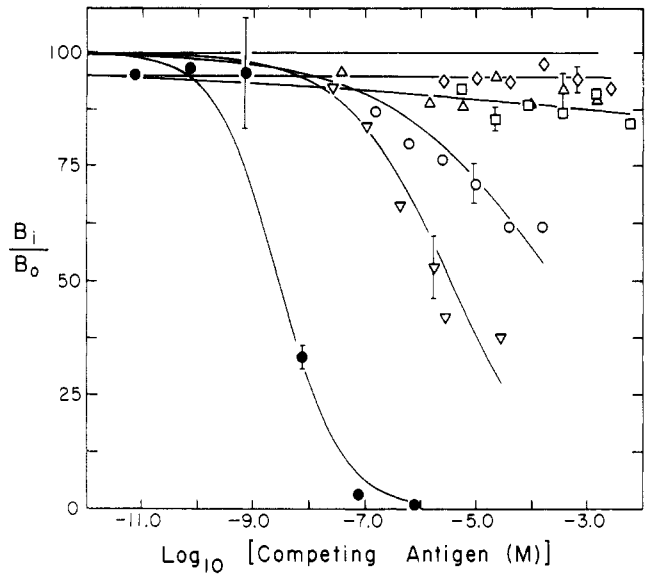

FIGURE 2: Competitive binding curves for anti-Bβ(20–28)$_N$ and ^{125}I-F in the presence of unlabeled F (●), DSK (▽), CNBrBβ (○), Bβ fragment 16–28 (◇), Bβ fragment 20–28 (□), and fibrinopeptide B (△). B_i is the percent of ^{125}I-F bound in the presence of a particular concentration of a competitive inhibitor, and B_0 is the percent of ^{125}I-F bound in the absence of any inhibitor. All samples were incubated for 24 h at 4 °C. The data have been normalized so that binding in the absence of any competitive inhibitor corresponds to 100% ^{125}I-F bound. The curves were drawn by analyzing the data with a "logit" analysis (Feldman & Rodbard, 1971; Rodbard & Hutt, 1974) as described by Nagy et al. (1982).

data (dashed line) was used to determine initial estimates of K_{assoc}. The best value of K_{assoc} was then found by using a nonlinear least-squares method described by Nagy et al. (1982). The value of K_{assoc} for anti-Bβ(20–28)$_N$ has thus been determined as $(5.1 \pm 1.8) \times 10^9$ M^{-1}. This value represents an average of four separate experimental determinations at several different concentrations of anti-Bβ(20–28)$_N$, viz., 2.5×10^{-10}, 3.8×10^{-10}, 5.0×10^{-10}, and 8.9×10^{-10} M. The solid curve in Figure 1 was calculated by using this best value of K_{assoc}.

The limiting value of r was found to be equal to 2, which is in agreement with the simulated Scatchard plots for the case of a bivalent ligand and a bivalent acceptor (Nichol & Winzor, 1976). In addition, the binding curve exhibits the characteristic nonlinearity predicted by the simulation studies of Calvert et al. (1979).

It should be noted that, for the case of a monovalent antigen and a bivalent antibody, deviations from linearity in the binding data represent either cooperativity of antibody combining sites or the presence of heterogeneous populations of antibody. However, in contrast, the experimental binding data for fibrinogen and the affinity-purified anti-Bβ(20–28)$_N$ antibody population are in agreement with the theoretically predicted nonlinear behavior for a system consisting of a bivalent antigen and a bivalent antibody, as shown by Calvert et al. (1979). The fit of the binding data to the theoretical formalism of Calvert et al. (1979) suggests, but does not prove, that the binding of fibrinogen to this affinity-purified anti-Bβ(20–28)$_N$ antibody subpopulation may be characterized by a single average affinity constant. It is these antibodies that were used to determine K_{conf} for the fragments of the Bβ chain.

Determination of K_{conf}. Inhibition of binding of ^{125}I-F to anti-Bβ(20–28)$_N$ by unlabeled F, the DSK, CNBrBβ, Bβ fragment 16–28, Bβ fragment 20–28, and FpB at 4 °C is shown in Figure 2. The error symbols represent 95% confidence limits for the experimental points (±2 standard deviations). The standard deviation of an experimental point, expressed as a percentage of that experimental value, was typically ±5%. The experimental points were 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 2 and other measurements (data not shown) and those calculated from the best fit curves for these data, respectively, are given in Table II. The values of K_{conf} for the *monovalent* competitive inhibitors CNBrBβ, Bβ fragment 16–28, Bβ fragment 20–28, and FpB were determined by using eq 6 of Nagy et al. (1982). The values of K_{conf} for the *divalent* competitive inhibitors, viz., F and the DSK, were determined by using eq 6 of Chavez & Scheraga (1980).

Table II: Values of K_{conf} for Derivatives of Fibrinogen Using Anti-Bβ(20–28)$_N$ at 4 °C, pH 8.3

inhibitor	concn (M)	$K_{conf,expti}$[a]	$K_{conf,calcd}$
F	$(0–8.0) \times 10^{-7}$	[b]	[b]
DSK	$(0–3.2) \times 10^{-5}$	$(5.9 \pm 3.5) \times 10^{-3}$	$(4.1 \pm 2.9) \times 10^{-3}$
CNBrBβ	$(0–6.3) \times 10^{-5}$	$(1.2 \pm 0.7) \times 10^{-3}$	$(1.2 \pm 0.9) \times 10^{-3}$
Bβ fragment 16–28	$(0–1.0) \times 10^{-2}$	[c]	[c]
Bβ fragment 20–28	$(0–3.2) \times 10^{-2}$	$(6.0 \pm 3.9) \times 10^{-7}$	$(6.0 \pm 3.9) \times 10^{-7}$
FpB	$(0–1.0) \times 10^{-2}$	[c]	[c]

[a] The values of K_{conf} represent the average of three separate determinations using ^{125}I-F concentrations of 1.3×10^{-9}, 5.6×10^{-9}, and 14.0×10^{-9} M. The Ab concentration was 2.5×10^{-10} 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 (1980), while the values of K_{conf} for CNBrBβ, Bβ fragment 16–28, and Bβ fragment 20–28 were determined by using eq 6 of Nagy et al. (1982). [b] Equation 6 of Chavez & Scheraga (1980) 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, because of the small differences between two large numbers in the denominator of eq 6 of Chavez & Scheraga (1980). [c] Below the limit of detection, hence $\leq 1.0 \times 10^{-7}$.

Inhibition by the DSK and CNBrBβ was less efficient in displacement of ^{125}I-F than that by unlabeled F by approximately 2 and 3 orders of magnitude, respectively, in the concentration of inhibitor (see Figure 2). Approximately a 100-fold increase in the DSK concentration relative to that of F_N is required to displace 50% of the ^{125}I-F, while a 1000-fold excess of CNBrBβ is required to achieve 50% displacement of ^{125}I-F. The Bβ fragment 20–28 shows only a slight (≤15%) inhibition at approximately a 10^6-fold excess in concentration. No inhibition of the binding of ^{125}I-F to anti-Bβ(20–28)$_N$ by Bβ fragment 16–28 or by FpB could be detected even at a 10^7-fold excess in concentration. Statistically significant inhibition of the binding of ^{125}I-F to anti-Bβ(20–28)$_N$ by a particular antigen occurs only when binding in the presence of the competing antigen is at least 2 standard deviations less than binding in the absence of competing antigen (Feldman & Rodbard, 1971). In our particular system, since the average standard deviation is 5%, B_i/B_0 in the presence of an inhibitor must be less than 90% for statistical significance.

The competitive RIA data indicate the relative ability to achieve inhibition of binding of a labeled antigen to an antibody population by a series of fragments of the unlabeled antigen as inhibitors. Sachs et al. (1972) have developed a method based upon competitive inhibition data, as well as on several assumptions [summarized by Anfinsen & Scheraga (1975) and reiterated in Nagy et al. (1982)], whereby quan-

titative estimates of the probability of a polypeptide chain attaining a particular conformation may be calculated from the immunochemical data. The values of K_{conf} calculated from the inhibition data represent the equilibrium constant for the interconversion of the nonnative and native conformations of a particular determinant within a series of fragments and serve as an index for easy comparison of these fragments (see the supplementary material for further discussion of this point).

As F is degraded progressively to the DSK, to CNBrBβ, and finally to those regions represented by the synthetic peptides, there is a decrease in the values of K_{conf} for the antigenic determinant within this region of the Bβ chain, as can be seen from the data of Table II. The values of K_{conf} calculated for each fragment from its inhibition data indicate that these fragments of F do not retain the native fibrinogen-like conformation within the antigenic determinant under study when the fragments are excised from the F molecule. Several of the values of K_{conf} determined for the various fragments of F are of particular interest. First, for the particular antigenic determinant within the Bβ chain under study, the value of K_{conf} for CNBrBβ differs from that for the DSK by less than 1 order of magnitude. Second, the value of K_{conf} for the Bβ fragment 16–28 is less than that for the Bβ fragment 20–28 despite the presence of an additional four residues at the N-terminus of Bβ fragment 16–28. The structural implications of these results are discussed below.

Discussion

Antigenic Region of Fibrinogen. The values of K_{conf} given in Table II lead to several conclusions concerning the structure of the antigenic determinant in the region of residues 20–28 within the Bβ chain of native bovine F and within this region in the fragments studied. Both FpB and Bβ fragment 16–28 show essentially no inhibitory capability, since binding in the presence of either of these two competing antigens is not significantly different from binding in the absence of these antigens (see Figure 2). The limits of detection in these experiments (Nagy et al., 1982) establish the value of K_{conf} for the Bβ fragment 16–28 and FpB as $\leq 10^{-7}$. The fact that FpB shows no inhibition even at 10^7-fold excess in concentration relative to unlabeled F indicates that the native antigenic determinant within Bβ fragment 20–28 either (1) spans the Arg_{21}–Gly_{22} bond or (2) requires a specific conformation within residues Ala_{20}–Arg_{21} that is stabilized by long-range interactions provided by residues not present in FpB. For the case of Bβ fragment 20–28, the observed inhibition of approximately 15% at a 10^6-fold excess in concentration of Bβ fragment 20–28 relative to unlabeled F is significantly greater than any inhibition that might be attributable to the experimental error in the measurement. The data yield a value of K_{conf} for this fragment of $(6.0 \pm 3.8) \times 10^{-7}$. The fact that B$\beta$ fragment 16–28 is a poorer inhibitor than Bβ fragment 20–28, as indicated by the lower value of K_{conf} for Bβ fragment 16–28 relative to that of Bβ fragment 20–28, implies that the presence of the additional residues Val_{16}-Gly-$_{17}$-Leu-$_{18}$-Gly_{19} disrupts those long-range stabilizing interactions present in Bβ fragment 20–28, which give Bβ fragment 20–28 its limited inhibitory capacity.

The value of K_{conf} for CNBrBβ is about 3 orders of magnitude greater than the corresponding value of K_{conf} for Bβ fragment 20–28. This larger inhibitory capability of CNBrBβ relative to that of Bβ fragment 20–28 supports the hypothesis that residues outside of Bβ fragment 20–28 are necessary for the stabilization of the native conformation in the region containing the site of hydrolysis by thrombin (Hageman & Scheraga, 1977).

The value of K_{conf} for CNBrBβ is of the same order of magnitude as that for the DSK. This result points out the fact that most of the residues contributing to the stabilization of the native conformation of this particular antigenic determinant within the DSK are also present within CNBrBβ. However, since approximately a 1000-fold molar excess of the DSK relative to unlabeled F is required to achieve 50% inhibition of ^{125}I-F, the DSK, as well as CNBrBβ, lack the contributions of additional conformation-stabilizing interactions provided by residues present in the intact native F molecule.

The decreased inhibitory activity of the DSK fragment relative to unlabeled F suggests that a conformational change accompanies the generation of the DSK by CNBr cleavage, at least in this particular region as reflected by the values of K_{conf} for the Bβ chain determinant in the DSK. Furthermore, the results of the present work concerning an antigenic determinant within the Bβ chain, in conjunction with our previous results concerning an antigenic determinant within the Aα chain (Nagy et al., 1982), support the conclusion that the presence of the entire intact F molecule is necessary to maintain the conformation in the N-terminal portions of both the Aα and Bβ chains of F required for efficient Ab binding.

In summary, one antigenic determinant within the Bβ chain of F has been localized among residues 20–28 that probably spans the Arg_{21}–Gly_{22} thrombin-cleavable bond. This antigenic region is accessible in the surface of the intact F molecule, and the conformational integrity of this region is essential for the binding of the antibodies isolated here.

Immunochemistry of Fibrinogen. The localization of an antigenic determinant in the region containing residues 20–28 of the Bβ chain of bovine fibrinogen correlates with the results of several other immunochemical investigations of fibrinogen [reviewed by Plow & Edgington (1982)]. Previous studies have identified antigenic determinants in the regions of the Bβ chain that are accessible on the surface of the native F molecule (Kudryk et al., 1974; Tanswell et al., 1977; Ciernewski & Edgington, 1979; Kudryk et al., 1982). The detailed analysis of Ciernewski & Edgington (1979) probed the spatial arrangement of the N-terminal segment of the Bβ chain corresponding to residues 1–118 of *human* fibrinogen. The results of those studies indicate that the amino acid residues near the thrombin cleavage site on the Bβ chain, i.e., residues Arg_{14}–Gly_{15} in *human* F, are exposed within the intact F molecule while Bβ fragment 54–118 is buried within the central domain of *human* F. In addition, two antigenic determinants were localized within the Bβ chain at residues 1–21 and at residues 43–53 of *human* F. Our localization of one antigenic determinant in the surface-accessible Bβ fragment 20–28 of *bovine* F (which corresponds to the Bβ fragment 13–21 of *human* F) is consistent with these results of Ciernewski and Edgington for *human* F. Furthermore, Ciernewski and Edgington found that thrombin cleavage at Arg_{14}–Gly_{15} destroys the antigenic determinant within the *human* Bβ fragment 1–42. This observation is consistent with our conclusion that the antigenic determinant in the *bovine* Bβ fragment 20–28 spans the Arg_{21}–Gly_{22} thrombin-cleavable bond. In addition, Ciernewski and Edgington found that plasmin cleavage at position Bβ Lys_{21}–Lys_{22} in *human* F did not destroy the antigenic determinant and, therefore, that this antigenic determinant could be localized to residues 1–21 in the Bβ chain of *human* F. (These residues would correspond to residues 1–28 in the Bβ chain of *bovine* fibrinogen.) In the present work, we have further localized this antigenic determinant to residues 20–28 of the Bβ chain of bovine F. (This

would correspond to residues 13–21 of the Bβ chain of human F.)

Finally, our results with purified antisera correlate with the recent work of Kudryk et al. (1983), who have prepared a monoclonal antibody that recognizes an antigenic determinant in or around the thrombin-susceptible Bβ Arg_{14}–Gly_{15} bond in *human* F. Using this monoclonal antibody in competitive inhibition studies, Kudryk et al. (1983) observed cross-reactivity with F, N-DSK, and human Bβ fragment (1–118). However, in vitro thrombin digestion of any of these competitors resulted in complete loss of cross-reactivity. In addition, neither FpB nor Bβ fragment 15–42 cross-react with this monoclonal antibody, providing further evidence that the antigenic determinant recognized by this monoclonal antibody spans the thrombin-hydrolyzable bond in the Bβ chain of human F, just as our purified anti-$B\beta(20–28)_N$ recognized an antigenic determinant spanning the thrombin-susceptible bond in the Bβ chain of *bovine* F.

Correlation between K_{conf} and K_M. Of the fragments of fibrinogen studied in this work, the Michaelis–Menten parameters, k_{cat} and K_M, have been determined only for CNBrBβ. The value of K_M for CNBrBβ is larger than the value of K_M for the Bβ chain of bovine and human F by a factor of about 10–20 (Martinelli & Scheraga, 1980; Hanna et al., 1984). The values of k_{cat} and K_M for CNBrBβ indicate that the kinetics of the thrombin-induced hydrolysis of the Arg–Gly bond within this fragment is less efficient as compared to that of native fibrinogen.

The value of K_{conf} for CNBrBβ indicates that this fragment does not have a completely native conformation around residues 20–28 of the Bβ chain. This implies that thrombin requires the native conformation of the Bβ chain at or near residues 20–28 for proper binding and efficient hydrolysis. A similar conclusion was drawn for the interaction of thrombin with the Aα chain of F (Nagy et al., 1982). However, in contrast to the Aα system (Nagy et al., 1982), the Bβ-chain regions within the DSK and CNBrBβ are more similar in conformation, as indicated by values of K_{conf} of the same order of magnitude for the antigenic determinant in the Bβ chain of the DSK and CNBrBβ, whereas values of K_{conf} differ by 2 ordrs of magnitude for the comparable antigenic determinant in the Aα chains of the DSK and CNBrAα. It should be noted that bovine CNBrAα, a 54-residue fragment, is heavily modified as part of its isolation procedure (four cysteine residues between 31 and 52 inclusive in bovine CNBrAα are carboxymethylated), and therefore, it was suggested (Hageman & Scheraga, 1974) and later confirmed (Nagy et al., 1982) that it is unlikely that CNBrAα retains its native conformation. In contrast, CNBrBβ, containing residues 1–131 of the Bβ chain in bovine F, undergoes carboxymethylation at only three cysteine residues between 72 and 86 inclusive, a proportionally smaller fraction of the entire CNBrBβ fragment. Modification in this small number of the residues in CNBrBβ may disrupt its conformation to a lesser extent than those modifications introduced in CNBrAα. It will be of interest to compare the kinetic parameters, k_{cat} and K_M, for the release of FpA from the DSK and CNBrAα and for the release of FpB from the DSK and CNBrBβ to the respective values of K_{conf} for the Aα and Bβ thrombin-cleavage sites for these fragments.

Kinetics of Release of FpB: Surface Accessibility. The surface accessibility of the N-terminal portion of the Aα chain has been demonstrated by its susceptibility to the proteolytic action of thrombin whereas the surface orientation of the N-terminal portion of the Bβ chain had previously been thought to be hindered by the presence of FpA (Blombäck et al., 1978). However, the results of the work of Martinelli & Scheraga (1980) and Hanna et al. (1984) imply that prior release of FpA is not necessary for release of FpB by thrombin. This conclusion is supported by the results of measurements of the kinetics of release of FpA and FpB by several enzymes other than thrombin. Using an RIA specific for human FpB to determine the rate of release of FpB, Bilzekian et al. (1975) have shown that the trypsin-catalyzed cleavage of FpB from F is more rapid than the trypsin-catalyzed cleavage of FpA. In addition, extract from the venom of ACC has also been shown to cleave FpB at a faster initial rate than FpA (Bilzekian et al., 1975). The results of the immunochemical studies presented here serve further to corroborate the fact that the thrombin-cleavage site within the Bβ chain is accessible on the surface of the native F molecule. Steric hindrance cannot serve to explain the initial slow rate of release of FpB by thrombin, and therefore, alleviation of this hindrance by removal of FpA cannot be used to explain the observed acceleration in the rate of release of FpB as the thrombin–fibrinogen reaction proceeds (Blombäck et al., 1978; Martinelli & Scheraga, 1980; Eckhardt et al., 1981; Hanna et al., 1984). The increase in the observed rate of release of FpB may, however, be due to an enhanced susceptibility of the Arg–Gly bond in the Bβ chain to cleavage by thrombin brought about by a conformational change accompanying the polymerization of fibrin monomer that follows the release of FpA. Indeed, Hurlet-Jensen et al. (1982) have shown that inhibition of polymerization of fibrin I by the tetrapeptide Gly-Pro-Arg-Pro eliminates the acceleration in the rate of release of FpB normally observed.

Conclusions

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 20–28 of the Bβ chain of bovine fibrinogen. Furthermore, the ability of native bovine fibrinogen to bind to anti-$B\beta(20–28)_N$ antibodies confirms the conclusion that the thrombin-cleavage site within the Bβ chain is accessible on the surface of the native molecule.

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Supplementary Material Available

Experimental details including one table and two figures of the preparation of peptides corresponding to residues 20–28 and 16–28, of the preparation and fractionation of anti-$B\beta(20–28)_N$ antibodies, and of the radioimmunoassays with ^{125}I-F (11 pages). Ordering information is given on any current masthead page.

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Phosphorus-31 Nuclear Magnetic Resonance of Highly Oriented DNA Fibers. 1. Static Geometry of DNA Double Helices†

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ABSTRACT: The static geometry of the phosphodiesters in oriented fibers of DNA and a variety of polynucleotides was investigated by solid-state ^{31}P nuclear magnetic resonance (NMR) spectroscopy. The structural parameters of the phosphodiester backbone expressed by two Euler angles β and γ were estimated on the basis of the NMR spectra of natural DNA, poly(dA)·poly(dT), poly(rA)·poly(dT), and poly(rA)·poly(rU). The Euler angles were calculated by using the known single crystal structures of a decamer, r(GCG)d(TATACGC), and a dodecamer, d(CGCGAATTCGCG). The distribution pattern of the Euler angles was quite different between these two oligonucleotides due to the different types of conformation, and it was fully consistent with the ^{31}P NMR results, showing that the conformation of the B form DNA is very heterogeneous while that of the A or A′ form is much more invariable with regard to the base composition. The structural parameters were also calculated by using various structures determined by the X-ray fiber diffraction studies, and they were evaluated on the basis of the ^{31}P NMR data. Notably, poly(dA)·poly(dT) fibers exhibited abnormal ^{31}P NMR spectra which were very broad in line width and were not appreciably perturbed by hydration; a coiled double-helical structure is proposed as the most plausible model for this polymer.

The polymorphism of the helical structures of deoxyribonucleic acid (DNA)[1] and synthetic polynucleotides has long been investigated mainly by the X-ray fiber diffraction method (Leslie et al., 1980, and references therein) and recently by

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[1] Abbreviations: NMR, nuclear magnetic resonance; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; RH, relative humidity; EDTA, ethylenediaminetetraacetate; DNase, deoxyribonuclease.

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