Isolation, Characterization, and Synthesis of Peptides from Human Fibrinogen That Block the Staphylococcal Clumping Reaction and Construction of a Synthetic Clumping Particle†

Donna D. Strong, Andrew P. Laudano,‡ Jacek Hawiger, and Russell F. Doolittle*

ABSTRACT: The 27-residue carboxy-terminal cyanogen bromide fragment of human fibrinogen γ chains inhibits the interaction between fibrinogen and those strains of *Staphylococcus* used in the "staphylococcal clumping reaction". Blocking activity was abolished by treatment of the fragment with trypsin and chymotrypsin, but digestion with staph protease generated a 15-residue peptide which retained all blocking activity. The pentadecapeptide, the activity of which is lost upon digestion with trypsin and chymotrypsin, corresponds to the carboxy-terminal 15 residues of the γ chain. The corresponding cyanogen bromide fragment was isolated from various fragments D generated by the action of plasmin on fibrinogen. Small ("late") fragments D, which are well-known to be lacking a substantial portion of the carboxy-terminal region of the γ chain, did not yield fragments with blocking activity, whereas large ("early") fragments D have γ chains that do yield fragments with blocking activity. Most of these large ("early") fragments D were found to have γ chains lacking the carboxy-terminal five to six residues, however, indicating that the carboxy terminus itself of native γ chains is not essential for clumping. These shortened fragments, which were significantly less active, not only were sensitive to trypsin but also lost their blocking activity upon digestion with staph protease. A series of peptides was synthesized that corresponded to various carboxy-terminal sections of the γ chain. Of these, a 15-residue peptide corresponding to the staph protease generated peptide exhibited blocking activity that was equivalent to and indistinguishable from native fragments by both biological and chemical criteria. Shorter peptides had progressively less activity, and peptides with fewer than 10 residues were not detectably active. Appropriate synthetic peptides were attached to bovine plasma albumin and the polyvalent conjugates shown to clump the staphylococci directly. Under the same conditions, a control nonclumping strain was not affected.

Certain strains of *Staphylococcus* bind preferentially to the fibrinogens of many species (Duthie, 1954). The interaction, which is highly selective in that most other plasma proteins do not bind to these bacteria, is the basis of the "staphylococcal clumping reaction", a highly sensitive agglutination test for those fibrinogens. The finding that the interaction of human fibrinogen and clumping strains of *Staphylococcus* involves the carboxy-terminal portion of the γ chain lost during the plasmin-degradation stage at which a larger fragment D ("early D") is converted into a smaller one ("late D") (Hawiger et al., 1982) led us to perform a series of experiments designed to pinpoint the interactive site more precisely. To this end, we examined a series of γ-chain CNBr fragments[1] to see if any of them could competitively inhibit the clumping of the bacteria by human fibrinogen. Indeed, the reaction was found to be blocked by the 27-residue carboxy-terminal fragment. This fragment was further degraded to a 15-residue segment, still containing the carboxy-terminal residue of the γ chain, which also exhibited the same degree of blocking activity. Smaller peptides generated by various digestions were devoid of activity, however. Accordingly, we undertook the solid phase synthesis of the active carboxy-terminal peptide from the carboxy terminus, removing resin portions at various stages in an effort to find the minimal fragment necessary for the interaction. Certain of these peptides were also attached to a carrier protein in an attempt to generate a polyvalent macromolecule with the ability to clump the appropriate strains of bacteria.

We also examined the CNBr fragments from γ chains of various D fragments to see which, if any, might be able to block the clumping reaction. In fact, only fragments from the population of larger fragments D, usually referred to as early D or big D, were able to block the reaction. Upon examination, the γ chains of most of these were found to lack the carboxy-terminal six residues of the native γ chain, however, and for a while we were confronted with a paradox as to exactly which residues were required for blocking and/or clumping, inasmuch as bonds whose cleavage eliminated blocking activity in some fragments were not even present in the active fragments from the early D γ chains. We were able to resolve the issue by showing that there is a sequence of nine residues that is absolutely required for all action. Beyond that, another set of residues is necessary, perhaps for reasons of bulk or folding, that may be situated on either the amino-terminal or carboxy-terminal side of the required set.

Experimental Procedures

Materials and Methods. All the fibrinogen used in this study was prepared by a previously described cold ethanol precipitation procedure (Doolittle et al., 1967) with blood obtained from the San Diego Blood Bank. The strains employed were the Newman D_2C variant of *Staphylococcus aureus* (clumping factor positive) and *Staphylococcus epi-*

† From the Department of Chemistry, University of California, San Diego, La Jolla, California 92093, and the Departments of Medicine and Pathology, Vanderbilt University, Nashville, Tennessee 37232. *Received August 11, 1981.* This work was supported by U.S. Public Health Service Grants HE-18,576, GM-17,702, HL-24,119, and HL-08,388.

* Address correspondence to this author at the Department of Chemistry, University of California.

‡ Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA.

[1] Abbreviations: CNBr, cyanogen bromide; BPA, bovine plasma albumin; SBTI, soybean trypsin inhibitor; DCCI, dicyclohexylcarbodiimide; DMF, dimethylformamide; BDB, bis-diazotized benzidine; Dns, (dimethylamino)naphthalenesulfonyl; Tos (tosyl), tolylsulfonyl; Boc, *tert*-butoxycarbonyl; Tris, tris(hydroxymethyl)aminomethane; Cbz, benzyloxycarbonyl.

0006-2960/82/0421-1414$01.25/0

dermis Zak (clumping factor negative); both were stored as a nonviable freeze-dried preparation (Hawiger et al., 1978). Tos-Phe-CH_2Cl-trypsin, α-chymotrypsin, and carboxypeptidase A were obtained from Worthington Biochemicals. Soybean trypsin inhibitor (SBTI) and bovine plasma albumin (BPA) were obtained from Sigma. Staph protease was purchased from Miles Laboratories, Inc.

The preparation of individual polypeptide chains from reduced and alkylated fibrinogen was conducted according to a previously described regimen (Dootlittle et al., 1977a). Purified γ chains were subjected to CNBr digestion, freeze-dried, and then gel filtered on a Sephadex G-75 column equilibrated and developed with 10% acetic acid (Figure 1); appropriate peaks were freeze-dried. They were lyophilized again after re-solution or resuspension in 0.05 M pyridine for 2 h at room temperature in order to convert all homoserine lactones to the carboxyl form. Pools were resuspended in H_2O and centrifuged, and the water-soluble portion was removed for redrying. Peptides were fully characterized according to procedures described in several previous reports from this laboratory (Takagi & Doolittle, 1975; Doolittle et al., 1977a). Indeed, the sequence of the region containing the blocking activity was long ago determined and reported (Chen & Doolittle, 1971; Sharp et al., 1972). Various fragments D were prepared from plasmin digests of fibrinogen and reduced and alkylated as previously described (Doolittle et al., 1977b). The fragment D α-, β-, and γ-chain mixtures were digested with CNBr, gel filtered on Sephadex G-75, and processed as described above.

Enzymatic Digestions. Trypsin digestions of synthetic and naturally occurring γ-chain and fragment D peptides were carried out in 0.1 M ammonium bicarbonate containing 0.1 mg/mL TPCK-treated trypsin for 2 h at 37 °C. Reactions were terminated with SBTI (final concentration 0.1 mg/mL). Chymotrypsin digests were conducted in the same fashion, except that reactions were stopped by adding 0.1 volume of 0.001 M phenylmethanesulfonyl fluoride. Staph protease digestions were carried out in 0.1 M ammonium bicarbonate containing 0.1 mg/mL enzyme for 16 h at 37 °C. Peptides were digested with carboxypeptidase A (60 units/mL) in 0.1 M ammonium bicarbonate for 18 h at 37 °C. The reaction products were boiled for 10 min in order to stop the digestion.

Staphylococcal Clumping Assay. The clumping reaction was performed by using a scaled down modification of a previously described procedure (Hawiger et al., 1978). The freeze-dried bacteria were suspended by vortex mixing in the usual way (10 mg/mL 0.05 M Tris-HCl buffer, pH 7.4, containing 0.01% bovine plasma albumin), but the agglutination reactions were conducted on a microtiter plate. The amounts of reagents used for testing fibrinogen stock solutions and solutions of carrier–peptide conjugates were 20 μL of fibrinogen or conjugate and 10 μL of suspended bacteria. The stock fibrinogen solution (0.25 mg/mL in 0.1 M NaCl–0.05 M Tris-HCl, pH 7.4) or conjugate solution was 2-fold serially diluted with the same buffer. The final concentrations of fibrinogen after the addition of all components ranged from 0.17 mg/mL to 0.3 μg/mL (5×10^{-7} to 9×10^{-10} M). The amounts of reagents used for blocking experiments were 10 μL of fibrinogen (10^{-7} M = 34 μg/mL), 10 μL of suspended bacteria, and 10 μL of appropriate additive. The plates were shaken for 30 s after the combination of reactants. Polaroid photographs were taken after 30–60 min.

Peptide Synthesis. Solid phase peptide synthesis was performed according to established Merrifield procedures (Stewart & Young, 1969; Erickson & Merrifield, 1976). Boc-glycine, Boc-L-alanine, Boc-L-valine, and Boc-L-leucine were obtained from Vega-Fox, as were *O*-benzyl Boc-L-aspartic acid and Boc-L-glutamine *p*-nitrophenyl ester. Tosyl-Boc-L-histidine and Cl-Cbz-L-lysine were purchased from Bachem. Radioactive glycine (^{14}C) was purchased from ICN, cold diluted with nonradioactive glycine to 0.1 mCi/mmol, and converted to its Boc derivative with 2-[(*tert*-butoxycarbonyl)oximino]-2-phenylacetonitrile (Itoh et al., 1975). The syntheses were initiated by attaching Boc-L-valine to chloromethylated polystyrene resin (Bio-Rad 1% cross-linked, 1.34 mequiv of Cl/g) by refluxing the amino acid derivative with the resin in ethanol containing triethylamine (Stewart & Young, 1969). The degree of substitution was determined by amino acid analysis after hydrolysis in a 1:1 mixture of propionic acid and HCl in a sealed evacuated tube for 24 h at 108 °C. Exact details of the synthesis procedures used for coupling each residue have been described in a recent article from this laboratory (Laudano & Doolittle, 1980). In essence, most of the Boc-amino acids were dissolved in dichloromethane and coupled with DCCI dissolved in that same solvent. Glutamine residues were attached by dissolving the Boc derivatives in DMF, however, with coupling being achieved by the reaction of the *p*-nitrophenyl ester. Peptides were cleaved from the resin with HBr–F_3AcOH for 90 min. Tosyl groups were removed from the histidine residues by catalytic hydrogenation with 10% palladium on charcoal in 90% acetic acid at a hydrogen pressure of 300 mmHg for 48 h. Peptides were purified by chromatography on Dowex 50-X2 columns that were equilibrated at pH 3.0 with 0.1 M ammonium formate and developed with various ammonium buffers depending on the composition of the particular peptide. Peptides were located in the effluent by counting aliquots, since [^{14}C]glycine was introduced as the third amino acid from the carboxy terminus and was therefore present in all peptides. Appropriate peaks were pooled and repeatedly freeze-dried until salt free. Aliquots were subjected to total acid hydrolysis and amino acid analysis. The peptides were also examined by paper electrophoresis at pH 6.4; papers were stained with ninhydrin, and the Pauli stain was for histidine; they were also scanned for radioactivity with a Packard Model 7201 strip counter. Amino terminals were established by the DnsCl procedure (Gray, 1967).

Attachment of Peptides to Bovine Plasma Albumin. Several of the synthetic peptides were chemically attached to a bovine plasma albumin (BPA) carrier. In cases where a tyrosine residue was added to the sequence, the bis-diazotized benzidine (BDB) procedure described by Bassiri et al. (1979) was used; the reagent was prepared fresh each time. Other peptides were attached by glutaraldehyde coupling (Kagan & Glick, 1979) or by a water-soluble carbodiimide (Hoare & Koshland, 1967). In all cases the BPA concentration was 5–7 mg/mL and the peptide present in a 30–50-fold molar excess. In the experiments using glutaraldehyde, the protein and peptide were dissolved in 0.5 mL of 0.1 M sodium phosphate, pH 7.5, and 0.25 mL of 0.02 M glutaraldehyde solution was added dropwise; stirring was continued for 30 min at room temperature. The peptide–protein conjugate was dialyzed against distilled water for 2 days and then freeze-dried. The preparation was redissolved in 0.1 M NaCl–0.05 M Tris-HCl, pH 7.4, at concentrations of 1–6 mg/mL. The attachment of peptides by the water-soluble carbodiimide method was accomplished by dissolving the peptide and protein in an unbuffered 0.1 M solution of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. The reaction was allowed to go on at 22 °C for 24 h. Citraconylated peptides were prepared by incubating a 10

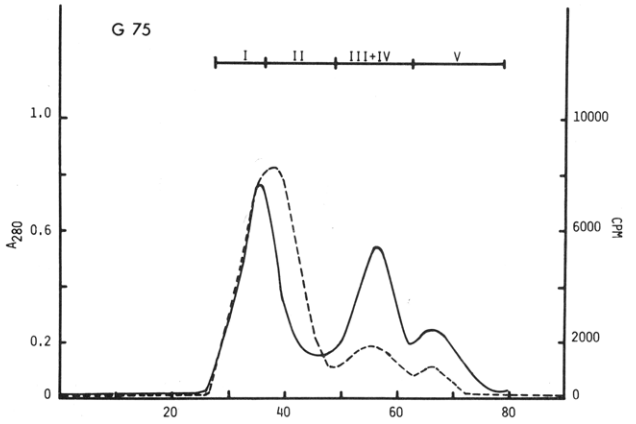

FIGURE 1: Gel filtration chromatography on Sephadex G-75 (2.5 × 70 cm) of 50 mg of CNBr-digested human fibrinogen γ chains. The column was equilibrated and eluted with 10% acetic acid. The flow rate = 60 mL/h and the fraction size = 4.0 mL. Solid line = A_{280}. Broken line = radioactivity from ^{14}C-carboxymethylated cysteine residues. The designated fractions were pooled and freeze-dried. The dried material was dissolved (or dispersed) in 0.05 M pyridine and incubated at room temperature for 2 h to convert homoserine lactones to homoserine. Pools were redried and redissolved in H_2O and aliquots tested for blocking activity.

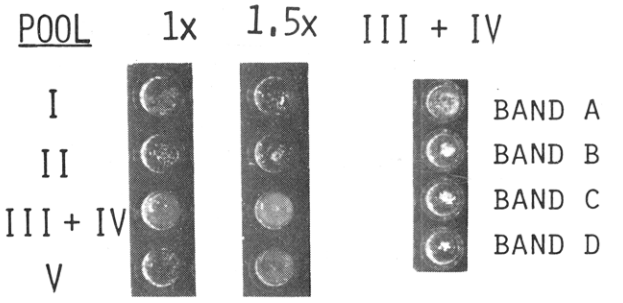

FIGURE 2: Blocking of the interaction of fibrinogen and staphylococci (strain Newman D_2C) by various CNBr fractions of human fibrinogen γ chains. (Left panels) Two different concentrations of each CNBr pool from the Sephadex G-75 column show in Figure 1. (Right panel) The material from pool III + IV was subjected to paper electrophoresis, and several bands were eluted. Band A, which corresponds to the 27-residue carboxy-terminal γ-chain peptide, blocks the clumping reaction; bands B and C correspond to other γ-chain CNBr peptides found in pool III + IV and do not block; band D corresponds to a blank section of paper.

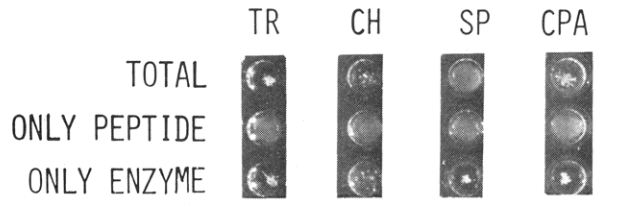

FIGURE 3: Effect of various enzyme digestions on the blocking activity of the 27-residue peptide isolated from pool III + IV. In each case the top wells contain a complete mixture (fibrinogen, bacteria, peptide, and enzyme). Note that trypsin, chymotrypsin, and carboxypeptidase all destroy the blocking activity, but staph protease does not. The bottom two rows are controls. The middle rows are controls that do not contain proteolytic enzyme, in which cases clumping is blocked. The bottom rows are controls that do not contain the peptide, in which cases clumping occurs in the usual fashion. TR = trypsin, CH = chymotrypsin, SP = staph protease, and CPA = carboxypeptidase A.

mg/mL peptide solution in 0.2 M borate buffer, pH 8.9, containing a 2.5-fold molar excess of citraconic anhydride for 30 min at room temperature. An additional 2.5-fold molar excess of citraconic anhydride was added, and incubation was continued for 1.5 h. Excess citraconic anhydride was separated from the citraconylated peptide by gel filtration on Sephadex G-15 equilibrated with 0.1 M ammonium bicarbonate. The citraconyl groups were subsequently removed from attached peptides by dialysis of conjugates against 1% acetic acid at 4 °C for 24 h.

No matter what the coupling regimen, the effectiveness of attachment was determined by counting the radioactivity in an aliquot. Usually 10–50% of a given peptide was successfully attached, with the average yield being about 10 mol of peptide bound per mol of albumin. Aliquots were examined by sodium dodecyl sulfate gel electrophoresis to ensure that the BPA carrier was not covalently aggregated.

Results

Isolation of Peptides with Blocking Activity. The various pools prepared from the G-75 gel filtration of a γ-chain CNBr digest (Figure 1) were tested to see if they would inhibit the interaction of fibrinogen and staphylococci. The freeze-dried pools were dissolved (or, in some cases, suspended) in distilled water, and the soluble material was added to the microtiter assay system. The agglutination reaction was found to be blocked by material present in pool "III + IV" (Figure 2).[2] Lesser amounts of blocking activity were also found in pools II and V but as far as could be determined were derived from the same peptide material found in pool "III + IV". The pool, which on the basis of previous experiments was known to contain various amounts of several CNBr fragments, was subjected to paper electrophoresis at pH 2.0. The major ninhydrin-positive band, which was also histidine positive, moved to the same position as does the well-characterized 27-residue CNBr carboxy-terminal fragment (Sharp et al., 1972). This band, as well as several minor components and a blank zone eluted as a control, was eluted with 10^{-2} M NH_4OH and freeze-dried. Eluates were tested in the agglutination system; only the major band was found to have inhibitory activity (Figure 2). Amino acid analysis of the eluates confirmed that this band contained the carboxy-terminal fragment (Table I).

The purified carboxy-terminal fragment was digested with trypsin, after which no inhibitory activity remained (Figure 3). Moreover, none of the constituent peptides obtained after electrophoresis at pH 6.4 exhibited any blocking activity (Table I). Treatment of the 27-residue peptide with chymotrypsin under the same conditions also abolished all the activity, as did sustained digestions with carboxypeptidase A (Figure 3).

In contrast to the results with trypsin and chymotrypsin, digestion of the 27-residue carboxy-terminal peptide with staph protease, which cleaves on the carboxy side of glutamic acid residues and to a lesser extent next to aspartic acid (Houmard & Drapeau, 1972), did not lead to a loss of inhibitory activity (Figure 3). The staph protease digest was electrophoresed at pH 5.5, and the peptide fragments were eluated, analyzed, and assayed (Table I). The only active component was a 15-residue peptide corresponding to the carboxy terminus. The molar ratio of pentadecapeptide to fibrinogen in a mixture just able to prevent agglutination under these conditions was approximately 5000:1 (Table I). Lower concentrations of the peptide appeared to have some initial effect but after 30 min were indistinguishable from controls.

CNBr peptides from large (early) and small (late) fragments D were also chromatographed on Sephadex G-75. Pools were tested for blocking activity as described for γ-chain CNBr

[2] The designation "III + IV" is a historical remnant from earlier experiments where the fractions were pooled somewhat differently (Sharp et al., 1972).

Table I: Amino Acid Compositions of γ-Chain CNBr Carboxy-Terminal Fragment and Enzymatically Derived Subfragments

	residues/molecule [a]					
	CNIII + IV A	TR1	TR4	TR5	SP1	SP2
aspartic acid [b]	2.16 (2)	1.10 (1)		0.97 (1)	1.23 (1)	1.14 (1)
threonine	1.14 (1)		1.04 (1)			0.95 (1)
glutamic acid [b]	4.26 (4)	1.09 (1)	3.57 (3)		1.47 (1)	3.17 (3)
proline	1.00 (1)		0.78 (1)		1.04 (1)	
glycine	4.61 (5)	1.06 (1)	4.56 (4)		1.15 (1)	3.79 (4)
alanine	2.23 (2)	1.00 (1)	1.29 (1)			1.92 (2)
valine	0.84 (1)	0.73 (1)				1.06 (1)
isoleucine	2.02 [c] (3)		1.43 [c] (3)		1.59 [c] (3)	
leucine	2.22 (2)		2.23 (2)		1.17 (1)	1.02 (1)
phenylalanine	1.25 (1)		0.99 (1)		1.22 (1)	
histidine	1.90 (2)		2.26 (2)			1.75 (2)
lysine	2.24 (2)		1.87 (2)		1.10 (1)	1.16 (1)
arginine	1.16 (1)			1.03 (1)	1.10 (1)	
total	(27)	(5)	(20)	(2)	(12)	(15)
residue no.	385–411	407–411	392–406	490–491	385–396	397–411
amino terminal	Lys	Glx	Lys, Leu	Asx	Lys	Gly
blocking activity	[2×10^{-4} M] [d]	NC	NC	NC	NC	[2×10^{-4} M]

[a] Values are the average of three or more 24-h hydrolyses. The values in parentheses are the number of residues as determined from the known sequence. [b] Aspartic and glutamic acid values include asparagine and glutamine, respectively. [c] Ile–Ile bond. [d] The values in brackets are the minimum peptide concentrations required to block the staphylococcal clumping reaction when the fibrinogen concentration is 3×10^{-8} M (11 μg/mL). NC = no clumping observed.

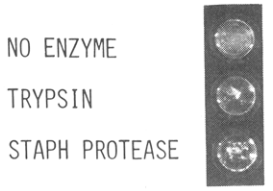

FIGURE 4: Blocking activity of the 21–22-residue CNBr peptide isolated from early fragment D. The top well shows the blocking activity of the fragment D peptide isolated after Sephadex G-75 chromatography and paper electrophoresis. In this case blocking activity was destroyed by both trypsin (middle well) and staph protease (bottom well). In all cases the fragment concentration was 2×10^{-3} M and the fibrinogen 3×10^{-8} M (molar ratio 67 000:1).

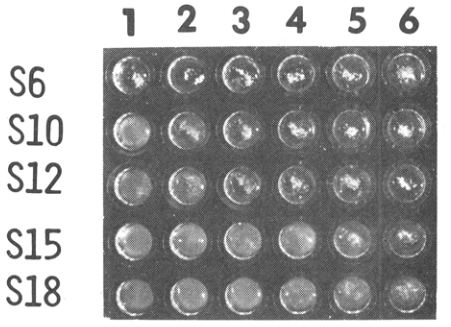

FIGURE 5: Blocking activity of synthetic peptides corresponding to the carboxy terminal of the γ chain. Synthetic peptides were 2-fold serially diluted. Peptide concentrations decrease from row 1 to 6. The highest concentrations were the following: S6, 4.5×10^{-2} M; S10, 1.6×10^{-2} M; S12, 4.8×10^{-3} M; S15, 1.9×10^{-3} M; S18, 1.6×10^{-3} M. See Figure 7 for the sequences of these peptides.

FIGURE 6: Comparison of the blocking activity of the naturally occurring and synthetic carboxy-terminal pentadecapeptide from human fibrinogen γ chain. N15 = pentadecapeptide isolated from γ chain. S15 = synthetic pentadecapeptide. In both cases solutions (4×10^{-4} M) of N15 and S15 were 2-fold serially diluted. Peptide concentrations decrease from left to right. Blocking was effective at a pentadecapeptide to fibrinogen molar ratio of 6700:1.

fragments. The agglutination reaction was blocked by material present in pool III + IV. After paper electrophoresis, it was found that the carboxy-terminal γ-chain peptides from large (early) fragments D were able to block the staphylococcal clumping reaction; the peptide existed in two forms, 21 and 22 residues long, respectively, with 6 and 5 residue-long segments being absent from the carboxy terminus. Digestion of these shortened γ-chain peptides with either trypsin or staph protease abolished all blocking activity (Figure 4). In the case of the staph protease digestion, the expected products were a dodecapeptide and 9- and 10-residue peptides, respectively.

Synthesis of Peptides Able To Prevent Agglutination. When it was clear that the 15-residue peptide obtained by staph protease digestion was able to block the interaction of fibrinogen and the bacteria, we undertook a reasonably large solid phase synthesis of that sequence in an attempt to pinpoint the minimum structure required. The synthesis was begun with 10 g of valine-substituted resin (4 mmol). We reasoned that, by removing aliquots of the resin during the course of the synthesis, we ought to be able to see at which point an active component was generated. The fact that trypsin abolished all activity in the case of the 15-residue peptide indicated that the active agent could not be contained in the carboxy-terminal 5 residues alone. Accordingly, we removed the first aliquot of resin (1 g) after the completion of six residues. The synthetic hexapeptide was purified and characterized, including amino acid analysis and amino-terminal identification; it was found to be devoid of inhibitory activity (Figure 5). Thereafter 0.5-g aliquots were removed and stored until the completion of the tenth residue, at which point 2.0 g of resin was removed and the decapeptide cleaved and carried through the entire workup. It, too, was found to have virtually no activity, although some blocking occurred when the concentration was increased 100-fold (Figure 5). An aliquot was removed after the 11th residue, and another after the 12th, with the latter being carried through the full characterization course. It demonstrated some weak inhibitory power. The synthesis was then carried through the full 15 amino acids before another preparation was prepared and purified. This product was found to be as active as the naturally derived carboxy-terminal pentadecapeptide (Figures 5 and 6); the product was electrophoretically indistinguishable from the naturally occurring pentadecapeptide. The minimum effective

Table II: Amino Acid Compositions of Various Synthetic Peptides Corresponding to Different Portions of the γ-Chain Carboxy Terminal[a]

	residues/molecule				
	S6	S10	S12	S15	S18′
aspartic acid	1.02 (1)	1.01 (1)	0.97 (1)	1.06 (1)	0.96 (1)
glutamic acid	1.03 (1)	1.03 (1)	1.01 (1)	2.94 (3)	3.86 (4)
glycine	1.02 (1)	3.19 (3)	3.26 (1)	4.24 (4)	5.12 (5)
alanine	1.04 (1)	2.02 (2)	1.98 (2)	1.93 (2)	2.12 (2)
valine	0.93 (1)	0.74 (1)	0.74 (1)	0.89 (1)	0.67 (1)
leucine		0.96 (1)	1.04 (1)	1.00 (1)	1.11 (1)
tyrosine					0.90 (1)
histidine			1.97 (2)	1.94 (2)	2.20 (2)
lysine	0.97 (1)	1.05 (1)	1.04 (1)	0.99 (1)	1.05 (1)
total	(6)	(10)	(12)	(15)	(18)
amino terminal	Lys	Leu	(His)	Gly	Tyr
blocking activity	NC	[2×10^{-2} M]	[5×10^{-3} M]	[3×10^{-4} M]	[2×10^{-4} M]

[a] See Table I for an explanation of values.

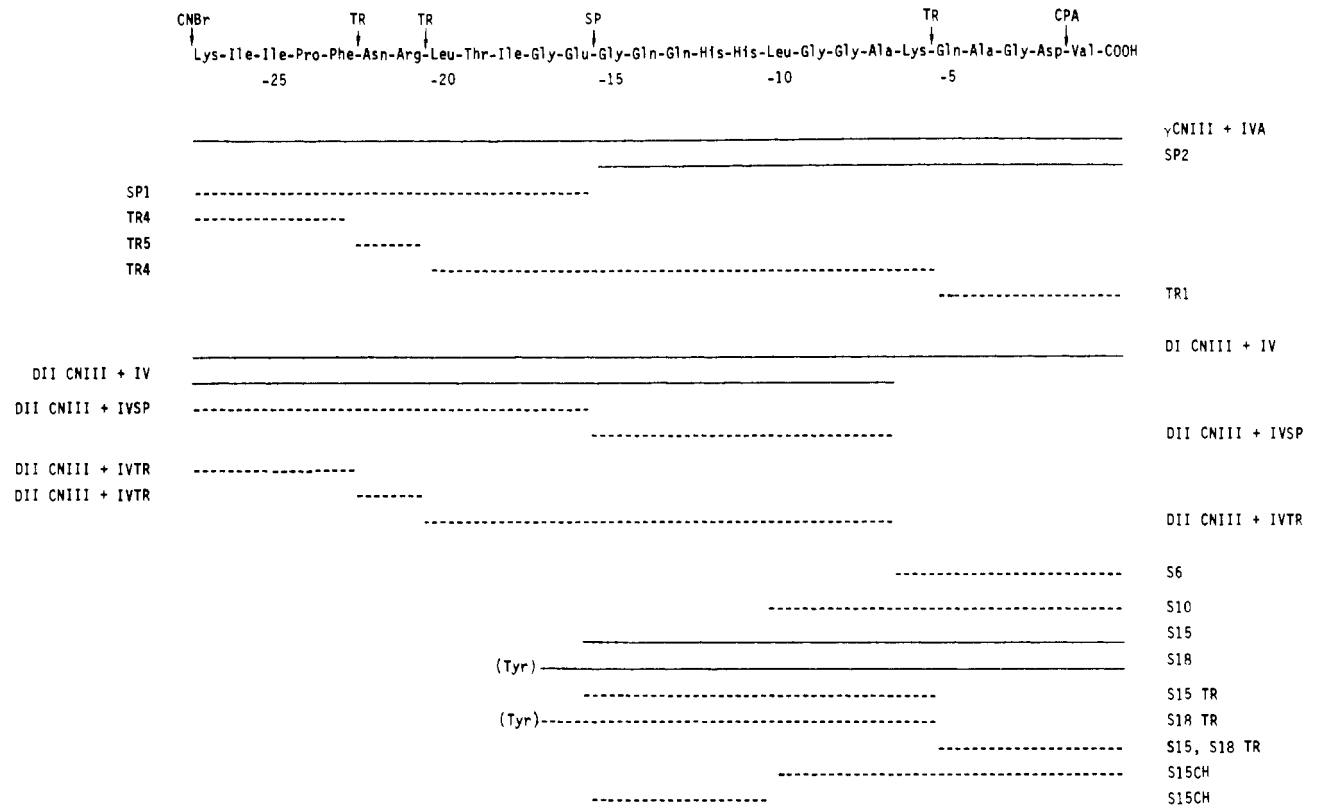

FIGURE 7: Key to naturally occurring and synthetic peptides whose blocking activity was studied. Those peptides exhibiting blocking activity are designated with a solid line corresponding to their sequence. Those peptides having insignificant blocking activity are designated with a dashed line. S indicates a synthetic peptide. All other peptides are derived from naturally occurring γ chains or D fragments. SP = staph protease, TR = trypsin, CH = chymotrypsin, and CPA = carboxypeptidase A.

concentration ranged from 2×10^{-4} to 4×10^{-4} M when the fibrinogen effective concentration was 3×10^{-8} M (5000–10 000-fold peptide excess). Treatment of the synthetic pentadecapeptide with trypsin or chymotrypsin abolished the inhibitory activity; sustained digestions with carboxypeptidase A also decreased the blocking activity. Finally, a synthetic peptide 18 residues long with a tyrosine at the amino terminus (instead of the naturally occurring isoleucine) was prepared. This product was slightly more active than the pentadecapeptide (Table II); a direct comparison of the blocking effectiveness of the five synthetic peptides (6, 10, 12, 15 and 18 residues in length) is presented in Figure 5. The amino acid compositions of the synthetic peptides are also given in Table II.

The sequences of the various naturally occurring and synthetic peptides tested and their effect on the staphylococcal clumping reaction are summarized in Figure 7. As it happens, all peptides exhibiting the ability to block the interaction of fibrinogen and the bacteria contain a critical nine-residue sequence: Gly-Gln-Gln-His-His-Leu-Gly-Gly-Ala. The nine residues alone are not capable of inhibiting the clumping reaction, however, and the inclusion of several residues on one side or the other is apparently essential for an effective blocking molecule.

Construction of an Artificial Clumping Molecule. A variety of synthetic peptides were attached to bovine plasma albumin (BPA) in an effort to construct a polyvalent molecule that could clump the bacteria. Our first attempt was with the 18-residue peptide that had a tyrosine inserted at its amino terminus (S18), with the BDB tyrosine-attachment procedure being employed. Although the preparation did in fact clump the bacteria, the results were somewhat erratic from day to day, probably because of side reactions with histidines in the essential region. Accordingly, we changed to the glutar-

Table III: Synthetic Peptides Attached to Bovine Plasma Albumin and Their Effectiveness in Clumping Two *Staphylococcus* Strains[a]

peptide	sequence	attachment method	*Staphylococcus* strain: Newman D_2C	*Staphylococcus* strain: ZAK
S6	K-Q-A-G-D-V	glutaraldehyde	no clumping	no clumping
S15′	G-Q-Q-H-H-L-G-G-A-K-Q-A-G-D-V	glutaraldehyde	clumps	no clumping
S15	G-Q-Q-H-H-L-G-G-A-K-Q-A-G-D-V	glutaraldehyde after citraconylation[b]	clumps	no clumping
S15TR	G-Q-Q-H-H-L-G-G-A-K/Q-A-G-D-V (mixture)	glutaraldehyde	no clumping	no clumping
S18	"Y"-G-E-G-Q-Q-H-H-L-G-G-A-K-Q-A-G-D-V	glutaraldehyde	clumps	no clumping
S18	"Y"-G-E-G-Q-Q-H-H-L-G-G-A-K-Q-A-G-D-V	bis(diazo)benzidine	(clumps)[c]	no clumping
X13	Y-T-P-N-D-F-A-L-R-T-L-H-S	glutaraldehyde	no clumping	no clumping
X13	Y-T-P-N-D-F-A-L-R-T-L-H-S	bis(diazo)benzidine	no clumping	no clumping

[a] Clumping results are shown in Figure 8. [b] In this case the attachment was exclusively by the α-amino group; in all other cases where the glutaraldehyde method was used, attachment involved the ϵ-amino group of lysine also. This peptide was also attached with water-soluble carbodiimide. [c] These results were erratic, probably because the BDB also reacts with the histidine residues.

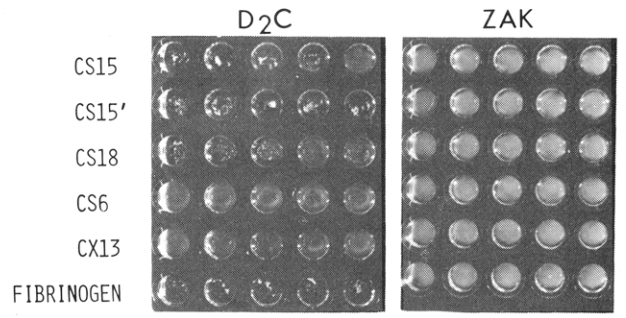

FIGURE 8: Clumping activity of BPA–peptide conjugates. Conjugates were 2-fold serially diluted. Conjugate concentrations decrease from left to right. The left-hand panel contains the staphylococcal strain (Newman D_2C) known to be clumped by human fibrinogen. The right-hand panel contains a strain (ZAK) that is not clumped by human fibrinogen. Clumping is observed with the conjugates CS15 (attached by the α-amino group only), CS15′, CS18, and fibrinogen. Clumping is not observed with CS6, the control conjugate CX13, or in any of the tests using the nonclumping strain of bacteria. The lowest concentrations of a clumping molecule able to clump the bacteria were the following: SC15, 3×10^{-6} M; CS15′, 8×10^{-7} M; CS18, 9×10^{-6} M; fibrinogen, 10^{-8} M. The end points for CS15′ and fibrinogen are not shown in the photo.

aldehyde and water-soluble carbodiimide attachment procedures. In these cases we were initially concerned about the lysine amino group, but it was subsequently found that its availability had no bearing on activity. Thus, the synthetic peptide S15 was used in two different forms, in one case citraconylated (CS15) and in one case not (CS15′). In the former case the blocked ϵ-amino group was exposed, after attachment of the peptide to BPA, by removing the citraconyl group as described under Materials and Methods. The observed clumping activity of the conjugates was about the same in both cases (Figure 8).

A number of control peptides were also attached to BPA, including a 13-residue peptide (X13) whose sequence was unrelated to the γ-chain peptide (Table III). In addition, S6, trypsin-treated S15, and trypsin–carboxypeptidase A treated S15 conjugates were not able to clump the bacteria (Figure 8). ZAK cells were not agglutinated by any of these BPA–peptide conjugates.

Discussion

The initial aim of the experiments described in this article was the localization, with as much precision as possible, of those features of the mammalian fibrinogen molecule required for the clumping of appropriate strains of *Staphylococcus*. We were especially intent on exploiting the experiments described by Hawiger et al. (1982) in which an immunologic approach implicated the carboxy-terminal region of the γ chain. To this end, we tested the CNBr fragments of the γ chains (and also α- and β-chain fragments) for their ability to competitively inhibit the interaction of fibrinogen and the bacteria. We were in fact able to isolate a pentadecapeptide from the carboxy terminus of the γ chain that exhibits such inhibition. At that point the problem became the determination of exactly what portions of that peptide were required for its blocking action. In particular, we wanted to find if the presence of the carboxy-terminal valine was required. Indeed, our early experiments performed with this material, as well as with a synthetic peptide of the same sequence, suggested that the carboxy-terminal valine was essential. The results with fragment D peptides, some of which were found to lack the γ-chain carboxy-terminal valine, indicated that the terminal residues per se were not required.

In the end we were able to resolve the apparent inconsistency as to which residues in the peptides were involved in the blocking operation by recourse to a need for bulk or mass that in some way allows proper folding. The major clue to this interpretation resides in the finding that the 27-residue peptide isolated from CNBr digests of native γ chains does not lose its activity upon digestion with staph protease, whereas the corresponding but shorter peptides from CNBr digests of large (early) fragments D do lose their blocking activity when digested with that enzyme under the same conditions. In all these experiments it must be kept in mind that peptides can assume a distribution of conformations (Sachs et al., 1972), and the presence of residues at either end of an active sequence may have quite unpredictable effects on the predominance of one conformational form over another.

The blocking activity of the various peptides is not exceptionally strong, and it is testimony to the exquisite sensitivity of the staphylococcal clumping assay that we were able to provide relative concentrations of peptide sufficiently high to effect complete blocking of the reaction. Even though a 5000–10000-fold excess is required, there is no doubt that the blocking activity is genuine, as convincingly demonstrated by the numerous controls. Moreover, the fact that we were able to construct a peptide–albumin conjugate that clumps the staphylococcal Newman D_2C strain and not the nonclumpable ZAK strain was unequivocal proof that we have identified the correct site.

It has been supposed for more than a decade that the carboxy-terminal segment of the fibrinogen γ chain is exposed (Doolittle et al., 1972). The basis of this supposition was the finding that the donor and acceptor sites for the reciprocal cross-linking that occurs during fibrin stabilization are situated 6 and 14 residues, respectively, from the carboxy terminus (Chen & Doolittle, 1971). Moreover, a variety of observations led to the proposal that these sites are situated at the ex-

tremities of a distended trinodular structure (Doolittle, 1973, 1977). As such, this region would seem, a priori, a reasonable recognition site for interaction with other large molecules or particles. It must be noted, however, that neither the carboxy-terminal pentapeptide nor the lysine residue at position −6, which is involved in the cross-linking of stabilized fibrin, is a feature of the clumping site. Indeed, cross-linked fibrin retains the ability to clump the bacteria, and it is therefore likely that the glutamine residue located 14 residues from the γ-chain carboxy terminus also is not directly involved in the interaction, even though it falls within the 9-residue span to which we have narrowed these events.

This region of the γ chain is highly conserved from species to species, with there being only three differences among the terminal 28 residues of human and bovine chains (Chen & Doolittle, 1971; Sharp et al., 1972). On the other hand, not all species have fibrinogens that interact with the particular staphylococcal strains used in these studies. Among mammals, for example, it has been reported that sheep, goats, guinea pigs, and a few other species studied do not react (Duthie, 1954; Lewis & Wilson, 1973). With regard to sheep fibrinogen, we have confirmed that this material does not cause the agglutination of these bacteria. Because sheep and cattle are closely related—much more so than humans and cattle—it was of interest to find what feature in the carboxy terminal might have changed during recent evolution. In fact, a preliminary study[3] of the equivalent sheep CNBr peptide revealed at least three interchanges, including the replacement of the glutamine at position −5 to lysine. A precise explanation of the relationship between the changes and the loss of activity will have to await the determination of the complete sequence.

Acknowledgments

We are pleased to note the assistance and cooperation of B. A. Cottrell, M. Riley, D. Trovato, and S. Timmons.

References

Bassiri, R. M., Dvorak, J., & Utiger, R. D. (1979) in *Methods of Hormone Radioimmunoassay* (Jaffe, B. M., & Behrman, H. R., Eds.) pp 46–47, Academic Press, New York.

Chen, R., & Doolittle, R. F. (1971) *Biochemistry 10*, 4486.

Doolittle, R. F. (1973) *Adv. Protein Chem. 27*, 109.

Doolittle, R. F. (1977) *Horiz. Biochem. Biophys. 3*, 164–191.

Doolittle, R. F., Schubert, D., & Schwartz, S. A. (1967) *Arch. Biochem. Biophys. 118*, 456.

Doolittle, R. F., Cassman, K. G., Chen, R., Sharp, J. J., & Wooding, G. L. (1972) *Ann. N.Y. Acad. Sci. 202*, 114.

Doolittle, R. F., Cassman, K. G., Cottrell, B. A., Firezner, S. J., Hucko, J. T., & Takagi, T. (1977a) *Biochemistry 16*, 1703.

Doolittle, R. F., Cassman, K. G., Cottrell, B. A., Friezner, S. J., & Takagi, T. (1977b) *Biochemistry 16*, 1710.

Duthie, E. S. (1954) *J. Gen. Microbiol. 10*, 427.

Erickson, B. W., & Merrifield, R. B. (1976) *Proteins (3rd Ed.) 2*, 256.

Gray, W. R. (1967) *Methods Enzymol. 11*, 139.

Hawiger, J., Hammond, D. K., Timmons, S., & Budzynski, A. Z. (1978) *Blood 51*, 799.

Hawiger, J., Timmons, S., Strong, D. D., Cottrell, B. A., Riley, M., & Doolittle, R. F. (1982) *Biochemistry* (preceding paper in this issue).

Hoare, D. G., & Koshland, D. E., Jr. (1967) *J. Biol. Chem. 242*, 2447.

Houmard, J., & Drapeau, G. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3506.

Itoh, M., Hagiwara, D., & Kamiya, T. (1975) *Tetrahedron Lett. 49*, 4393.

Kagan, A., & Glick, S. M. (1979) in *Methods of Hormone Radioimmunoassay* (Jaffe, B. M., & Behrman, H. R., Eds.) pp 328–329, Academic Press, New York.

Laudano, A. P., & Doolittle, R. F. (1980) *Biochemistry 19*, 1013.

Lewis, J. H., & Wilson, J. H. (1973) *Thromb. Res. 3*, 419.

Sachs, D. H., Schechter, A. N., Egstlake, A., & Anfinsen, C. B. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3790.

Sharp, J. J., Cassman, K. G., & Doolittle, R. F. (1972) *FEBS Lett. 25*, 334.

Stewart, J. M., & Young, J. D. (1969) *Solid Phase Peptide Synthesis*, W. H. Freeman, San Francisco, CA.

Takagi, T., & Doolittle, R. F. (1975) *Biochemistry 14*, 940.

[3] R. F. Doolittle and B. A. Cottrell, unpublished data.