Studies on Synthetic Peptides That Bind to Fibrinogen and Prevent Fibrin Polymerization. Structural Requirements, Number of Binding Sites, and Species Differences†

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ABSTRACT: Short peptides beginning with the sequence Gly-L-Pro-L-Arg... can bind to fibrinogen and prevent the polymerization of fibrin monomers [Laudano, A. P., & Doolittle, R. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3085]. The sequence corresponds to the first three amino acids of fibrin α chains exposed by the thrombin-catalyzed release of the fibrinopeptide A in all vertebrate species examined. The peptide beginning with the corresponding mammalian β-chain sequence, Gly-L-His-L-Arg-L-Pro, binds to fibrinogen but does not prevent polymerization. Competition studies indicate that the second class of peptides binds to a different set of sites. The tetrapeptide Gly-L-Pro-L-Arg-L-Pro actually binds to both sets of sites, although less tightly to the β-chain type. The binding and/or inhibition activities are very sensitive to simple structural alterations. Thus, acetylation of the α-amino group and substitution of Gly by L-Ala at the first position, L-Pro by D-Pro at the second position, or L-Arg by L-Lys at the third position all completely abolish binding. The Gly-L-Pro-L-Arg peptides also bind to lamprey fibrinogen and prevent fibrin formation in that species. The fact that clotting can be effected in that primitive system by the exclusive release of the fibrinopeptide B led us to synthesize the corresponding β-chain segment, which begins Gly-L-Val-L-Arg. This peptide bound to lamprey fibrinogen but not to human, emphasizing an extraordinary specificity of binding that is sensitive to very subtle amino acid substitutions.

Vertebrate fibrinogen is transformed into fibrin by the thrombin-catalyzed release of small polar peptides (fibrinopeptides A and B) from the amino terminal of the α and β chains. Upon release of these peptides, polymerization of the fibrin monomer units occurs spontaneously to form a noncovalently bonded gel. The polymer can be covalently cross-linked by another enzyme, factor XIII, itself activated by thrombin.

Recently, we reported that synthetic peptides beginning with the sequence glycyl-L-prolyl-L-arginine (Gly-Pro-Arg), which corresponds to the amino-terminal segment of the fibrin α chain after the release of the fibrinopeptide A, can prevent the polymerization of fibrin monomers (Laudano & Doolittle, 1978). Furthermore, these peptides bind to fibrinogen (2 mol/mol) and to the plasmin-generated fragment D (1 mol/mol). Although Gly-Pro-Arg itself was found to be an effective inhibitor of polymerization, we found that the addition of a fourth residue—particularly proline or sarcosine—significantly increased both the binding and the inhibitory activity. On the other hand, we also synthesized a number of peptides that neither bind nor inhibit polymerization, including glycyl-L-prolyl-L-seryl-L-proline (Gly-Pro-Ser-Pro), which corresponds to the Arg/Ser replacememt reported for a variant fibrinogen that exhibits defective polymerization (Blombäck et al., 1968).

The sequence Gly-Pro-Arg... has been found at the amino termini of fibrin α chains of all species examined, from the primitive lamprey to birds to mammals (Table I). This evolutionary constancy, coupled with the observation that Gly-Pro-Arg appears to be the minimum structural unit that has both binding and inhibitory activity, reinforces early notions that this moiety is functionally important as a key polymerization site.

Table I: Amino Acid Sequences at the Amino Termini of Fibrin α and β Chains from Various Species

	1	2	3	4	5	6	7
human α chain[a]	Gly-	Pro-	Arg-	Val-	Val-	Glu-	Arg. . .
bovine α chain[b]	Gly-	Pro-	Arg-	Leu-	Val-	Glu-	Lys. . .
dog α chain[c]	Gly-	Pro-	Arg-	Ile -	Val-	Glu-	Arg. . .
chicken α chain[d]	Gly-	Pro-	Arg-	Ile -	Leu-	Glu-	Asn. . .
lamprey α chain[b]	Gly-	Pro-	Arg-	Leu -	? -	Glx-	Glx. . .
human β chain[a]	Gly-	His-	Arg-	Pro -	Leu-	Asp-	Lys. . .
bovine β chain[b]	Gly-	His-	Arg-	Pro -	Tyr-	Asx-	Lys. . .
dog β chain[c]	Gly-	His-	Arg-	Pro -	Leu-	Asp-	Lys. . .
chicken β chain[d]	Gly-	His-	Arg-	Pro -	Leu-	Asp-	Lys. . .
lamprey β chain[b]	Gly-	Val-	Arg-	Pro -	Leu-	Pro -	? . . .

[a] Iwanaga et al. (1967). [b] Cottrell & Doolittle (1976). [c] Birken et al. (1975). [d] Murano et al. (1977).

The peptide glycyl-L-histidyl-L-arginyl-L-proline (Gly-His-Arg-Pro), which corresponds to the tetrapeptide sequence at the amino terminus of mammalian fibrin β chains, was also synthesized. The peptide binds to fibrinogen (2 mol/mol) and to fragment D (1 mol/mol) but does not prevent the polymerization of fibrin monomers. On its own this suggested that Gly-His-Arg-Pro binds to a different set of sites than the peptides corresponding to the fibrin α chain. To reinforce the point, however, we have now performed a series of competitive binding studies that confirm the existence of the second set of sites.

The peptide glycyl-L-valyl-L-arginyl-L-proline (Gly-Val-Arg-Pro), which is the sequence found at the amino terminus of the lamprey β chain, was also synthesized since it has been reported that lamprey fibrinogen can be clotted by the release of the fibrinopeptide B alone (Doolittle, 1965; Cottrell & Doolittle, 1976), a circumstance in which the terminal Gly-Pro-Arg... sequence of the α chain is not exposed by the action of thrombin. We have also extended the study to include a variety of simple modifications of those peptides that bind to human fibrinogen, including the replacement of individual residues by similar amino acids, as well as acetylation and esterification of certain of the peptides.

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Experimental Section

Materials and Methods. Human blood plasma was obtained from the San Diego Blood Bank, and fibrinogen was prepared according to a previously described cold ethanol precipitation procedure (Doolittle et al., 1967). Fragments D and E were prepared by plasmin digestion of fibrinogen followed by DEAE chromatography (Nussenzweig et al., 1961; Doolittle et al., 1977). Lampreys (*Petromyzon marinus*) were collected in various New England rivers and streams during the 1974 spring spawning runs. Blood collection and fibrinogen preparation were carried out as described previously (Doolittle, 1965; Cottrell & Doolittle, 1976).

Fibrin monomers (human) were prepared by dispersing fibrin in 1 M NaBr–0.05 M NaOAc, pH 5.3 (Donnelly et al., 1955). In this regard, 2 mL of human fibrinogen (10–15 mg/mL) in 0.3 M NaCl and 0.005 M phosphate, pH 7.0, was clotted by the addition of 2 units of bovine thrombin (Parke-Davis) in 2 mL of 10^{-3} M EDTA, pH 6.8. After the clot had been allowed to form at room temperature for 30 min, it was wound out on a glass rod and dispersed in 1 mL of the sodium bromide solution. The dispersion was continued with gentle stirring for 8 h, at which point it was centrifuged to remove any undispersed material. The A_{280} of the dispersed fibrin preparation was generally ~17 (11 mg/mL). Reaggregation of the fibrin was effected by adding 25 μL of the fibrin monomer preparation to 0.5 mL of 0.08 M phosphate buffer, pH 6.3, containing various peptide additives in a 1-cm path length cuvette. The solution was mixed immediately, and reaggregation was monitored by measuring the scattered light in a Zeiss spectrophotometer at 350 nm (Latallo et al., 1962).

Peptide Synthesis. Solid-phase peptide synthesis was conducted according to established Merrifield procedures (Stewart & Young, 1969; Erickson & Merrifield, 1976) both manually and with an automated peptide synthesizer of our own construction. Boc[1] amino acids were purchased from Vega and Peninsula. [^{14}C]Glycine and [^{14}C]alanine were purchased from ICN, cold diluted with nonradioactive glycine or alanine to 0.1 mCi/mM, and converted to their Boc derivatives by using 2-[(*tert*-butoxycarbonyl)oximino]-2-phenylacetonitrile purchased from Aldrich (Itoh et al., 1975). The first amino acid was attached to chloromethylated polystyrene resin (Bio-Rad, 1% cross-linked, 1.34 mequiv/g) by refluxing the appropriate Boc amino acids with the resin in ethanol containing triethylamine as described by Stewart & Young (1969). The degree of substitution attained was generally ~0.4 mmol/g. Subsequent amino acids were attached to the resin in a standard solid-phase peptide synthesis vessel with a fritted glass bottom. The resin was washed 3 times with the appropriate solvent (10 mL/g) before and after deblocking, neutralization, and coupling steps. The Boc group was removed by a 30-min treatment with either 4 N HCl–dioxane or 50% F_3AcOH in dichloromethane. The resin was neutralized prior to each coupling by shaking the resin with 10% triethylamine–chloroform. Couplings were performed by shaking the resin with a 2.5-fold molar excess of the appropriately blocked amino acid (Boc-Gly, Boc-L-Pro, Boc-OBzl-L-Ser, Boc-L-Val, Boc-L-Ala, and Boc-D-Pro) dissolved in dichloromethane (6 mL/g of resin) for 5 min. Boc-NO_2-L-Arg and Boc-Tos-His were dissolved in a minimal volume of Sequanal grade DMF (Pierce). Dichloromethane was added to a final volume of 6 mL/g of resin; this solution was shaken 5 min with the neutralized resin that had been washed with Sequanal grade DMF. A 2.5-fold molar excess of DCCD was added as a 50% solution in dichloromethane and shaking continued for 2 h. The order of addition of DCCD and Boc amino acid solution was reversed on the coupling of the third amino acid when proline was the first amino acid on the resin in order to minimize loss of peptide from the resin due to dioxopiperizine formation (Erickson & Merrifield, 1976). Each amino acid was double-coupled with another 2.5-fold excess of Boc-amino acid–DCCD in order to ensure high coupling efficiency. A ninhydrin color test (Kaiser et al., 1970) was used to test for completeness of coupling. Peptides were cleaved from the resin with HBr–F_3AcOH for 90 min at room temperature (Stewart & Young, 1969). The nitro group of nitroarginine-containing peptides and the Tos group from imidazole Tos-His peptides were removed by catalytic hydrogenation using 10% Pd–charcoal in 90% acetic acid at a hydrogen pressure of 300 mmHg for 48 h. Arginine-containing peptides were purified either by paper electrophoresis at pH 6.4 or by chromatography on Bio-Rex 70 (Bio-Rad). Usually 0.5 mmol of peptide was dissolved in 0.05 M ammonium acetate, pH 6.0, and applied to a Bio-Rex 70 column (1.2 × 20 cm, 50–100 mesh, ammonium form) previously equilibrated with the same buffer. The column was washed with 100 mL each of 0.05 M ammonium acetate, pH 6.0, 0.05 M ammonium acetate, pH 4.2, and 0.1% acetic acid, all at a flow rate of 1 mL/min. The peptide was eluted with a linear gradient of acetic acid (200 mL of 0.1% acetic acid and 200 mL of 10% acetic acid). Peptides that did not contain arginine were purified on Dowex 50 W-X2 isocratically with 0.1 M ammonium formate, pH 3.1. Peptide peaks were located by liquid scintillation counting or by quantitative ninhydrin analysis of aliquots, pooled, and lyophilized several times. Stock solutions of various peptides were prepared in H_2O and their concentrations determined by amino acid analysis on a Spinco Model 119 amino acid analyzer after total acid hydrolysis (5.7 N HCl, 108 °C, 24 h in evacuated sealed tubes). Purity was also checked by paper electrophoresis at pH 6.4 using ninhydrin and an arginine stain (Yamada & Itano, 1966) as well as radioactivity scanning in a Packard scanner. A summary describing the peptides synthesized, yields, methods of purification, and amino acid compositions is presented in Table II.

Equilibrium Dialysis. Equilibrium dialysis was conducted in small test tubes using standard No. 8 Visking dialysis tubing. In the case of fibrinogen binding studies, 1.0 mL of fibrinogen solution (10 mg/mL) in 0.3 M NaCl and 0.005 M phosphate buffer pH 7.2, was dialyzed against 5 mL of a solution containing 0.2 M NaCl and 0.01 M phosphate buffer, pH 7.2, and various concentrations of the radioactive peptides under study. Binding studies with fragments D and E were performed similarly except that the protein concentration was reduced to 5 mg/mL. Competitive binding studies were limited to human fibrinogen. In this regard, 3.4×10^{-5} M radioactive peptide solutions ([^{14}C]Gly-Pro-Arg-Val, [^{14}C]-Gly-His-Arg-Pro, and [^{14}C]Gly-Pro-Arg-Pro) containing various concentrations of nonradioactive peptides (0–10^{-3} M) were subjected to equilibrium dialysis. Studies comparing the binding of peptides to human and lamprey fibrinogen were performed at 10 °C. Binding studies dealing only with human fibrinogen, fragment D, or fragment E were performed at room temperature. After 8 h of equilibration on a mechanical shaker, the dialysis bags were cut open and 0.5-mL aliquots of the inside and outside solutions were removed for liquid scintillation counting in 10 mL of Aquasol-2 (New England

[1] Abbreviations used: Boc, *tert*-butyloxycarbonyl; DMF, dimethylformamide; DCCD, dicyclohexylcarbodiimide; F_3AcOH, trifluoroacetic acid; Tos, *p*-toluenesulfonyl.

Table II: Methods of Purification, Yields, and Amino Acid Compositions of Synthetic Peptides

peptide	method of purifn	yield (%)[a]	amino acid composition (residues/mol)[b]
Gly-L-Pro-L-Arg-Sar	pH 6.4 electrophoresis	2	Gly (1.13); Pro (0.83); Arg (1.04); Sar (1)
L-Pro-L-Arg	pH 6.4 electrophoresis	27	Pro (0.95); Arg (1.05)
Gly-L-Pro-L-Arg	Bio-Rex 70	44	Gly (1.02); Pro (0.98); Arg (1.00)
Gly-L-Pro	Dowex 50 X2	77	Gly (0.96); Pro (1.04)
Gly-L-Pro-L-Ser-L-Pro	Dowex 50 X2	10	Gly (1.12); Pro (1.99); Ser (0.89)
Gly-L-Pro-L-Arg-L-Val	Bio-Rex 70	35	Gly (0.95); Pro (1.07); Arg (0.86); Val (1.12)
Gly-L-Val-L-Arg-L-Pro	Bio-Rex 70	31	Gly (1.02); Val (0.90); Arg (0.86); Pro (1.22)
Gly-L-Pro-L-Arg-L-Pro	Bio-Rex 70	36	Gly (0.93); Pro (2.12); Arg (0.94)
L-Ala-L-Pro-L-Arg-L-Pro	Bio-Rex 70	30	Ala (0.97); Pro (2.08); Arg (0.96)
Gly-L-His-L-Arg-L-Pro	Bio-Rex 70	19	Gly (1.10); His (1.03); Arg (0.88); Pro (0.99)
Gly-D-Pro-L-Arg	Bio-Rex 70	46	Gly (0.97); Pro (1.02); Arg (1.01)
Gly-L-Pro-L-Lys-L-Pro	Bio-Rex 70	94	Gly (1.05); Pro (1.93); Lys (1.01)

[a] After purification; based on number of millimoles of initial residue on starting resin. [b] After total acid hydrolysis.

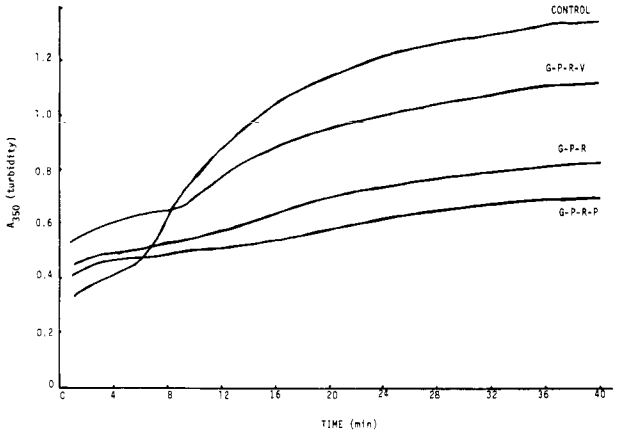

FIGURE 1: Inhibition of the reaggregation of fibrin monomers by the synthetic peptides Gly-L-Pro-L-Arg-L-Val (G-P-R-V), Gly-L-Pro-L-Arg (G-P-R), and Gly-L-Pro-L-Arg-L-Pro (G-P-R-P). All peptides were at concentration = 8×10^{-4} M.

Nuclear). Final concentrations of protein solutions were determined at this point by measuring the A_{280}. Bovine plasma albumin (Armour) was used as a control protein in the binding studies.

Thrombin–Fibrinogen Clotting Assays. These tests were conducted by adding a solution of bovine thrombin (Parke-Davis) to human and lamprey fibrinogen solutions containing peptide additives and then determining the clotting time. The final solution contained 2.0 mg/mL fibrinogen, 2–5 units/mL bovine thrombin (Parke-Davis), 0.15 M NaCl, and 0.00075 M phosphate buffer, pH 7.2.

Results

Peptides That Bind to Human Fibrinogen and Prevent Polymerization. Four different peptides were synthesized that bound to fibrinogen and also effectively blocked the polymerization of fibrin monomers. All of these began with the sequence Gly-L-Pro-L-Arg. The most effective of these were Gly-L-Pro-L-Arg-L-Pro and Gly-L-Pro-L-Arg-Sar (Table III). Gly-L-Pro-L-Arg was a good binder and inhibitor, whereas Gly-L-Pro-L-Arg-L-Val, which corresponds to the naturally occurring sequence in human fibrin (Table I), was somewhat less active than the other three. The degree of interference of the four peptides with thrombin–fibrinogen clotting times (Table IV) was roughly proportional to their effectiveness in preventing the aggregation of fibrin monomers (Figure 1). In this regard, experiments with Gly-L-Pro-L-Arg-L-Pro showed that whole blood can be rendered incoagulable more or less indefinitely at concentrations of 10^{-2} M.

In all cases the number of primary binding sites was ~2/ molecule of fibrinogen (Figure 2). As previously reported

Table III: Binding of Various Synthetic Peptides to Human and Lamprey Fibrinogens

peptide	human fibrinogen[a] K (M^{-1})	human fibrinogen[a] n	lamprey fibrinogen[b] K (M^{-1})	lamprey fibrinogen[b] n
Gly	does not bind			
Gly-L-Pro	does not bind			
Gly-L-Pro-L-Arg	2×10^4	1.8		
Gly-L-Pro-L-Arg-L-Pro	4×10^4	2.2	9×10^4	3.0
Gly-L-Pro-L-Arg-L-Val	1×10^4	2.0		
Gly-L-His-L-Arg-L-Pro	7×10^3	1.8	1×10^5	1.8
Gly-L-Val-L-Arg-L-Pro	does not bind		2×10^4	2.2
Gly-L-Pro-L-Ser-L-Pro	does not bind		does not bind	
L-Ala-L-Pro-L-Arg-L-Pro	does not bind			
Gly-D-Pro-L-Arg	does not bind			
Gly-L-Pro-L-Lys-L-Pro	does not bind			

[a] Temperature = 22 °C. [b] Temperature = 10 °C.

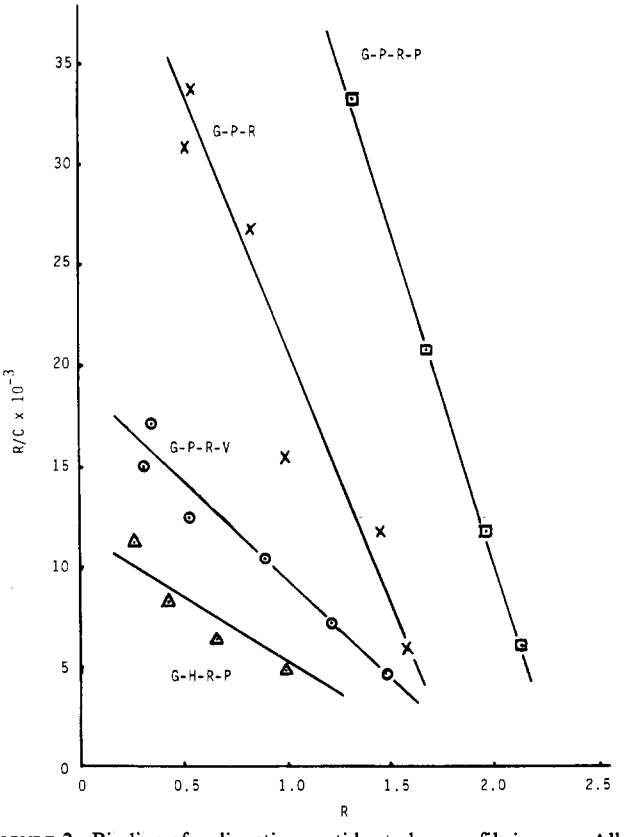

FIGURE 2: Binding of radioactive peptides to human fibrinogen. All peptides were synthesized with [^{14}C]glycine. Gly-L-Pro-L-Arg-L-Pro (□); Gly-L-Pro-L-Arg (×); Gly-L-Pro-L-Arg-L-Val (○); Gly-L-His-L-Arg-L-Pro (△). R = amount of peptide bound per mole of protein; C = concentration of unbound peptide solution. All studies were performed at room temperature (t = 22 °C).

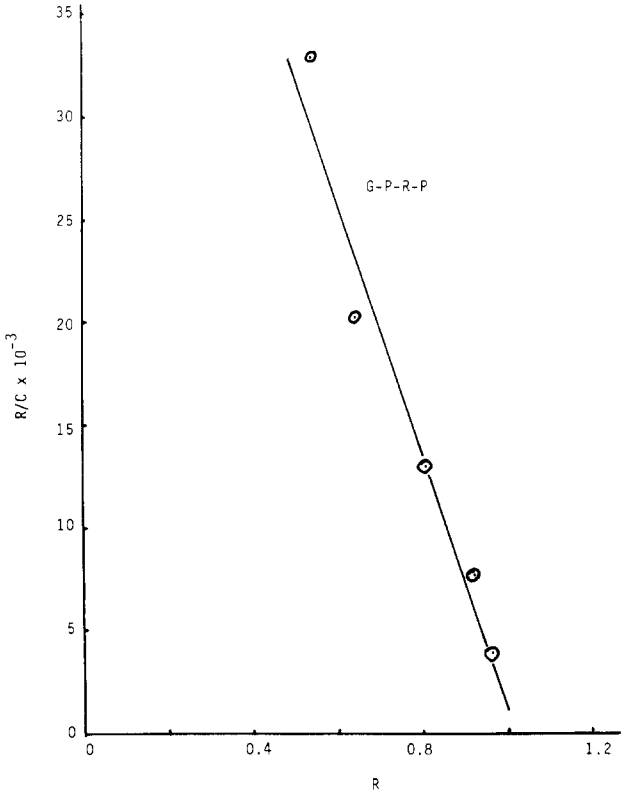

FIGURE 3: Binding of Gly-L-Pro-L-Arg-L-Pro to fragment D (5 mg/mL).

(Laudano & Doolittle, 1978), the binding sites are located on fragment D (Figure 3); no binding was observed with fragment E or to control proteins such as bovine plasma albumin.

A Peptide That Binds to Fibrinogen but Does Not Prevent Polymerization. The human fibrin β chain begins with the sequence Gly-L-His-L-Arg-L-Pro (Table I). This tetrapeptide was found to bind to fibrinogen but did not prevent the reaggregation of fibrin monomers (Table III). Nor did it increase the clotting time of thrombin–fibrinogen mixtures (Table IV). Because these observations suggested the existence of a separate set of sites for the exposed amino terminus of the fibrin β chain, we undertook a series of competition experiments in which increasing amounts of nonradioactive

Table IV: Effect of Various Synthetic Peptides on the Clotting Times of Human and Lamprey Fibrinogen with Bovine Thrombin

peptide[a]	human fibrinogen clot time (s)[b]	human fibrinogen % inhibn	lamprey fibrinogen clot time (s)[b]	lamprey fibrinogen % inhibn
Gly-L-Pro-L-Arg	65	85	240	91
Gly-L-Pro-L-Arg-L-Pro	>360	100	>360	100
Gly-L-Pro-L-Arg-L-Val	20	50	180	88
Gly-L-His-L-Arg-L-Pro	10	0	21	0
Gly-L-Val-L-Arg-L-Pro	9	0	35	40
control	10	0	21	0

[a] Concentration of all peptides = 1.33×10^{-3} M. [b] Concentration of fibrinogen = 2 mg/mL (5.9×10^{-6} M); thrombin = 5 units/mL.

Gly-L-Pro-L-Arg-L-Val (the natural fibrin α-chain segment) were used to compete with the binding of radioactive peptides. The binding of radioactive Gly-L-Pro-L-Arg-L-Pro and Gly-L-Pro-L-Arg-L-Val was markedly reduced, whereas the binding of Gly-L-His-L-Arg-L-Pro was only slightly diminished under the same conditions (Figure 4), confirming the existence of a separate set of binding sites. Conversely, nonradioactive Gly-L-His-L-Arg-L-Pro only prevented the binding of its radioactive twin, having virtually no effect on the binding of the other two peptides. On the other hand, Gly-L-Pro-L-Arg-L-Pro was not only able to displace Gly-L-Pro-L-Arg-L-Val but also competed for the sites occupied by the β-chain analogue (Figure 4).

Comparisons of Binding to Human and Lamprey Fibrinogens. The peptides that bind to human fibrinogen and prevent polymerization also bind to lamprey fibrinogen (Table III). These experiments were performed at 10° C because lamprey fibrinogen is easily denatured and cannot tolerate 8 h at room temperature. As in the case of the human system, the most effective peptide was Gly-L-Pro-L-Arg-L-Pro. Not only was it a very strong binder ($K = 10^5$ M^{-1}) but also the number of binding sites observed per molecule was consistently greater, averaging ~3 sites/M_r 360 000. Although fibrin monomer reaggregation studies were not undertaken, the clotting times of thrombin (bovine)–lamprey fibrinogen systems were prolonged by the same peptides as those of the human fibrinogen experiments (Table IV).

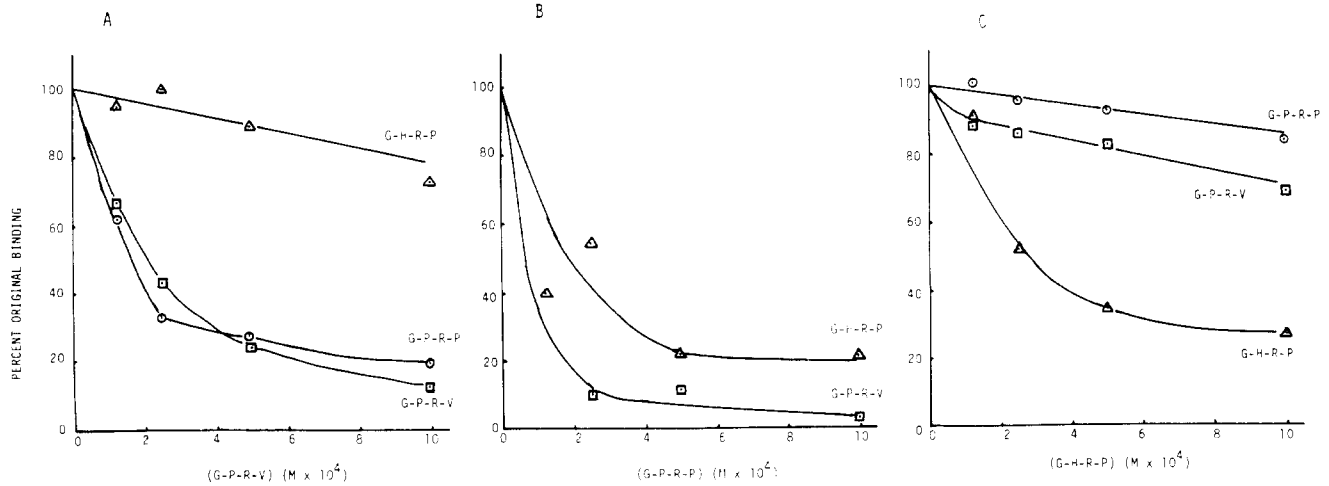

FIGURE 4: Competitive binding study showing displacement of radioactive peptides by nonradioactive congeners. (A) Binding of radioactive Gly-L-Pro-L-Arg-Pro (○), Gly-L-Pro-L-Arg-Val (□), and Gly-L-His-L-Arg-L-Pro (△) in the presence of increasing amounts of nonradioactive Gly-L-Pro-L-Arg-L-Val. (B) Binding of radioactive Gly-L-His-L-Arg-L-Pro (△) and Gly-L-Pro-L-Arg-L-Val (□) in the presence of increasing amounts of nonradioactive Gly-L-Pro-L-Arg-L-Pro. (C) Binding of radioactive Gly-L-Pro-L-Arg-L-Pro (○), Gly-L-Pro-L-Arg-L-Val (□), and Gly-L-His-L-Arg-L-Pro (△) in the presence of increasing amounts of nonradioactive Gly-L-His-L-Arg-L-Pro.

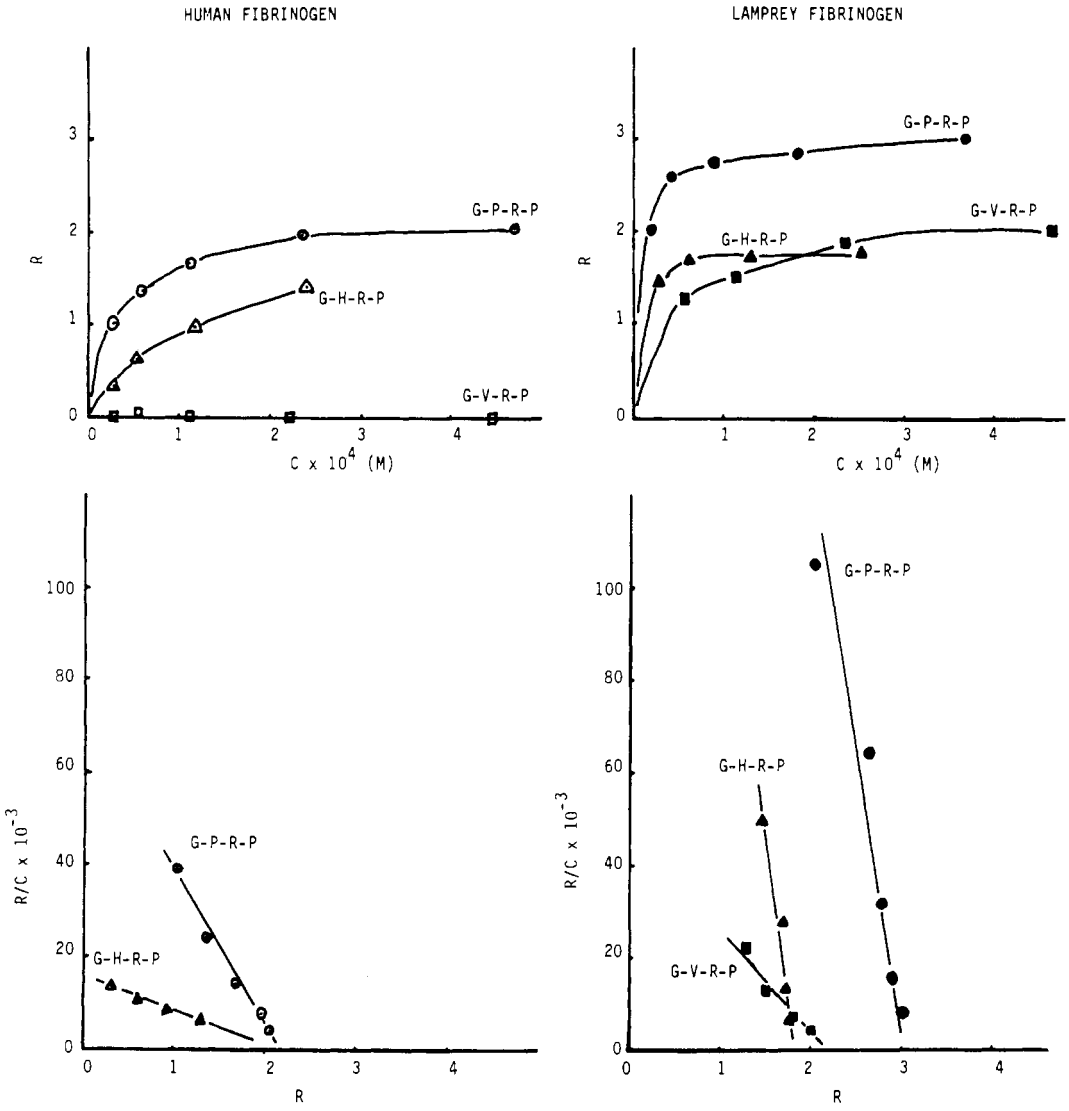

FIGURE 5: Comparison of the binding of three synthetic peptides to human (left side, open symbols) and lamprey (right side, closed symbols) fibrinogens. Gly-L-Pro-L-Arg-L-Pro (○ and ●); Gly-L-His-L-Arg-L-Pro (△ and ▲); Gly-L-Val-L-Arg-L-Pro (□ and ■). All studies were at 10 °C.

Because the lamprey fibrin β chain ends with a different sequence than mammalian fibrins (Table I), the histidine at position 2 being instead a valine, the corresponding tetrapeptide Gly-L-Val-L-Arg-L-Pro was synthesized and tested. The peptide bound to lamprey fibrinogen (2 sites/M_r 360000) but not to human fibrinogen (Figure 5). On the other hand, the mammalian-type peptide Gly-L-His-L-Arg-L-Pro bound to both lamprey and human fibrinogens (Figure 2 and 5). Gly-L-Val-L-Arg-L-Pro exhibited a modest inhibitory action on thrombin–lamprey fibrinogen clotting times, and Gly-L-His-L-Arg-L-Pro had no effect at all (Table IV).

Effect of Temperature on Binding. Inasmuch as the lamprey fibrinogen binding experiments had to be conducted at 10 °C, we undertook a study of the binding of serveral of these peptides to human fibrinogen at 10, 22, and 37 °C. Although Gly-L-Pro-L-Arg-L-Pro binds less tightly at 37 °C, the difference between 10 and 22 °C was too small to measure reliably.

Effect of pH on Peptide Binding and Inhibition of Fibrin Monomer Reaggregation. In our initial report (Laudano & Doolittle, 1978) our observations on fibrin monomer reaggregation were limited to the conventional system where reaggregation occurs at pH 6.3 (Latallo et al., 1962). The presence of a histidine residue in the β-chain analogue, coupled with the observation that this peptide bound to fibrinogen but did not interfere with reaggregation, prompted us to reexamine the influence of various peptides on reaggregation at pH 7. Indeed, in the case of binding, Gly-L-His-L-Arg-L-Pro binds less effectively at pH 6.5 ($K = \sim 2 \times 10^3$ M^{-1}) than at pH 7.2 ($K = 7 \times 10^3$ M^{-1}).

The nature of fibrin reaggregation at the higher pH makes the turbidity measurements more difficult to follow. Higher concentrations of fibrin monomer are required, and, as a consequence, more peptide is necessary. In all cases, however, the net results were identifcal with those found at pH 6.3 (we are indebted to R. Lane for conducting these experiments).

Influence of Substitutions and/or Derivatizations on the Binding of Peptides to Fibrinogen. The most effective of the synthetic peptides, with regard to both binding and polymerization inhibition, was Gly-L-Pro-L-Arg-L-Pro. Accordingly, we undertook a series of experiments to find exactly what features were essential to its effectiveness. Its separate parts, as exemplified by free glycine, Gly-L-Pro, L-Pro-L-Arg, and free arginine, were without activity, excepting the tripeptide Gly-L-Pro-L-Arg.

Substitution of the terminal glycine by L-alanine led to complete abolition of binding (Figure 6). At position 2, replacememt of the L-proline by D-proline also eliminated all

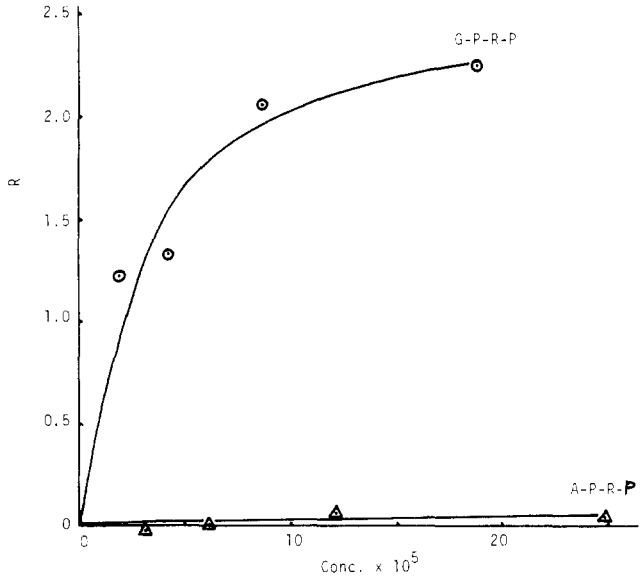

FIGURE 6: Comparison of binding of Gly-L-Pro-L-Arg-L-Pro (○) and L-Ala-L-Pro-L-Arg-L-Pro (△) to human fibrinogen (t = 22 °C).

binding. Also, the effects of replacing L-proline by L-histidine and L-valine are noted in previous sections. At residue 3, we have already reported that replacement of L-arginine by L-serine destroys all activity (Laudano & Doolittle, 1978). We can now report that the corresponding L-lysine peptide is also inactive (Table III). As noted above, peptides that have L-proline or sarcosine at position 4 are more effective than "no amino acid" (Gly-L-Pro-L-Arg) or valine (Gly-L-Pro-L-Arg-L-Val).

Acetylation of the various peptides completely eliminates all binding, indicating the essential nature of the glycyl α-amino group. Esterification of the peptide's α-carboxyl groups had only a slight negative impact on binding and polymerization in the thrombin–fibrinogen system.

Discussion

A generation ago it was hypothesized that the newly exposed amino terminals in fibrin were involved as contact sites in polymerization (Bettelheim & Bailey, 1952). The fact that there were two different pairs of fibrinopeptides released at different rates—A faster than B—led to the idea that removal of A allowed a lengthwise polymerization leading to intermediate polymers, whereas removal of fibrinopeptide B allowed lateral interactions leading to the aggregation of the smaller polymers (Blombäck & Laurent, 1958). Since then, it has been found that all four of these thrombin-exposed sites are located near each other in a single domain that can be isolated as a cyanogen bromide fragment (N-terminal disulfide knot) or as the plasmin-derived fragment E (Marder, 1971; Kowalska-Loth et al., 1973). Moreover, the overwhelming bulk of present-day evidence favors a three-domained structure for fibrinogen, the central focus of which includes the amino-terminal segments. As such, it was proposed that the release of fibrinopeptides from the central domain allows intermolecular contact between that domain and the terminal domains of other molecules (Doolittle, 1973). Experimental verification of such a scheme was provided by experiments in which both fibrinogen and fragment D were bound to fibrin immobilized on Sepharose (Kudryk et al., 1974). What has been needed, however, are experiments that demonstrate unequivocally the existence of two different sets of receptor sites, one for interaction with sites exposed by the release of fibrinopeptide A and the second for interaction with the site exposed by the release of B. In this regard, we reported that synthetic peptides corresponding to the amino-terminal segments of fibrin α and β chains can bind to fibrinogen and, in particular, to its fragment D portions (Laudano & Doolittle, 1978). Moreover, the α-chain-type peptides, characterized by an L-proline at position 2, are powerful antipolymerants and inhibit fibrin formation, whereas the β-chain-type peptides only bind.

Some of our results were unanticipated. For example, Gly-L-Pro-L-Arg-L-Pro is a more effective agent than Gly-L-Pro-L-Arg-L-Val, even though the latter is the exact sequence occurring in the human fibrin α chain (Table I). There may be several reasons for this. First, the two prolines in Gly-L-Pro-L-Arg-L-Pro introduce more rigidity to the structure and the additional restraint may favor a preferred conformation. Or, seen from the opposite view, Gly-L-Pro-L-Arg-L-Val is more flexible and likely exhibits a wider variety of conformations. Second, the fourth position in the natural α-chain sequences observed in various species is occupied by different residues (Table I), indicating that the nature of the side chain is not such a critical factor at this position. Finally, the fact that the β-chain sequence invariably has proline at position 4 may be a factor. The data indicate weak binding by Gly-L-Pro-L-Arg-L-Pro at the other (i.e., β-chain type) set of sites. This binding was most readily shown by competitive binding experiments in which Gly-L-Pro-L-Arg-L-Pro was able to displace Gly-L-His-L-Arg-L-Pro (Figure 4). The binding at this site must be significantly weaker than at its major sites, however, as evidenced by the fact that Gly-L-Pro-L-Arg-L-Pro binding gives a Scatchard plot that extrapolates to two rather than four sites (Figure 2). In the case of the lamprey, the data indicate strong binding to at least 3 sites/molecule (Figure 5).

This latter observation may also explain why lamprey fibrinogen is so readily clotted by the exclusive removal of its fibrinopeptide B. In this case the β-chain peptide Gly-L-Val-L-Arg-Pro was not nearly as effective a clotting inhibitor as Gly-L-Pro-L-Arg-L-Pro, even though the exposed fibrin "knob" is Gly-Val-Arg-Pro. An explanation for this may be that in the lamprey the two hypothetical modes of polymerization—lengthwise and lateral—are not as clearly differentiated. As a result, polymerization can be initiated by the exclusive exposure of the β-chain amino terminus. It should be kept in mind that at reduced temperatures (<25 °C) even human fibrinogen can be clotted by the exclusive release of fibrinopeptide B (Shainoff & Dardik, 1979).

Nature of the Binding Sites. Several inferences can be made about the nature of the receptor sites on the basis of the peptide binding studies reported in this article. The data indicate that a terminal glycine residue with a free α-amino group and an arginine side chain at position 3 are required for binding in the case of two different sets of sites. The major distinction between the two is made on the basis of the residue located at position 2. If it is L-proline (sequence Gly-L-Pro-L-Arg...), then binding occurs at the primary polymerization sites. If residue 2 is L-histidine, then binding occurs at another set of sites and does not interfere with primary polymerization.

A space-filling model of Gly-L-Pro-L-Arg-L-Pro reveals that the positive charge on the arginine guanidino group can be situated on the same side (face) of the peptide as the α-amino group, much like a two-pronged socket. The molecule is quite rigid because of the restraints imposed by the two proline residues. Given this model, the most obvious conjecture to be made about the sites is that they are likely negatively charged cages of some sort.

It will not be surprising if the two sets of sites (i.e., α-chain types and β-chain types) involve homologous peptide segments.

The rationale, over and beyond the structural similarity of the two types of synthetic peptide ligands, is based on evolutionary considerations. Although all three chains of the fibrinogen molecule have descended from a common ancestral type, the homology between the β- and γ-chain segments over the course of the carboxy-terminal two-thirds (which is to say, the parts of those chains that comprise the bulk of fragment D) is especially strong (Henschen & Lottspeich, 1977; Watt et al., 1978). The strong sequence homology must reflect spatial equivalence, and, as such, equivalent structural features in the binding sites—whether they be exclusively associated with one chain or the other or the result of their cooperative interaction—can be expected. At an early stage in the evolution of the system, the two sets of binding sites may have been identical, as would be the two set of sites exposed by thrombin during fibrinopeptide release. An historical vestige of this phenomenon may be what is occurring in the lamprey system, in which case Gly-Pro-Arg-Pro binds to more than 2 sites/molecule and in which case, also, clotting ensues with the release of either set of fibrinopeptides.

Acknowledgments

We are grateful to Marcia Riley for preparing the fibrinogen and fragments D and E and to Dennis Trovato for operation of the amino acid analyzer.

References

Bettelheim, F. R., & Bailey, K. (1952) *Biochim. Biophys. Acta 9*, 578.

Birken, S., Wilner, G. D., & Canfield, R. E. (1975) *Thromb. Res. 7*, 599.

Blombäck, M., Blombäck, B., Mammen, E. F., & Prasad, A. S. (1968) *Nature (London) 218*, 134.

Cottrell, B. A., & Doolittle, R. F. (1976) *Biochim. Biophys. Acta 453*, 426.

Donnelly, T. H., Laskowski, M., Jr., Notley, N., & Scheraga, H. A. (1955) *Arch. Biochem. Biophys. 56*, 369.

Doolittle, R. F. (1965) *Biochem. J. 94*, 735.

Doolittle, R. F. (1973) *Adv. Protein Chem. 27*, 1.

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

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

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

Henschen, A., & Lottspeich, F. (1977) *Thromb. Res. 11*, 869.

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

Iwanaga, S., Wallén, P., Gröndahl, N. J., Henschen, A., & Blombäck, B. (1967) *Biochim. Biophys. Acta 147*, 606.

Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595.

Kowalska-Loth, B., Gårdlund, B., Egberg, N., & Blombäck, B. (1973) *Thromb. Res. 2*, 423.

Kudryk, B. J., Collen, D., Woods, K. R., & Blombäck, B. (1974) *J. Biol. Chem. 249*, 3322.

Latallo, Z., Fletcher, A. P., Alkjaersig, N., & Sherry, S. (1962) *Am. J. Physiol. 202*, 675.

Laudano, A. P., & Doolittle, R. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3085.

Laudano, A. P., & Doolittle, R. F. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 792 (abstract).

Marder, V. (1971) *Scand. J. Haematol., Suppl. 13*, 21.

Murano, G., Walz, D., Williams, L., Pindyek, J., & Mosesson, M. W. (1977) *Thromb. Res. 11*, 1.

Nussenzweig, V., Seligman, M., Pelmont, J., & Grabar, P. (1961) *Ann. Inst. Pasteur, Paris 100*, 377.

Shainoff, J. R., & Dardik, B. N. (1979) *Science 204*, 200.

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

Watt, K. W. K., Takagi, T., & Doolittle, R. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1731.

Yamada, S., & Itano, H. A. (1966) *Biochim. Biophys. Acta 130*, 538.