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A double-headed Gly-Pro-Arg-Pro ligand mimics the functions of the E domain of fibrin for promoting the end-to-end crosslinking of γ chains by factor $XIII_a$

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ABSTRACT **The E domain of fibrinogen represents the central region of the protein that, after the removal of fibrinopeptides from the N-termini of its α chains by thrombin, orders the noncovalent assembly of fibrin units into a half-staggered array. This structural organization is accomplished purely through noncovalent binding between the E domain of one molecule and the distal D domains of two others. The process of assembly has a physiologically important up-regulatory effect on the next enzymatic phase of blood coagulation, which is the factor $XIII_a$-catalyzed end-to-end ligation of the γ chains at the D domains of the protein. Fibrin assembly, as well as the acceleration of the factor $XIII_a$ reaction, could be prevented by Gly-Pro-Arg-Pro, a homologue of the natural sequence of amino acids at the N termini of α chains in the E domain. We have now succeeded with a simple double-headed ligand, bis(Gly-Pro-Arg-Pro-amido)polyethylene glycol, in fully replacing the regulatory functions of the large E domains of the native protein.**

The clotting of fibrinogen in human plasma is regulated by a remarkable series of biochemical controls (for a recent summary, see ref. 1). The key for unlocking the self-assembly potential of fibrinogen in its conversion to fibrin is the limited proteolytic attack by thrombin that, through removal of the N-terminal fibrinopeptide moieties (2, 3), generates a new set of Gly end groups (4, 5). The short sequence of amino acids including and following the newly exposed N-terminal Gly residues of the two α chains of fibrin (6, 7) seems to be the necessary ligand for causing the noncovalent, reversible aggregation of fibrin into a clot (5, 8, 9). Gly-Pro-Arg-Pro, which resembles the natural Gly-Pro-Arg-Val sequences (knobs) in the fibrinopeptide A-denuded central **E** domain of fibrin, can prevent clot formation (10). The tetrapeptide blocks the polymerization pockets or holes located in the γ chains at the two **D** end domains of the protein (11–16). It is important to bear in mind that these polymerization pockets are present not only in fibrin but also in the parent fibrinogen molecule, i.e., thrombin action is not required for opening them for Gly-Pro-Arg-Pro to bind. Thus, the two N-terminal knobs in the central **E** domain of the protein unmasked by thrombin seem to act as a bifunctional ligand for the end-to-end, noncovalent assembly of fibrin units into the well known, half-staggered, double array of protofibrils seen in the electron microscope (17, 18). This brings together each **E** domain with the **D** domains of two other molecules.

The assembly process, as organized by the thrombin-modified **E** domains, imparts a major advantage for the next enzymatic phase of the coagulation cascade, the factor $XIII_a$-catalyzed covalent stabilization of fibrin filaments by N^{ε}(γ-glutamyl)lysine crosslinks. Fibrinogen in solution is about an order of magnitude weaker substrate for factor $XIII_a$ than clotted fibrin (19, 20), which, from the physiological point of view, is obviously an important feed-forward regulatory control. We have recently succeeded in reproducing this unique substrate-level control mechanism by complementation of fibrinogen, and even the purified **D** fragments, with the thrombin-modified central **E** portion of the protein (21). The Gly-Pro-Arg-Pro tetrapeptide prevented complex formation between **E** and fibrinogen, as well as between **E** and **D,** and it also abolished the enhancing effect of **E** on the crosslinking of fibrinogen or **D** fragments by factor $XIII_a$.

We reasoned that a synthetic, double-headed Gly-Pro-Arg-Pro ligand might serve as a polymerizing agent for fibrinogen and as a dimerizing agent for **D** fragments, and could thereby act as a functional substitute for the **E** domains in boosting the factor $XIII_a$-dependent crosslinking reactions with intact fibrinogen or with the **D** fragments as substrates. This report bears out the correctness of the assumption.

MATERIALS AND METHODS

Chemical Synthesis of the Double-Headed Gly-Pro-Arg-Pro Ligand. Reagents and solvents were purchased from Aldrich and Sigma, blocked amino acid derivatives were from Bachem, and the amine-derivatized polyethylene glycol (peg) was from Fluka. Thin-layer chromatography (TLC) was performed on Whatman K_6F-Silica gel glass plates (0.25 mm; Alltech Associates) by using the following solvent systems (vol/vol): (A) chloroform/methanol/2-propanol (10:4:4); (B) *n*-butanol/glacial acetic acid/water (15:6:5); (C) 1-propanol/water (7:3); and (D) 1-propanol/water/concentrated ammonium hydroxide/ethanol (7:4:2:3). Plates were viewed under UV light (at 254 nm) for detection of UV-absorbing moieties or were developed by ninhydrin (0.25% in 1-butanol for N-deblocked peptides) or by hypochlorite (10%) followed by starch/KI spray for N-blocked peptides (22). Melting points were determined with a Büchi apparatus and are uncorrected. After acid hydrolysis, amino acid analyses were kindly carried out by Thomas J. Lukas of the Department of Molecular Pharmacology and Biochemistry, Northwestern University Medical School, Chicago. Peptides were synthesized by the solution method according to published methods (23, 24).

Z-Gly-Pro-Arg $(Z)_2$-Pro. First, Boc-Arg$(Z)_2$ was coupled with Pro-OMe.HCl, neutralized with *N*,*N*-diisopropylethylamine according to the published procedure (23, 24) to give Boc-Arg$(Z)_2$-Pro-0Me, yield 97% mp, 48–51 °C, R_f = 0.95 (A), 0.91 (B), 0.81 (C), and 0.87 (D). Analysis by proton NMR (300 MHz, $CDCl_3$) was in agreement with the structure of the

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Abbreviations: Z, benzyloxycarbonyl; Boc, tert-butyloxycarbonyl; Me, methyl; peg, polyethylene glycol; $CDCl_3$, deutero chloroform; TLC, thin layer chromatography; TFA, trifluoroacetic acid; factor $XIII_a$, the thrombin- and Ca^{2+}-activated form of coagulation factor XIII.

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compound, which was next deblocked by trifluoroacetic acid (TFA) and coupled to Z-Gly-Pro to give Z-Gly-Pro-Arg$(Z)_2$-Pro-0Me, yield 70%, R_f = 0.96 (A), 0.81 (B), 0.73 (C), and 0.88 (D). Amino acid analysis gave Gly 1.18 (1), Arg 1 (1), and Pro 1.76 (2). Deblocking of the methyl ester group was accomplished by saponification with 0.2 M NaOH in 70% aqueous ethanol for 2 h at room temperature to give a 52% yield of Z-Gly-Pro-Arg$(Z)_2$-Pro, R_f = 0.21 (A), 0.58 (B), 0.59 (C), and 0.70 (D).

Bis(Gly-Pro-Arg-Pro-amido)peg. [CH_3CH(NH-Pro-Arg-Pro-Gly)-CH_2-$(OCH_2CH_2)_{18}$-OCH_2-CH(NH-Pro-Arg-Pro-Gly)-CH_3. First, the commercial 0,0′-bis(2-aminopropyl)polyethylene glycol of MW 900, containing 2.16 milliequivalent (meq) of amino function per g, was coupled with an equivalent amount of Z-Gly-Pro-Arg$(Z)_2$-Pro by published procedures (23, 24) to give the bis-derivatized polyethylene glycol, bis[Z-Gly-Pro-Arg-$(Z)_2$-Pro-amido]peg, which was isolated from a chloroform extract of the product after processing (23) as an oily compound. It was reprecipitated from a mixture of ethanol-anhydrous ether-petroleum ether (1:20:10 vol/vol/vol) to give an 80% yield of product homogeneous by TLC (UV positive, but ninhydrin negative) with R_f = 0.55 (A), 0.15 (B), 0.21 (C), and 0.81 (D). Catalytic hydrogenation (23, 24) of this material in 75% aqueous ethanol followed by precipitating the final product as the hydrochloride salt with 1 M HCl in 2-propanol-anhydrous ether gave a white solid. This was reprecipitated from absolute ethanol-anhydrous ether to give the hydrochloride derivative of the titled compound in about 82% yield, homogeneous in TLC (ninhydrin positive), R_f = 0.05 (A), 0.1 (B), 0.1 (C), and 0.57 (D). Amino acid analysis gave Gly 1 (1), Arg 1 (1), Pro 1.7 (2).

Enzyme-Catalyzed Covalent Ligation with Fibrinogen or with D Fragments as Substrates. Plasminogen-free human fibrinogen (American Diagnostica, Greenwich, CT) was dissolved by addition of water (40 mg/2 ml) and dialyzed against a buffer of 50 mM Tris·HCl, 150 mM NaCl, pH 7.5 (4 liters, 4°C, 20 h). Protein concentration was estimated on the basis of absorbance of $E_{1\%,\ 280\ nm}$ = 15.1 (25), and the solution was stored at −70°C. Preparation of **D** fragments, from the plasmin digest of fibrinogen (26), was carried out by E. Ang of Northwestern University as previously described (21). The material was dissolved in the above Tris-NaCl buffer; protein concentration was measured by $E_{1\%,\ 280\ nm}$ = 20 (27), and the solution was stored at −20°C. Recombinant human factor XIII A subunits [rA_2; MW = 160,000 (28)] was a gift from P. D. Bishop (Zymogenetics, Seattle, WA); the lyophilized powder was taken up in water (12 mg/ml) and stored in small aliquots at −70°C. Human α-thrombin, a gift from J. W. Fenton III (New York State Dept. of Health, Albany, NY), was stored at −70°C in 75 mM Tris·HCl, 1 mM EDTA, pH 7.5, in aliquots at 500 units/ml. Hirudin (650 anti-thrombin units/mg) was purchased from Sigma and stored at −20°C at 1,000 units/ml in 50 mM Tris·HCl, 150 mM NaCl, pH 7.5. The double-headed bis(Gly-Pro-Arg-Pro-amido)peg ligand was dissolved in water to yield a 40-mM stock solution, which was stored at −20°C.

Before each experiment, the factor XIII rA_2 zymogen (1.56 μM) was activated by treatment with thrombin (25 National Institutes of Health units/ml) at room temperature (ca. 22°C) for 30 min, when a 2-fold excess of hirudin units over thrombin (50 units of hirudin per ml) was added to quench thrombin activity. Aliquots from this thrombin-activated rA_2 preparation (40 μl) were used in the crosslinking experiments.

Solutions (24 μl) of **D** fragments were incubated with bis(Gly-Pro-Arg-Pro-amido)peg of varying concentrations at room temperature (≈22°C) for 60 min in the presence of $CaCl_2$ (2 mM). Then, thrombin-activated rA_2 (1 μl) was added, which, on account of Ca^{2+} in the mixtures, would function immediately as a transamidase to initiate crosslinking (i.e., 2 μM **D** fragment with 0.06 μM enzyme). The reaction was terminated at 90 min by addition of EDTA (to 4 mM). Control mixtures were prepared with EDTA (2 mM) replacing $CaCl_2$ or by omitting the double-headed Gly-Pro-Arg-Pro compound.

In the experiments with fibrinogen, 23-μl solutions of the protein were incubated with varying concentrations of bis(Gly-Pro-Arg-Pro-amido)peg at 37°C for 40 min, when crosslinking was initiated by adding 6 μl of $CaCl_2$ and 1 μl of thrombin-activated rA_2 with final concentrations of 3 μM for fibrinogen, 0.02 μM activated rA_2, and 2 mM $CaCl_2$. Reactions were allowed to proceed for 30 min and were terminated with EDTA (to 4 mM). Appropriate controls without $CaCl_2$ or the double-headed compound were employed.

For analyzing the crosslinked products of fibrinogen, samples were solubilized in 2% SDS with 40 mM DTT (37°C, 30 min), whereas the formation of crosslinked **DD** dimers was examined by solubilization in SDS without DTT. SDS/PAGE (29) was performed in a Mini-Protean II system (Bio-Rad) by using 8 or 10% acrylamide. Scanning of gels and calculation of crosslinking percentages were carried out as previously reported (21).

Electrophoresis of the crosslinked fibrinogen products in 2% agarose (SeaKem, FMC, Rockland, ME) was performed by the method of Moroi *et al.* (30) with tube gels in a Bio-Rad Model 150A apparatus (4 h, room temperature, 100 mA for 12 tubes) after solubilizing the samples in 100 mM sodium phosphate, pH 7.1, and 0.1% SDS.

RESULTS AND DISCUSSION

We have shown in an earlier study (21) that mixing nearly equal amounts of the thrombin-modified **E** fragments, which represent the central domains of fibrin, with intact fibrinogen caused a significant increase in the reactivities of the distal **D** domains of the protein for crosslinking by coagulation factor $XIII_a$. A similar up-regulatory effect was observed in the presence of **E** for the reactions of the proteolytically derived **D** fragments of fibrinogen for the formation of crosslinked **DD** dimeric products. Because the **E** fragments did not react with the enzyme, this remarkable substrate-level control was attributed to complex formations between fibrinogen and **E**, and between **D** and **E**. Displaying sufficient stability in nondenaturing electrophoresis, the existence of such complexes could, in fact, be demonstrated in solution. Inasmuch as the kinetic boosting effect of **E** on the enzymatic crosslinking of fibrinogen or **D**, and also the formation of fibrinogen:**E** or **D**:**E** complexes was abolished by admixture of the Gly-Pro-Arg-Pro tetrapeptide (21), it was concluded that contact between the N termini of the thrombin-modified α chain duplex in the central **E** domains of fibrinogen, comprising the homologous Gly-Pro-Arg-Val sequences, and the distal **D** domains of the protein was of critical importance. Recently, in a parallel, electron-microscopic examination of mixtures of fibrinogen with **E** fragments, linear daisy-chain co-assemblies were seen stretching to lengths of more than 30 fibrinogen units (Y. Veklich, E. Ang, J. Weisel, and L.L., unpublished results).

We reasoned that a double-headed Gly-Pro-Arg-Pro derivative might very well replace the functions of the thrombin-modified **E** domain of the native clotting protein of plasma in coordinating the binding of two distal **D** domains. If so, the best test would be to evaluate the effect of the new compound for promoting the crosslinking of fibrinogen or **D** fragments by factor $XIII_a$. Fig. 1 illustrates the ideas underlying our experimental approach.

The double-headed Gly-Pro-Arg-Pro, synthesized as the functional fibrin **E** domain mimic, was constructed with a polyethylene glycol tether to which the carboxyl ends of the two C-terminal Pro residues were connected by amide bonds (see *Materials and Methods*). Good water solubility of the bis(Gly-Pro-Arg-Pro-amido)peg derivative was an obvious advantage, and flexibility of the long polyethylene glycol tether was presumed to facilitate positioning of the Gly-Pro-Arg-Pro

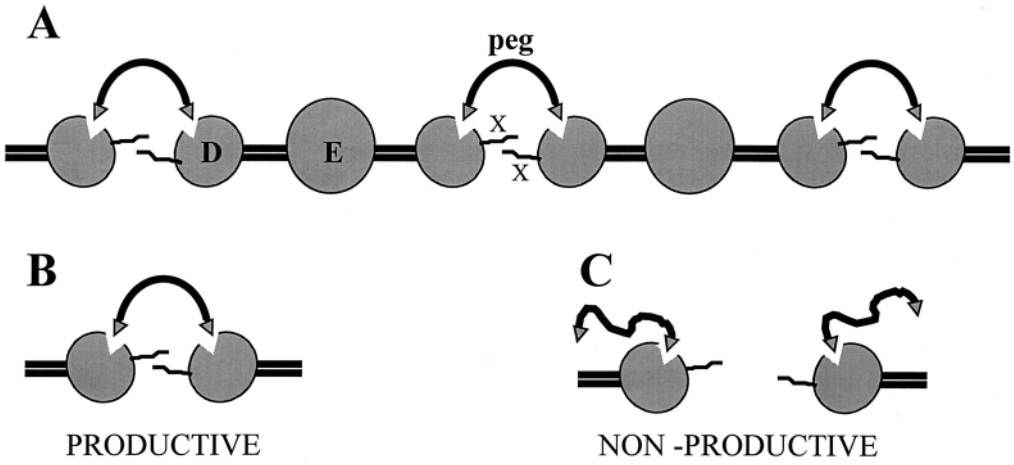

FIG. 1. The double-headed bis(Gly-Pro-Arg-Pro-amido)peg ligand is thought to promote the factor $XIII_a$-catalyzed crosslinking of fibrinogen or of its plasmin-derived **D** fragments by forming noncovalent complexes, illustrated by *A* and *B*. The central **E** and the distal **D** domains of the fibrinogen molecule are marked; the Gly-Pro-Arg-Pro heads of the ligand are represented by triangles (▽) and the 0,0′-bis(2-aminopropyl)polyethylene glycol tethers (denoted as **peg**) by solid lines. In the productive mode of binding into the polymerization pockets in the γ chains (triangular indentations in the **D** domains) the double-headed ligand brings together two **D** domains, apparently a prerequisite for optimal reaction for crosslinking (X) by factor $XIII_a$. Nonproductive mode of binding of the tethered ligand, when present in excess over the concentration of **D** in the medium, is illustrated in *C*.

ligands into the polymerization pockets of the **D** domains without strain. An **E**-fragment-like enhancement for the enzymatic crosslinking of **D** domains could be expected only with the proper mole ratios between the concentrations of the double-headed ligand and substrates, be they fibrinogen or **D** fragments. Formation of ternary complexes necessary to promote the reactions would occur only with a productive mode of binding (see Fig. 1 *A* and *B*). Thus, the influence of bis(Gly-Pro-Arg-Pro-amido)peg on the crosslinking of the substrates, tested with 2 μM **D** or 3 μM fibrinogen, had to be explored over a large range of concentrations of the ligand (up to 2 mM). The findings in Fig. 2*A* immediately proved that the synthetic ligand was an effective substitute for the large **E** fragment in boosting the crosslinking of **D** fragments. The rate of enzymatic crosslinking (measured for 90 min at ≈22°C by monitoring the formation of nonreducible, crosslinked **DD** dimers with SDS/PAGE; see *Materials and Methods*) increased about 4-fold with increasing concentrations of the ligand. Though there seemed to be an apparent leveling off in the rate of crosslinking to approximately 20 μM of the ligand, experiments performed at higher concentrations (Fig. 2*B*) clearly showed that a nonproductive mode of binding set in, which brought crosslinking rates down to control values.

Very similar findings were obtained with intact fibrinogen as crosslinking substrate (Figs. 3 *A* and *B*). The reactions were monitored by production (37°C, 30 min) of nonreducible γγ dimers on SDS/polyacrylamide gel. A 5- to 7-fold boosting of reaction rates was recorded at the lower concentrations of the

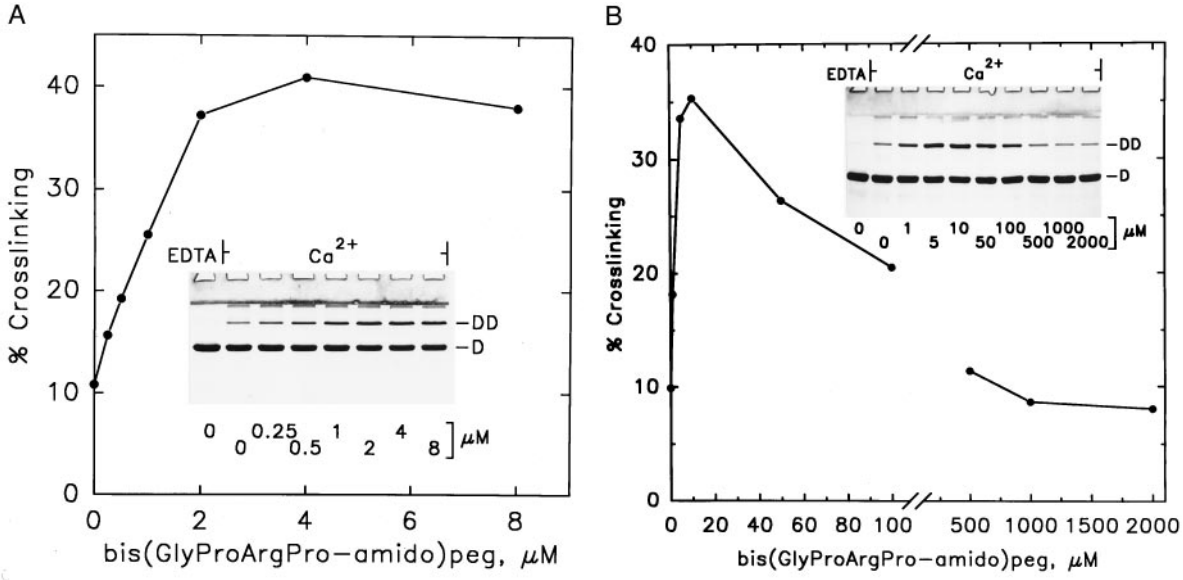

FIG. 2. Influence of the bis(Gly-Pro-Arg-Pro-amido)peg derivative on the factor $XIII_a$-catalyzed covalent dimerization of **D** fragments from fibrinogen. Various concentrations of the double-headed ligand (abscissa, 0–8 μM in *A* and 0–2 mM in *B*) were mixed with **D** fragments (2 μM) purified from the plasmin digest of human fibrin. For details, see *Materials and Methods*. The crosslinking reaction was initiated by addition of thrombin-activated recombinant rA_2 (0.06 μM) in the presence of Ca^{2+} (2 mM) and was allowed to proceed for 90 min at room temperature (≈22°C), and it was terminated by adding EDTA (4 mM). SDS/PAGE, with ca. 4.5 μg of protein per lane, was carried out in 10% acrylamide for *A* and in 8% acrylamide for *B*. After staining and drying of gels, densitometry was performed, and the extent of crosslinking as percentages was calculated from the measured intensities of monomeric **D** and dimeric **DD** species as $\mathbf{DD}/(\mathbf{D}+\mathbf{DD}) \times 100$.

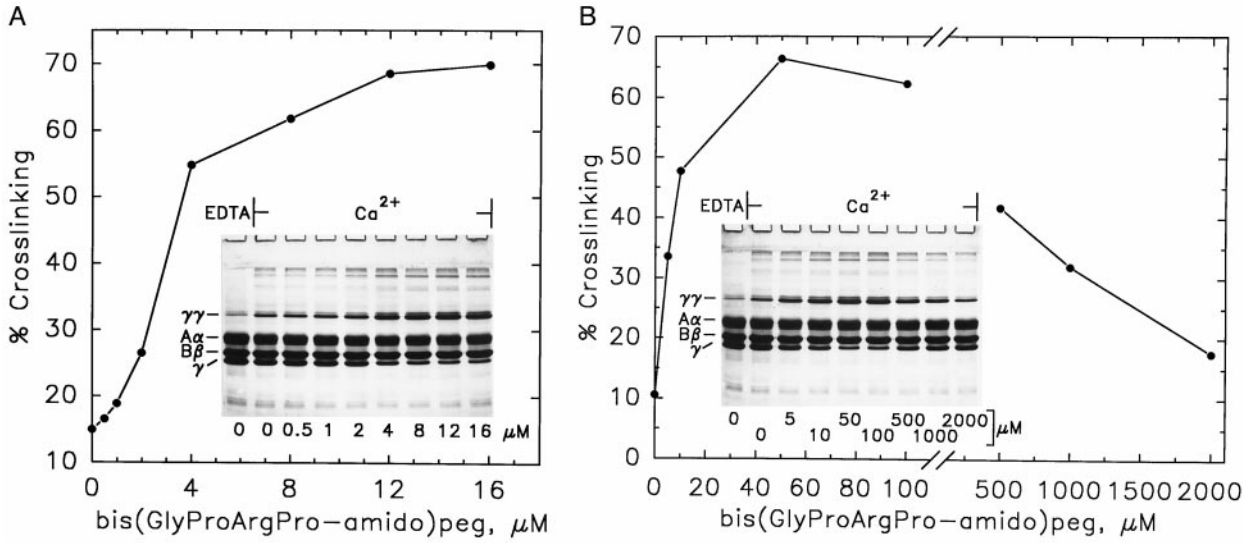

FIG. 3. The effect of the bis(Gly-Pro-Arg-Pro-amido)peg ligand on the factor $XIII_a$-catalyzed crosslinking of fibrinogen γ chains. Different concentrations of the ligand (abscissa, 0–16 μM in *A* and 0–2 mM in *B*) were mixed with human fibrinogen (3 μM). For details, see *Materials and Methods*. Crosslinking in the presence of Ca^{2+} (2 mM) by activated factor XIII (0.02 μM) was allowed to proceed at 37°C for 30 min when it was terminated by admixture of EDTA (4 mM). After reduction of the samples with DTT, SDS/PAGE was performed (applying ca. 12 μg of protein per lane) in 10% acrylamide, followed by staining, drying, and densitometric scanning. The extent of the crosslinking of γ chains was calculated from the intensities of the monomeric γ and dimeric $\gamma\gamma$ chains as $\gamma\gamma/(\gamma+\gamma\gamma) \times 100$.

double-headed ligand (up to 50 μM), but, at higher concentrations, reaction rates fell gradually to the control value seen in the absence of the ligand.

In the previous study on the effects of complementation with **E** fragments (21), for technical reasons, mole ratios of **E** to fibrinogen or **E** to **D** could not be raised high enough beyond demonstrating an apparent saturation by **E** for enhancing the enzymatic crosslinking reactions. Thus, the downward slopes, seen with bis(Gly-Pro-Arg-Pro-amido)peg as modulator (Figs. 2*B* and 3*B*), could not be documented with **E**. Apart from this, however, the boosting effect of the new, relatively small ligand was similar in every respect to that observed with the protein-derived, large **E** fragment. It is also interesting that both modulators promoted only the end-to-end crosslinking of the γ chains of fibrinogen without enhancing the enzyme-catalyzed reaction for the side-by-side crosslinking of α chains. We surmise that the transfer or rolling over of the enzyme from the γ chain to the α chain sites in the protein may require the formation of a three-dimensional lattice rather than the linear daisy chain structures generated in mixtures of fibrinogen with **E** or with the bis(Gly-Pro-Arg-Pro-amido)peg derivative.

Given that fibrinogen $[A\alpha B\beta\gamma]_2$ is a disulfide-bonded symmetrical duplex molecule composed of three different constituent chains that are also connected to each other by disulfides (6, 7), SDS/PAGE analysis after reduction with DTT is of very limited use for providing information about the true extent of end-to-end crosslinking. Figs. 3 *A* and *B* show that, in the presence of the double-headed ligand, as much as 70% of the monomeric γ chains could be crosslinked to form nonreducible $\gamma\gamma$ dimers, compared with ca. 10% in the control. This measure, however, sheds no light on the size of the covalently fused protein structures, and the finding would be compatible with the crosslinking of 70% of the starting fibrinogen monomers into $2 \times [A\alpha B\beta\gamma]_2$ dimeric structures or into higher-order polymers with lesser percentages.

An SDS-agarose electrophoretic method, without reduction by DTT (30), was employed for analyzing the crosslinked products formed in the enzymatic reaction of fibrinogen (3 μM) in the presence of varying concentrations (0–2 mM) of double-headed Gly-Pro-Arg-Pro. The results presented in Fig. 4 clearly demonstrate the biphasic influence of the ligand, as predicted from the model for its concentration-dependent, productive and nonproductive mode of binding with fibrinogen (Fig. 1*A*). Crosslinked oligomeric bands of fibrinogen up to pentamers are marked as ϕ_2–ϕ_5 on the gels in Fig. 4, and ϕ_1 denotes the starting monomer. The reference material (EDTA control), contained a small amount of dimeric ϕ_2, often seen in commercial preparations. Crosslinked fibrinogens larger than pentamers are marked collectively as ϕ_n in Fig. 4, but, on the gel relating to the reaction in the presence of 10 μM ligand, even bands representing ϕ_{15} ($M_r \approx 5 \times 10^6$) and higher polymers could be discerned with great clarity. With 20 and 50 μM of the double-headed ligand, virtually no ϕ_1 and ϕ_2

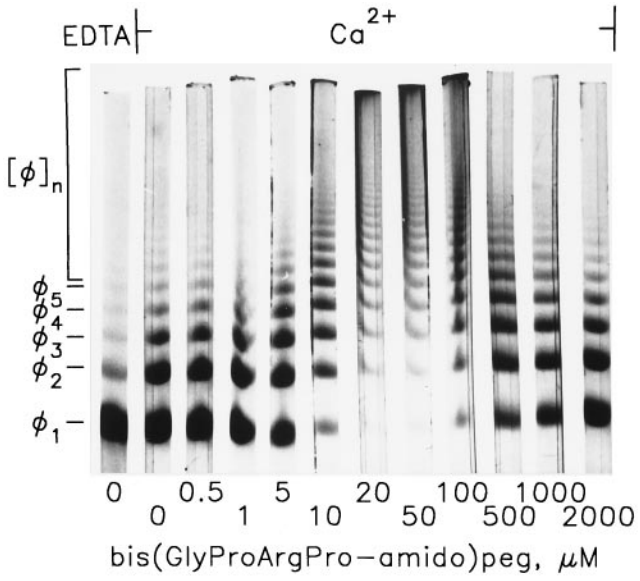

FIG. 4. Influence of the double-headed bis(Gly-Pro-Arg-Pro-amido)peg ligand on the degree of covalent polymerization of fibrinogen with factor $XIII_a$. This experiment was carried out according to the procedure described in the legend to Fig. 3. However, the reaction products (ca. 40 μg per tube) were analyzed, without reduction, by electrophoresis in agarose (2%)/0.1% SDS, as given in *Materials and Methods*.

remained by the end of the enzymatic reaction (30 min, 37°C), and a significant portion of the crosslinked polymers was present in very large structures piled up on the top of the gels. These findings are fully in accord with the idea that addition of the bis(Gly-Pro-Arg-Pro-amido)peg derivative to fibrinogen, similar to the observations for mixtures of **E** fragments with fibrinogen, causes the fibrinogen molecules to assemble noncovalently into long chains. Apparently, this is a prerequisite for favorable reaction with the enzyme at the γ chain crosslinking sites of the **D** domains. Proper alignment of these sites may lower the K_M for the enzyme and/or increase V_{max} *for the catalytic turnover.*

The thrombin- and Ca^{2+}-activated coagulation factor XIII belongs to a larger family of transamidases commonly referred to as transglutaminases. Some function extracellularly, others in the intracellular milieu, and they display one of two basic motifs of operation. Crosslinking (x) of proteins (P) in solution by N^{ε}(γ-glutamyl)lysine side-chain bridges: $nP \rightarrow xP_n$ is the simpler motif, exemplified by the formation of copulation plug in rodents by a prostatic transglutaminase (31) or by the clotting of *Homarus* plasma where the enzyme is released from exploding amoebocytes (32). The other, more complicated motif is typified by the clotting of human (i.e., vertebrate) fibrin (F) where the noncovalent, reversible assembly of the substrate precedes the enzymatic ligation step: $nF \rightleftharpoons F_n \rightarrow xF_n$. As shown in our previous work (21) and also in the present paper, the preassembly of the substrate can be an important trigger for increasing the rate of the crosslinking reaction. In terms of physiological significance, the **D**-to-**E** contact-dependent up-regulation of the factor $XIII_a$-catalyzed crosslinking of γ chains matches the rate difference for thrombin generation between a normal and a hemophilic plasma. It is remarkable that this complex-regulatory mechanism, built around the large **E** segment of the native fibrin molecule, could be so effectively reproduced with a relatively small and simple double-headed ligand.

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