# Strategy for Recombinant Multichain Protein Synthesis: Fibrinogen B$\beta$-Chain Variants as Thrombin Substrates[†]

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ABSTRACT: Thrombotic disease has been found in patients with congenital dysfibrinogens that have abnormalities in the amino terminal domain of the fibrinogen B$\beta$-chain. Surprisingly, these fibrinogens are poor substrates for thrombin. In order to examine the molecular basis for this impaired thrombin−fibrinogen interaction, we synthesized three fibrinogens with single amino acid substitutions in this domain: B$\beta$ A68T, B$\beta$ P70S, and B$\beta$ L72S. B$\beta$-chain expression vectors were altered by PCR-directed mutagenesis of the B$\beta$ cDNA. The altered vectors were transfected into a Chinese hamster ovary (CHO) cell line that was constructed as a first step in recombinant fibrinogen synthesis; this CHO line synthesizes fibrinogen A$\alpha$- and $\gamma$-chains. More than 86% of the stably selected clones expressed significant levels of fibrinogen, confirming that a two-step strategy permitted efficient synthesis of variant fibrinogens. In large-scale cultures variant fibrinogen accumulation in serum-free medium fluctuated between 1 and 15 $\mu$g/mL. Normal and variant recombinant fibrinogens were compared to plasma fibrinogen by following the time course of thrombin-catalyzed release of fibrinopeptides. Only the variant B$\beta$ A68T, a change identified in a congenital dysfibrinogen, showed significantly impaired kinetics. The rate of fibrinopeptide A release was decreased 27-fold, and the rate of fibrinopeptide B release was decreased 45-fold relative to normal fibrinogen. Fibrinopeptide release was not significantly altered by the substitutions B$\beta$ P70S or B$\beta$ L72S. These results suggest that B$\beta$ residue Ala68 has a novel and critical role in the interaction between thrombin and fibrinogen.

Fibrinogen is a soluble plasma protein that consists of six polypeptide chains, two each of the A$\alpha$-, B$\beta$-, and $\gamma$-chains [reviewed in Doolittle (1984)]. During coagulation, soluble fibrinogen is converted to insoluble fibrous polymers. This conversion is catalyzed by the serine protease thrombin, which cleaves four short fibrinopeptides from fibrinogen, two fibrinopeptides A (FpA)[1] from the amino termini of the A$\alpha$-chains and two fibrinopeptides B (FpB) from the amino termini of the B$\beta$-chains. The remaining products are fibrin monomers that aggregate spontaneously to form ordered polymers.

The interaction between thrombin and fibrinogen is remarkable because thrombin binds not only to the substrate but also to the product, fibrin. The binding of thrombin to fibrin is thought to have critical physiological consequences. Clinically significant thrombotic disease has been found in patients with congenital dysfibrinogens that demonstrate impaired thrombin−fibrin interactions *in vitro* (Liu et al., 1985; Koopman et al., 1992). Thus, the thrombin−fibrin interaction probably restricts the active enzyme to the site of injury *in vivo* and limits further pathologic, thrombus formation. These dysfibrinogens have abnormalities within the amino terminal domain of the B$\beta$-chain. In fibrinogen New York I, B$\beta$ amino acids 9−72 are missing (Liu et al., 1985). In fibrinogen Naples, the change is more subtle with a single amino acid substitution replacing Ala68 with Thr (Koopman et al., 1992).

The interaction between thrombin and fibrinogen is further remarkable because thrombin is an unusual serine protease. Several high-resolution structures have been determined by X-ray analysis of crystals of thrombin−inhibitor complexes (Bode et al., 1989; Grutter et al., 1990; Rydel et al., 1990; Bode et al., 1992; Martin et al., 1992). These structures demonstrate that the substrate binding site of thrombin consists of three domains: the active site with the Ser, His, Asp catalytic triad that is typical of serine proteases, an apolar pocket that provides specificity for substrate residues on the amino terminal side of the scissile bond, and an extended groove that forms the domain called exosite I or the fibrinogen recognition site. This groove is thought to interact with fibrinogen residues on the carboxy terminal side of the scissile bond and further to enable thrombin binding to fibrin. From these structures, Stubbs et al. (1992) have proposed a model for multiple interactions between thrombin and fibrinogen. These interactions include binding of A$\alpha$ residues 33−42 to the fibrinogen recognition site, indicating that these residues are critical for both fibrinogen and fibrin binding. This model does not specify any interactions between thrombin and the fibrinogen B$\beta$-chain. The model,

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[1] Abbreviations: A68T, B$\beta$ residue Ala68 replaced with Thr; P70S, B$\beta$ residue Pro70 replaced with Ser; L72S, B$\beta$ residue Leu72 replaced with Ser; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; FpA, fibrinopeptide A; FpB, fibrinopeptide B; HPLC, high-performance liquid chromatography; MLP, major late promoter; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; TBS, Tris-buffered saline; Tris, tris(hydroxymethyl)-aminomethane.

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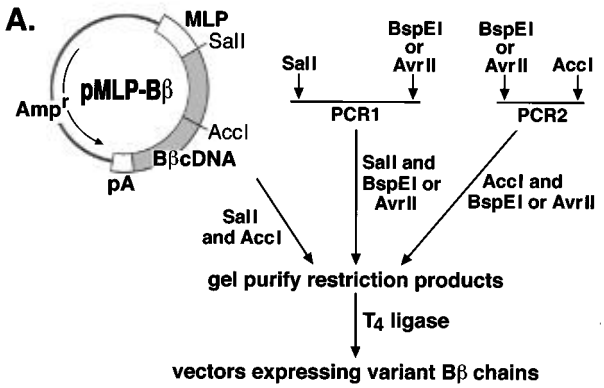
however, is readily adapted to include interactions with B$\beta$ residues 66−75. These residues are similar in nature to A$\alpha$ 33−42, and the regions must be proximate as B$\beta$ Cys 65 is disulfide-bonded to A$\alpha$ residue 36. Taken together, this model and the analyses of fibrinogens New York I and Naples indicate that the B$\beta$-chain domain either directly participates in critical interactions with thrombin, or influences the structure of the proximate A$\alpha$-chain domain. Thus, the B$\beta$-chain variants would either directly disrupt interaction with thrombin or indirectly disrupt binding by altering the structure of the proximate A$\alpha$-chains.

In order to further examine the role of this B$\beta$-chain domain in thrombin−fibrinogen interactions, we have synthesized three recombinant human fibrinogens with substitutions in this region, including the Ala68 to Thr change as the prototype. This analysis is an extension of our previous protein-engineering experiments to examine the residues of fibrinogen that interact with thrombin. In the previous experiments we altered A$\alpha$-chain residues expressed in a model fusion protein synthesized in *Escherichia coli* (Lord et al., 1990; Binnie & Lord, 1993). In the work described here we have synthesized intact recombinant human fibrinogen in Chinese hamster ovary (CHO) cells, using a two-step procedure that facilitates synthesis of variant fibrinogens, as previously described (Binnie et al., 1993). In the first step, two expression plasmids with cDNAs for the A$\alpha$- and $\gamma$-chains were cotransfected with a plasmid encoding neomycin resistance into CHO cells. Stable CHO cell lines that are resistant to G418, a neomycin analog, were screened by immunoblotting to identify clones that synthesize high levels of both the A$\alpha$- and the $\gamma$-chains. In the second step, the CHO cells that express both A$\alpha$- and $\gamma$-chains were cotransfected with an expression plasmid encoding the B$\beta$ cDNA and a vector, pMSVhis, that enabled selection with histidinol. Cells resistant to G418 and histidinol were selected, and individual clones expressing fibrinogen were identified by immunoassay. In the studies presented here we have synthesized three variant fibrinogens with changes in the B$\beta$-chain: Ala68 replaced with Thr (A68T), Pro70 replaced with Ser (P70S), and Leu72 replaced with Ser (L72S). These changes were introduced by PCR-directed mutagenesis of the B$\beta$ expression plasmid that was used for the second step in this two-step procedure. We purified the normal and variant recombinant fibrinogens from tissue culture media and examined the time course for thrombin-catalyzed fibrinopeptide release.

## EXPERIMENTAL PROCEDURES

*Materials.* The plasmid vectors, CHO cells, culture medium, and immunologic reagents have been previously described (Binnie et al., 1993). Human plasma fibrinogen was purchased from Calbiochem. Human α-thrombin was a generous gift from Dr. Frank Church, Department of Pathology, University of North Carolina at Chapel Hill.

*Construction of Variant Expression Vectors.* The strategy for PCR-directed mutagenesis of the B$\beta$ chain cDNA is outlined in Figure 1. Three altered cDNAs were constructed, each from two PCR products: PCR1, a 500 bp product beginning at the unique *Sal*I site of the vector polylinker and ending with the codons surrounding the mutation, and PCR2, a 300 bp product beginning with the codons surrounding the mutation and ending at the *Acc*I site in the




**B.**

**PCR primers to incorporate mutations:**

**BβA68T - fibrinogen**

AvrII
CCCAGA[C$^{\nabla}$CTAGG]GGTGTTGT → PCR2
PCR1 ← AGAAGTG**TGA**CTGGGTCT[GGATC$_{\Delta}$C]CCACA

**BβP70S - fibrinogen**

BspEI
GCTGA[T$^{\nabla}$CCGGA]C**TCG**GGGGTGT → PCR2
PCR1 ← AGTGCGTACT[AGGCC$_{\Delta}$T]CGACCCC

**BβL72S - fibrinogen**

AvrII
CCCAGA[C$^{\nabla}$CTAGG]GGTGTTGT → PCR2
PCR1 ← CGACTG**AGT**CT[GGATC$_{\Delta}$C]CCACAA

FIGURE 1: Construction of the altered B$\beta$-chain expression vectors. A. Schematic representation of the DNA fragments assembled into the three altered plasmid expression vectors. B. PCR primer sequences used to introduce the mutations into the B$\beta$ chain cDNA. The novel codons for the variant residues are noted in underlined bold type. The new restriction sites are enclosed in brackets.

codons for residues Val238 and Tyr239. The forward primer for all PCR1 products was 5′-TTGAGATCTGGCCATA-CACTTGAGT-3′, which is the sequence just upstream from the *Sal*I site. The reverse primer for all PCR2 products was 5′-AACCTCTACAAATGTGGTATGGCTG-3′, which is the reverse sequence just downstream from the *Acc*I site. The reverse primers for PCR1 were as follows: for A68T, 5′-ACACCCCTAGGTCTGGGTCAGTGTGAAGA-3′, for P70S, 5′-CCCCAGCTCCGGATCAGCGTGA-3′, and for L72S, 5′-AACACCCCTAGGTCTGAGTCAGC-3′. The forward primer for PCR2 to incorporate both A68T and L72S was 5′-CCCAGACCTAGGGGTGTTGT-3′. The forward primer for PCR2 to incorporate P70S was 5′-GCTGATCCG-GACTCGGGGGTGT-3′. The primers, which introduced the altered codons, also introduced restriction sites that were used to link the two PCR products without changing the encoded sequence or the reading frame of the altered cDNAs. For the A68T and L72S changes, an *Avr*II site was introduced; for the P70S change, a *Bsp*EI site was introduced. The expression plasmid, pMLP-B$\beta$, served as the template for the PCR reactions that used taq polymerase from Promega and reaction conditions recommended by the manufacturer. The reactions were cycled for 45 s at 95 °C, 45 s at 55 °C, and 30 s at 72 °C for 30 cycles. The PCR products were precipitated with ethanol, and the precipitates were dissolved in water and digested with the appropriate restriction enzymes. The digested bands were purified from agarose gels by electroelution and cloned into the parent vector, which was digested first with *Sal*I and then with *Acc*I, and purified by electroelution from an agarose gel. Cloning procedures were as described by Lord et al. (1990). Ligation products were transfected by electroporation into competent

DH5α-F′ cells. Colonies were screened by restriction digest analyses, and the B$\beta$ cDNAs were sequenced using sequenase V2.0 as described by the manufacturer (US Biochemicals). No inadvertent changes arose during the mutagenesis procedures.

*Recombinant Protein Expression.* CHO cells expressing the normal Aα- and $\gamma$-chains of human fibrinogen were prepared as previously described (Binnie et al., 1993) but using a new vector, pMLP-$\gamma$, to expresses normal fibrinogen $\gamma$-chain. The new vector was shown by DNA sequence analysis to contain the complete, normal $\gamma$-chain cDNA. The expressed $\gamma$-chain product reacts with the monoclonal antibody 4A5, which is specific for the C terminal residues of $\gamma$-chain (Blumenstein et al., 1992), in immunoblot analysis. Antibody 4A5 was kindly provided by Gary Matsueda, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ. One G418 resistant cell line expressing Aα- and $\gamma$-chains was used for all the B$\beta$ transfections. The normal B$\beta$ expression vector, pMLP-B$\beta$, or one of the variant B$\beta$ expression vectors described below was cotransfected with pMSVhis, and histidinol resistant cells were selected as previously described (Binnie et al., 1993). Cell lysates and culture medium were analyzed by immunoblotting and ELISA, respectively. CHO lines producing useful concentrations were subcloned from single cells and grown in roller bottles for preparation of recombinant fibrinogens (Binnie et al., 1993).

*Purification and Characterization of Recombinant Fibrinogen.* CHO cells producing recombinant fibrinogens were grown in roller bottles with adherent microcarrier beads in a total of 200 mL of serum-free medium: DMEM/F12 , 10 IU of penicillin/mL, 10 mg of streptomycin/mL, 10 units of aprotinin/mL, and 10 $\mu$g/mL each of insulin, sodium selenite, and transferrin (Boehringer Mannheim). Starting 3 weeks after shifting to serum-free conditions, medium was harvested every 4−7 days by removing 100 mL from the culture and replacing it with 100 mL of fresh medium. Usually five or more roller bottles were grown from the same cell line. Harvested medium was pooled, phenylmethylsulfonyl fluoride (PMSF) was added to 0.15 mM, and the fibrinogen concentration was determined by ELISA. Pooled medium was stored at −80 °C.

Prior to purification, the media was thawed in a 37 °C water bath just until the last solid disappeared. Thereafter, all manipulations were either on ice or at 0−4 °C. Fibrinogen was precipitated from the media by addition of 40% saturated ammonium sulfate, as follows. In a 2 L flask, 1200 mL of media was mixed with 30 mL of 40× buffer (50 mM Tris-HCl, pH = 7.6, 100 mM NaCl, 200 mM $\epsilon$-aminocaproic acid ($\epsilon$-ACA), 80 mM ethylenediaminetetraacetic acid (EDTA), 400 units of aprotinin/mL, 40 $\mu$M pepstatin, 40 $\mu$M leupeptin, and 200 mM benzamidine). With gentle, continuous stirring, 820 mL of saturated ammonium sulfate was added slowly by dripping through Whatman 3mm paper. The suspension was left overnight without stirring, and the precipitate was collected in 250 mL bottles by centrifugation at 16 000*g* for 30 min. After each spin, the supernatant was removed, fresh ammonium sulfate sample was added to the existing pellet, and centrifugation was repeated. Thus, the precipitate from each 2.05 L sample was collected in two bottles. The pellets were dissolved in 0.04 times the original media volume of buffer A1: 50 mM Tris-HCl, pH = 7.3, 150 mM NaCl, 5 mM $\epsilon$-ACA, 2 mM EDTA, 10 units of aprotinin/mL, 1 $\mu$M pepstatin, 1 $\mu$M leupeptin, 100 $\mu$M PMSF, and 5 mM benzamidine. The pellets were left on ice for several hours to dissolve, and centrifuged for 30 min at 16 000*g* to remove undissolved material. The supernatant was applied to a protamine sepharose column, prepared as previously described (Dempfle & Heene, 1987), and equilibrated with buffer A1. On the basis of the concentration measured by ELISA analysis of the media, 1 mL of resin was used for each 2 mg of fibrinogen. The column was washed, and fibrinogen eluted at pH = 4.5 and was neutralized and dialyzed as previously described (Binnie et al., 1993) with the addition that every buffer, aside from the pH = 4.5 elution buffer, contained the protease inhibitors: 5 mM $\epsilon$-ACA, 2 mM EDTA, 10 units of aprotinin/mL, 1 $\mu$M pepstatin, 1 $\mu$M leupeptin, 100 $\mu$M PMSF, and 5 mM benzamidine. The pH = 4.5 elution buffer contained all the inhibitors except benzamidine, as this inhibitor obscured monitoring of protein elution by absorption at 280 nm. Inhibitors were added to buffers just prior to their use in each step. Purified fibrinogen was stored in the presence of inhibitors at −80 °C. Aliquots of purified fibrinogen were monitored by SDS−PAGE and immunoblot analysis as described by Binnie et al. (1993).

*Thrombin-Catalyzed Release of Fibrinopeptides.* The thrombin-catalyzed release of FpA and FpB was measured by HPLC essentially as described by Ng et al. (1993) but using the HPLC buffers described in Haverkate et al. (1986). Prior to thrombin analysis, recombinant fibrinogens were dialyzed (MWCO = 12 000−14 000) at 0−4 °C for 20 h against three changes of TBS (50 mM Tris-HCl, pH = 7.4, 150 mM NaCl) to remove protease inhibitors. The concentration of fibrinogen was determined by measuring the $\Delta A_{280-320}$ and assuming 10 mg of fibrinogen/mL gives a result of 15.1 (Mihalyi, 1968). Aliquots of approximately 2 mL with $\Delta A = 0.2$ were prepared in polypropylene tubes and equilibrated to ambient temperature. Human α-thrombin, the generous gift of Dr. Frank Church, was diluted on ice in TBS in a polypropylene tube to a concentration of 4.3 units/mL. At time 0 min, thrombin was added to the fibrinogen sample to a final concentration of either 0.43 units/mL or 0.043 units/mL, as indicated in results. The samples were mixed by gentle vortexing, and aliquots of 260 $\mu$L were dispensed as rapidly as feasible into seven 1.5 mL microfuge tubes. At the indicated times, reactions were terminated by incubating the aliquoted samples at 97−100 °C for >8 min. To prepare an infinite time point, 1 $\mu$L of thrombin at 4300 units/mL was added to the last aliquot and incubated at room temperature for approximately 25 min and heated at 97−100 °C for 5 min. After being heated, the tubes were kept on ice until all reactions were terminated. The tubes were centrifuged at 13 000*g* for 15 min at 4 °C, and the supernatants transferred to fresh tubes and stored at −20 °C prior to HPLC analysis.

*Data Analysis.* The concentrations of fibrinopeptides were determined by measuring the areas under the peaks on the HPLC chromatograms using the program provided by Isco (Chemresearch Chromatographic Data Management, Isco, Inc., Lincoln, NE), and comparing to areas from standard concentrations of fibrinopeptides. The peak areas for the infinite time points were consistent with the release of 2 mol of fibrinopeptide per mole of fibrinogen. The HPLC detector monitored absorbance at 205 nm, where the molar absorptivities for FpA and FpB are slightly different (Ng et al.,

1993); we did not incorporate this difference into our analysis. HPLC chromatograms were also run for "zero" time points by mixing fibrinogen aliquots heated to 97−100 °C with thrombin also heated to 97−100 °C, and these samples routinely did not have peaks eluting at the times for the fibrinopeptides. The data were plotted as progress curves. Using the program Enzfitter (Elsevier-Biosoft, Cambridge, U.K.), we initially fitted the FpA data to a first-order rate equation using the areas of the six time points plus a point of 0 min and 0 area plus the area of the infinite time point set arbitrarily at 300 min. From this fit we determined a "limit" area that was used as the area for maximal release of either fibrinopeptide. Each area was divided by this limit area, and plotted as the percent of fibrinopeptide release, using the software Sigma Plot (Jandel Scientific Software, San Rafael, CA). The FpA data were fitted to a simple first-order rate equation, and the FpB data were fitted to the standard equation that describes two sequential steps, each of which is a simple first-order reaction. The basis for using these equations is summarized in Ng et al. (1993). The Enzfitter program fitted the data by a nonlinear least-squares analysis and determined the standard error.

## RESULTS

*Synthesis and Characterization of Recombinant Fibrinogens.* Using the procedures previously described (Binnie et al., 1993), we have constructed a G418 resistant CHO cell line that expresses the normal Aα- and γ-chains of human fibrinogen. To obtain normal recombinant fibrinogen, we cotransfected this CHO cell line with a plasmid that confers resistance to histidinol and the plasmid vector, pMLP-Bβ, that directs synthesis of the normal Bβ-chain. We selected cells in histidinol and G418 and screened the media of these cells for fibrinogen. We identified the clone expressing the highest level of fibrinogen and subcloned this cell line by infinite dilution. As described in Materials and Methods, this clone was grown in roller bottles with the surface area expanded by the addition of carrier beads. After the cells reached confluence, serum-free media supplemented with selenium, transferrin, insulin, and aprotinin was added. Media containing fibrinogen was collected as described in Experimental Procedures.

We synthesized three variant fibrinogens incorporating substitutions in the Bβ-chain expressed from plasmid vectors altered by PCR-primer directed mutagenesis of the Bβ cDNA in pMLP-Bβ. The steps in this mutagenesis are summarized in Figure 1A. The cDNA segment encoding the first 239 residues of the Bβ-chain was removed from pMLP-Bβ by digestion with *Sal*I and *Acc*I followed by electrophoretic purification of the larger fragment, which includes the segment conferring ampicillin resistance. The altered cDNAs were prepared as two PCR fragments, PCR1 and PCR2. PCR1 was prepared using a forward primer upstream from the *Sal*I site and a reverse primer that incorporated the desired nucleotide changes. PCR2 was prepared using a reverse primer downstream from the *Acc*I site and a forward primer that incorporated the desired nucleotide changes. The mutagenic primers are shown in Figure 1B. The PCR products were digested with the appropriate enzymes, purified electrophoretically, and ligated to the purified vector. The resulting plasmid clones were sequenced to confirm that the PCR-generated fragments did not contain unintended changes. The three encoded changes were Bβ Ala68 to Thr, Bβ Pro70 to Ser, and Bβ Leu72 to Ser.

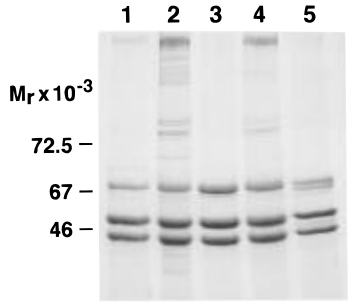



FIGURE 2: SDS−PAGE analysis. Samples in reducing Laemmli buffer were run on an 8% gel that was stained with Coomassie Blue. Lane 1, normal recombinant fibrinogen; lane 2, Bβ A68T-fibrinogen; lane 3, Bβ P70S-fibrinogen; lane 4, Bβ L72S−fibrinogen; lane 5, plasma fibrinogen.

The altered vectors were transfected into the CHO line that expresses normal Aα- and γ-chains, as described above. The three transfections were performed together, and clones resistant to G418 and histidinol were concurrently picked, expanded, and assayed for fibrinogen. We found that 19 of 22 colonies from the A68T transfection, 25 of 29 colonies from the P70S transfection, and 14 of 15 colonies from the L72S transfection synthesized significant levels of fibrinogen. Thus, in all three transfections more than 86% of the colonies were fibrinogen positive. This is in marked contrast to the transfection that produced the CHO line that expresses normal Aα- and γ-chains. Here only 18% of the clones (four of 22 colonies) expressed both Aα- and γ-chains. The consistently high percent of positive clones, which expressed fibrinogens with abnormalities in a single chain, substantiated our choice of a two-step strategy.

Fibrinogen expression varied among these four CHO lines. Fibrinogen concentration in pooled samples for normal fibrinogen varied from 3 to 15 μg/mL; for A68T, from 1 to 6 μg/mL; for P70S, from 4 to 13 μg/mL; and for L72S, from 2 to 15 μg/mL. The fibrinogen concentration fluctuated in an apparently random manner, but frequently the highest synthesis was 20−40 days after the initial collection. Cultures were maintained and medium harvested until fibrinogen concentrations clearly decreased, which varied from 35 to 60 days.

Fibrinogen was purified as described in methods, incorporating two significant changes into the previously described procedure (Binnie et al., 1993). First, the fibrinogen was concentrated from the media by precipitation with 40% saturated ammonium sulfate. The precipitate was collected, dissolved in buffer, and applied to protamine sepharose. Second, every step of the purification was carried out in the presence of a cocktail of protease inhibitors. As previously reported (Binnie et al., 1993), fibrinogen was eluted from protamine sepharose at pH = 4.5, neutralized, dialyzed, and stored at −80 °C in the presence of protease inhibitors. We found that even with these improvements, the yield of pure fibrinogen was unacceptably low when the fibrinogen concentration in the culture medium was ≤1 μg/mL. As shown in Figure 2, SDS−PAGE analysis of the purified fibrinogens indicated that the proteins are reasonably pure and the individual chains are not proteolytically degraded. Prior to thrombin kinetic analysis, the recombinant fibrinogens were dialyzed to remove the protease inhibitors.

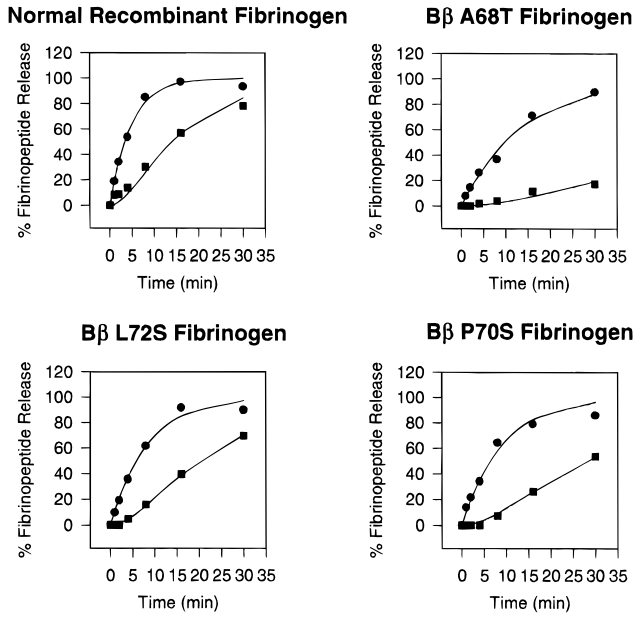



FIGURE 3: Progress curves for fibrinopeptide release. The thrombin-catalyzed release of FpA (●) and FpB (■) was monitored by HPLC. All reactions were run at ambient temperature in 50 mM Tris-HCl, pH = 7.4, 0.15 M NaCl; the fibrinogen concentration was 0.4 $\mu$M; for normal fibrinogen, B$\beta$ P70S-fibrinogen and B$\beta$ L72S-fibrinogen, α-thrombin was 0.043 units/mL; for B$\beta$ A68T-fibrinogen, α-thrombin was 0.43 units/mL. The line through the data for FpA is from eq 1; the line for FpB is from eq 2.

*Time Course of Thrombin-Catalyzed Fibrinopeptide Release.* All kinetic reactions were in 50 mM Tris-HCl, pH = 7.4, 0.15 M NaCl, with the fibrinogen concentration at 0.4 $\mu$M, or approximately $0.1K_{mA}$, to simplify the kinetic analysis. The thrombin concentration was 0.043 units/mL for the reactions with normal fibrinogen, B$\beta$ P70S-fibrinogen, and B$\beta$ L72S-fibrinogen; it was 0.43 units/mL for the reactions with B$\beta$ A68T-fibrinogen. The thrombin-catalyzed release of fibrinopeptides was measured by HPLC essentially as described in detail by Ng et al. (1993). The quantity of fibrinopeptide was measured as the peak area, and the data were plotted as the % fibrinopeptide release where 100% is the maximum value for FpA release, as described in Materials and Methods. Representative progress curves for each of the four recombinant fibrinogens are presented in Figure 3.

The data for the time dependent release of FpA were fitted, as previously described (Ng et al., 1993), to the first-order rate equation

$$\% \text{ FpA} = (1 - e^{-k_1 t}) \times 100 \quad (1)$$

The data for the time-dependent release of FpB were fitted, as previously described (Ng et al., 1993), to the standard equation for two successive first order reactions; that is, the release of FpB is first order and follows the release of FpA:

$$\% \text{ FpB} = (1 + [k_2/(k_1 - k_2)]e^{-k_1 t} - [k_1/(k_1 - k_2)]e^{-k_2 t}) \times 100 \quad (2)$$

In these equations, $k_1$ is the first-order rate constant for the release of FpA and $k_2$ is the first-order rate constant for the release of FpB. To determine $k_2$ for FpB release, we inserted the value of $k_1$ determined from the fit of FpA and we assumed that the maximal release of FpB was equal to that determined from the data for FpA.

The values for $k_1$ and $k_2$ are presented in Table 1. We found that only B$\beta$ A68T−fibrinogen was a poor substrate for thrombin, so we increased the concentration of thrombin 10-fold for the reactions with this variant fibrinogen. The time course experiments were usually run in pairs, using a common diluted thrombin sample. As this dilution appeared to be a major variant in the experimental protocol, experiments using the same diluted thrombin sample are noted by superscript numerals. Because the substrate concentration is less than $0.1K_m$ for FpA, and the $K_m$ for FpB is approximately the same as the $K_m$ for FpA (Martinelli & Scheraga, 1980), we determined the values for the specificity constant, $k_{cat}/K_m$, by dividing the kinetic constants by the concentration of thrombin. These data are presented in Table 2. Analysis of the specificity constants clearly demonstrated that fibrinopeptide release for B$\beta$ A68T−fibrinogen was markedly impaired: the release of FpA from B$\beta$ A68T−fibrinogen was 27-fold slower than from normal recombinant fibrinogen, and the release of FpB from B$\beta$ A68T−fibrinogen was 45-fold slower than from normal fibrinogen. Analysis of the specificity constants also showed that FpA release from B$\beta$ P70S−fibrinogen or B$\beta$ L72S−fibrinogen was about 0.6 times the rate of normal recombinant fibrinogen but was not different from the rate of FpA release from plasma fibrinogen. For FpB release, plasma fibrinogen and normal recombinant fibrinogen were essentially the same, while B$\beta$ L72S−fibrinogen is about 0.8× and B$\beta$ P70S-fibrinogen is about 0.6× that of normal recombinant fibrinogen. Thus, the rates of thrombin catalyzed fibrinopeptide release from these two variants were not substantially different from those of normal fibrinogen, whether plasma fibrinogen or normal recombinant fibrinogen. We note that these experiments indicated that FpA release from normal recombinant fibrinogen is about twice that of plasma fibrinogen. Subsequent experiments show that this difference is not reproducible (Gorkun et al., 1995).

## DISCUSSION

The experiments described here demonstrate that the two-step procedure for synthesis of recombinant fibrinogen enabled efficient synthesis of multiple variants with changes in a single polypeptide chain, in this case the B$\beta$-chain. For each transfection we identified the clone with the highest synthesis. Nevertheless, the synthesis of fibrinogen varied among the clones. We have not examined the basis for this difference in expression, but we presume that it results from differences in the site of plasmid integration and the copy number of integrated expression vector. In order to enhance the efficiency of purification, we concentrated the fibrinogen by ammonium sulfate precipitation prior to chromatography. We found that the concentrated protein was more labile, presumably due to increased concentration of proteases as well as their substrate. We therefore added a mixture of inhibitors during the precipitation and in all steps thereafter. These new steps improved both the yield and the quality of the pure protein relative to the previous protocol (Binnie et al., 1993). We continued to find that the level of fibrinogen in the pooled media was critical for effective purification. When the concentration was ≤1 $\mu$g/mL, the yield of pure protein was less than 20% and the individual chains were significantly degraded.

The present study was initiated to examine the basis for the observation that fibrinogen Naples is a poor substrate

Table 1: First-Order Rate Constants for Fibrinopeptide Release[a]

| | plasma fib. | recombinant | Bβ A68T[b] | Bβ P70S | Bβ L72S |
|---|---|---|---|---|---|
| expt 1 | | | | | |
| FpA | 0.19 ± 0.02[1 c] | 0.23 ± 0.04 | 0.084 ± 0.005[3] | 0.11 ± 0.02[2] | 0.16 ± 0.03[1] |
| FpB | 0.062 ± 0.002 | 0.035 ± 0.001 | 0.012 ± 0.001 | 0.043 ± 0.005 | 0.037 ± 0.002 |
| expt 2 | | | | | |
| FpA | 0.12 ± 0.03[2] | 0.21 ± 0.02[4] | 0.071 ± 0.005[4] | 0.11 ± 0.02[5] | 0.12 ± 0.01[5] |
| FpB | 0.064 ± 0.008 | 0.077 ± 0.009 | 0.013 ± 0.001 | 0.038 ± 0.001 | 0.062 ± 0.002 |
| expt 3 | | | | | |
| FpA | 0.082 ± 0.03[6] | 0.19 ± 0.02[4] | 0.077 ± 0.005[4] | 0.14 ± 0.02[3] | 0.094 ± 0.011[6] |
| FpB | 0.093 ± 0.007 | 0.098 ± 0.011 | 0.016 ± 0.001 | 0.041 ± 0.002 | 0.068 ± 0.011 |
| av | | | | | |
| FpA | 0.13 ± 0.02 | 0.21 ± 0.02 | 0.077 ± 0.004 | 0.12 ± 0.01 | 0.12 ± 0.01 |
| FpB | 0.067 ± 0.002 | 0.063 ± 0.004 | 0.014 ± 0.001 | 0.038 ± 0.002 | 0.051 ± 0.002 |

[a] Values are $k_1$ from eq 1 for FpA and $k_2$ from eq 2 for FpB. Data are in $min^{-1}$. [b] Data from Bβ A68T−fibrinogen were obtained with 0.43 units of thrombin/mL, while all other reactions were with 0.043 units of thrombin/mL. [c] Superscript numerals (1−6) indicate experiments performed concurrently.

Table 2: Specificity Constants, $k_{cat}/K_m$, for FpA and FpB Release[a]

| substrate | FpA | FpB |
|---|---|---|
| plasma fibrinogen | 5.4 | 2.8 |
| recombinant fibrinogen | 8.8 | 2.6 |
| Bβ A68T-fibrinogen | 0.32 | 0.058 |
| Bβ P70S-fibrinogen | 5.0 | 1.6 |
| Bβ L72S-fibrinogen | 5.0 | 2.1 |

[a] Values are $\times 10^6$ $M^{-1}$ $s^{-1}$.

for thrombin. The previous work demonstrated that a single amino acid substitution, Bβ A68T, disrupted the interaction between thrombin and either fibrinogen or fibrin. Current models of thrombin indicate that the interaction between the substrate and thrombin's fibrinogen recognition site is critically impaired (Koopman et al., 1992; Binnie & Lord, 1993). We hypothesized that this substitution did not break one critical thrombin−fibrinogen interaction. Rather, this substitution altered the local fibrinogen conformation such that multiple thrombin−fibrinogen contacts are broken. We made two variant fibrinogens to test the hypothesis that single substitutions in this region would alter this local conformation. We chose substitutions on the basis of primary sequence information. A comparison of the three human polypeptide chains in this region, aligned by the Cys residues that link the chains, indicated that Pro70 differs from the comparable Glu found in Aα and γ. Thus, this residue may have a significant role, unique to Bβ-chain structure. Further comparison of the three chains in this region shows that Leu72 in Bβ, Trp41 in Aα, and Phe15 in γ are all hydrophobic side chains. Previous experiments had indicated that Aα Trp41 has a role in thrombin binding (Lord & Binnie, 1990). Across-species comparison of this region of the Bβ chain, presented in Figure 4, also indicated that Leu72 may have a critical role. Leu is conserved in bovine, chicken, and lamprey fibrinogens at positions comparable to the human Bβ72. In rat, however, this Leu is replaced by Met. We noted that Asp69 and Asp71 are highly conserved across species as well as between the human Aα- and Bβ-chains. As we and others have found that substitutions at analogous charged residues did not substantially alter thrombin binding, we chose not to change these residues (Binnie & Lord, 1993; Stone et al., 1989). As our goal was to imitate the disruption associated with the A68T change, we chose to substitute Pro70 and Leu72 with Ser in order to incorporate the hydrophilic $^-$OH found in the A68T substitution and also to minimize the bulk of the substitution. We also synthesized recombinant A68T as the prototype for these new variants.

```
60  -PDAGGCLHADPDLGVLCPTGCQLQEA-  85    Human
     *** ***************** **

32  -PDADGCLHADPDLGVLCPTGCKLQDT-  57    Bovine
     ****** * * * **** *** *

92  -PDAGGCVHGDGDMGVLCATGCELRQT-  117   Rat
     ****** *    ********* **

64  -PDAGGCKHPLDELGVLCPTGCELQTT-  89    Chicken
      * ***     ********** * *

43  -RDEGGCMLPESDLGVLCPTGCELREE-  68    Lamprey
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FIGURE 4: Alignment of Bβ chain sequences from five species. The amino acid sequences were aligned using data from GenBank and the GCG program BESTFIT (Genetics Computer Group, Inc., Madison, WI). The numbers refer to the amino acid position relative to residue 1 at the amino terminus of the circulating protein. The "*" indicates positions in the species on the line below the symbol that are identical to the human sequence.

We found that only A68T was a poor substrate, with FpA release decreased 27-fold and FpB release decreased 45-fold. Thus, the recombinant fibrinogen maintained the characteristics found for the dysfibrinogen Naples. This single substitution clearly accounted for the biochemical data obtained with fibrinogen Naples. The new kinetic data presented here indicate that the A68T substitution impaired FpB release more strongly than FpA release. Impaired FpB release was also found with the other variants. That is, the rate of FpB release from P70S and L72S was slightly impaired relative to FpA release. This implies that all three Bβ-chain substitutions have a direct, but small, effect on FpB cleavage. Because the P70S and L72S changes do not significantly impair fibrinopeptide cleavage, we conclude that Bβ Ala68 has a novel role in the interaction between thrombin and fibrinogen. The data do not support our hypothesis that A68T alters the local conformation of the Bβ-chain domain and thereby impairs thrombin binding. Of course, it is possible that we chose residues that do not contribute to the local conformation that is critical for thrombin recognition. We plan to further characterize the recombinant A68T fibrinogen to determine the basis of this unique finding. For example, we will examine whether the disulfide pairs in this variant fibrinogen differ from normal fibrinogen.

In summary, we have efficiently synthesized three variant recombinant fibrinogens with changes in the Bβ-chain. The

proteins have been purified and characterized as substrates for thrombin. We found significantly impaired fibrinopeptide release with the A68T substitution, confirming that this single substitution accounts for the *in vitro* changes found with the patient's sample. Fibrinopeptide release with either the P70S or L72S substitution was not significantly different from normal.

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## REFERENCES

Binnie, C. G., & Lord, S. T. (1993) *Blood 81,* 3186−3192.
Binnie, C. G., Hettasch, J. M., Strickland, E., & Lord, S. T. (1993) *Biochemistry 32,* 107−113.
Blumenstein, M., Matsueda, G. R., Timmons, S., & Hawiger, J. (1992) *Biochemistry 31,* 10692−10698.
Bode, W., Turk, D., & Karshikov, A. (1992) *Protein Sci.* 1, 426−471.
Bode, W., Mayr, I., Huber, R., Stone, S. R., & Hofsteenge, J. (1989) *EMBO J. 8,* 3467−3475.
Dempfle, C. L., & Heene, D. L. (1987) *Thromb. Res. 46,* 19−27.
Doolittle, R. F. (1984) *Annu. Rev. Biochem. 53,* 195−229.
Gorkun, O. V., Lord, S. T., Veklich, Y. I., & Weisel, J. W. (1995) *Blood 86* (Suppl. 1), Abstr. 3477.
Grutter, M. G., Priestle, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., & Stone, S. R. (1990) *EMBO J. 9,* 2361−2365.
Koopman, J., Haverkate, F., Lord, S. T., Grimbergen, J., & Mannucci, P. M. (1992) *J. Clin. Invest. 90,* 238−244.
Liu, C. Y., Koehn, J. A., & Morgan, F. J. (1985) *J. Biol. Chem. 260,* 4390−4396.
Lord, S. T., & Binnie, C. G. (1990) *Blood Coagulation Fibrinolysis 1,* 461−463.
Lord, S. T., Byrd, P. A., Hede, K. L., Wei, C., & Colby, T. J. (1990) *J. Biol. Chem. 265,* 838−843.
Martin, P. D., Robertson, W., Turk, D., Huber, R., Bode, W., & Edwards, B. F. P. (1992) *J. Biol. Chem. 267,* 7911−7920.
Martinelli, R. A., & Scheraga, H. A. (1980) *Biochemistry 19,* 2343−2350.
Mihalyi, E. (1968) *Biochemistry 7,* 208−223.
Ng, A. S., Lewis, S. D., & Shafer, J. A. (1993) *Methods Enzymol. 222,* 341−358.
Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., & Fenton, J. W. (1990) *Science 249,* 277−280.
Stone, S. R., Dennis, S., & Hofsteenge, J. (1989) *Biochemistry 28,* 6857−6863.
Stubbs, M. T., Oschkinat, H., Mayr, I., Huber, R., Angliker, H., Stone, S. S., & Bode, W. (1992) *Eur. J. Biochem.* 206, 187−195.

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