Severely Impaired Polymerization of Recombinant Fibrinogen γ-364 Asp → His, the Substitution Discovered in a Heterozygous Individual* 

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During blood coagulation, soluble fibrinogen is converted to fibrin monomers that polymerize to form an insoluble clot. Polymerization has been described as a two-step process: the formation of double-stranded protofibrils and the subsequent lateral aggregation of protofibrils into fibers. Previous studies have shown that γ chain residues Tyr-363 and Asp-364 have a significant role in polymerization, most likely in protofibril formation. To better define the role of these residues, we synthesized three fibrinogens with single substitutions at these two positions: Tyr-363 → Ala, Asp-364 → Ala, and Asp-364 → His. We found that the release of fibrinopeptides A and B was the same for these variants and normal recombinant fibrinogen, showing that all variants had normal fibrin formation. In contrast, we found that polymerization was significantly delayed for both Ala variants and was almost nonexistent for the His variant. Clottability for the Ala variants was only slightly reduced, and fibrin gels were formed. Surprisingly, clottability of the His variant was substantially reduced, and fibrin gels were not formed. Our data suggest that both protofibril formation and lateral aggregation were altered by these substitutions, indicating that the C-terminal domain of the γ chain has a role in both polymerization steps. 

Fibrinogen is a plasma glycoprotein composed of a pair of three polypeptide chains, Aα, Bβ, and γ. The six N termini form a central domain, called E, which can be isolated as a single fragment from a plasmin digest of fibrinogen. The six chains divide into two three-chain sets that emanate in opposite directions from the central E domain as coiled-coil rods that terminate with the C-terminal residues of each chain forming separate domains. The peripheral domains can also be isolated from plasmin digests as the D fragments, which contain residues from all three chains but consist primarily of the C-terminal domains of the Bβ and γ chains. 

During blood coagulation, fibrinogen is converted to an insoluble fibrin clot by the serine protease thrombin, which cleaves four peptide bonds, releasing two fibrinopeptides A (FpA, Aα 1–16) and two fibrinopeptides B (FpB, Bβ 1–14) that fibrin monomers that polymerize spontaneously. The association of fibrin monomers into a fibrin clot has long been described as a two-step process, where the first step involves half-staggered, end-to-end interactions leading to double-stranded protofibrils and the second step, usually called lateral aggregation, involves the assembly of protofibrils into thick, multi-stranded fibers that branch to form a fibrin network. The final product is a fibrin gel (1, 2). The interactions that promote protofibril formation occur between N-terminal α chain residues in the D domain on one fibrin molecule and C-terminal γ chain residues in the D domain on a second fibrin molecule. The interactions that promote lateral aggregation are less well known, although it has been shown that FpB cleavage enhances lateral aggregation (1, 3). The enhanced lateral aggregation may, however, be an indirect result of strengthened protofibril interactions that accompany FpB release (4). It has also been shown that the C-terminal domains of the α chain (the αC domains) participate in lateral aggregation and that calcium binding to fibrinogen increases during lateral aggregation (5–11). 

Recent experiments have identified two specific residues in the D domain of the γ chain as participants in protofibril formation. Tyr-363 in the γ chain was identified by photoaffinity labeling with a peptide that mimics the N-terminal α chain residues of the E domain of fibrin (12). Asp-364 in the γ chain was identified in fibrinogen Matsumoto I (13), a dysfibrinogen that was found in a heterozygous individual. Fibrinogen Matsumoto I is a mixture of molecules with normal and variant γ chains that have His at position 364. Polymerization of fibrinogen Matsumoto I is markedly delayed, and this delay can be partially compensated by mixing with normal fibrinogen (13). The results suggest that the normal molecules of this fibrinogen support polymerization, whereas the variant molecules do not. 

In the studies described, here we examined the roles of these two residues by analysis of genetically engineered variants. Using the previously described two-step procedure for synthesis of variant fibrinogens, we synthesized three variants with single amino acid substitutions in the γ chain: Tyr-363 → Ala (Y363A), Asp-364 → Ala (D364A), and Asp-364 → His (D364H). We determined the clottability of these variants and followed the kinetics of thrombin-catalyzed fibrinopeptide release and thrombin-catalyzed polymerization. We compared the variants to one another and to normal recombinant fibrinogen, which has polymerization characteristics comparable to normal plasma fibrinogen (14). 

EXPERIMENTAL PROCEDURES 

Materials—The plasmid vectors, Chinese hamster ovary (CHO) cells, and culture medium have been previously described (15). Poly-

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1 The abbreviations used are: CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis.
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Purity of proteins were monitored by SDS-PAGE and immunoblot analysis as described (15). After dialysis, fibrinogen was aliquoted, frozen, and stored at −80 °C.

Thrombin-catalyzed Release of Fibrinopeptides—The reactions were performed in 20 mM HEPES, pH 7.4, 0.1 m NaCl, and 10 mM CaCl₂ at ambient temperature with human α-thrombin. The final concentrations of thrombin and fibrinogen were 0.01 unit/ml (0.11 nM) and 0.09 mg/ml (260 nM), respectively. We chose these concentrations of thrombin and fibrinogen to readily detect the sequential release of FpB following FpA (21). The reactions were performed in duplicate for normal and each variant fibrinogen. Fibrinopeptide release was monitored by reverse phase-high performance liquid chromatography, and the quantity of each fibrinopeptide was determined from the peak area. The data were fitted to two equations, and the specificity constants were calculated as described (18).

Thrombin-catalyzed Fibrin Polymerization—Polymerization was followed by turbidity changes in time at 350 nm using a BioSpec-1601 spectrophotometer (Shimadzu Corp., Tokyo, Japan). Reactions were performed in a final volume of 100 μl, as described (14). Briefly, fibrinogen (90 μl at 0.1 or 0.5 mg/ml) in 20 mM HEPES, pH 7.4, 0.12 mM NaCl, and 10 mM CaCl₂ was mixed with human α-thrombin (10 μl at 5 or 0.5 unit/ml), and changes in turbidity were monitored at ambient temperature. The final concentrations of thrombin and fibrinogen were indicated in the figure legends for each experiment. Two parameters were calculated from the turbidity curves, as described previously (22): the lag-period, which reflects the rate of prototol fibrin formation, and the maximum slope of the turbidity, which is characteristic of the rate of lateral aggregation of prototobils. The reactions were performed in triplicate for normal and each variant fibrinogen.

Fibrinogen Clottability—Clottability of the purified fibrinogens was determined essentially as described (23), mixing human α-thrombin (final concentration 0.05 unit/ml) and fibrinogen (final concentration 0.4 mg/ml) in 20 mM HEPES, 0.12 mM NaCl, and 10 mM CaCl₂. Samples were incubated for 18 or 48 h at ambient temperature, and fibrin clot or fibrin aggregates were removed by centrifugation at 13000 × g for 15 min. Fibrin that was not incorporated into the pellet was determined from the A₂₈₀ of the supernatant, and clottability was calculated as (ΔA₂₈₀ at zero time − ΔA₂₈₀ of the supernatant)/ΔA₂₈₀ at zero time) × 100%. No correction was made for absorbance from the added thrombin.

RESULTS

Synthesis and Characterization of Recombinant Fibrinogens—We synthesized three variant fibrinogens with single amino acid substitutions in the C-terminal domain of the γ chain: Tyr-363 → Ala (Y363A), Asp-364 → Ala (D364A), and Asp-364 → His (D364H). The substitutions were introduced by oligonucleotide-directed mutagenesis of the γ chain cDNA cloned in the previously described expression vector pMLP-γ (18). We sequenced the entire γ chain cDNA to identify the correctly changed codons and to confirm that no unanticipated coding changes were incorporated (data not shown). Each al- tered vector was cotransfected with pMSVhis into a CHO cell line that expressed the normal As and Bβ chains of fibrinogen. Histidinol-resistant colonies were picked and expanded, and the culture medium was assayed for fibrinogen as described (15). We found that 8 of 19 colonies from the Y363A transfection, 10 of 11 colonies from the D364A transfection, and 8 of 18 colonies from the D364H transfection synthesized significant levels of fibrinogen. Cell lines that expressed the highest levels of fibrinogen were used for large scale production of each fibrinogen variant.

Serum-free medium containing recombinant fibrinogen was harvested repetitively for several weeks from CHO cell cultures grown in roller bottles. Harvested aliquots were pooled, and the fibrinogen concentration was determined by enzyme-linked immunosorbent assay. The concentration of secreted fibrinogen varied between cell lines. Pooled medium contained 1–4 μg/ml Y363A fibrinogen, 2–7 μg/ml D364A fibrinogen, and 4–7 μg/ml D364H fibrinogen. Fibrinogen was concentrated from the medium by ammonium sulfate precipitation and purified by immunoaffinity chromatography with the Ca⁴⁺-dependent monoclonal antibody IF-1 (19). We found that the D364H variant, in

clonal antisem to human fibrinogen was from Dako, Carpenteria, CA, and phospha- tase-conjugated secondary antibodies were from Pierce. Monoclonal antibody 9C3, which reacts with As chain residues 240–268 (10), was a generous gift from Dr. Andrei Budzynski, Temple University, Philadelphia, PA. Human α-thrombin was a generous gift from Dr. Andrei Budzynski, Temple University, Philadelphia, PA. Human α-thrombin was a generous gift from Dr. Andrei Budzynski, Temple University, Philadelphia, PA. Human α-thrombin was a generous gift from Dr. Andrei Budzynski, Temple University, Philadelphia, PA. Human α-thrombin was a generous gift from Dr. Andrei Budzynski, Temple University, Philadelphia, PA. Human α-thrombin was a generous gift from Dr. Andrei Budzynski, Temple University, Philadelphia, PA. Human α-thrombin was a generous gift from Dr. Andrei Budzynski, Temple University, Philadelphia, PA.
contrast to normal fibrinogen, did not bind to this immunoaffinity resin in the presence of 1 mM CaCl$_2$. This variant did bind to the immunoaffinity column in the presence of 10 mM CaCl$_2$. We therefore immunopurified all three $\gamma$ chain variants in the presence of 10 mM CaCl$_2$, eluting the variant fibrinogen that was retained on the column with 50 mM EDTA, as described under “Experimental Procedures.” A representative chromatographic elution profile and an SDS-PAGE analysis of the purified fibrinogens are presented in Fig. 1. The data show that all three variant proteins were pure and that each variant had the normal $\alpha$, $\beta$, and $\gamma$ chain composition. There was also no evidence of unusual protein degradation.

Thrombin-catalyzed Fibrinopeptide Release—The thrombin-catalyzed release of fibrinopeptides from normal fibrinogen and the three variant fibrinogens was followed with time, as described (18). The data were plotted as progress curves (data not shown) and were fitted to two successive first-order rate equations, assuming FpB release follows FpA release (24). The results, presented in Table I, clearly showed that the thrombin cleavage of FpA and FpB for all three variant fibrinogens was not significantly different from normal fibrinogen.

| Substrate                    | FpA      | FpB      |
|-----------------------------|----------|----------|
| Normal recombinant fibrinogen | $10.6 \pm 1.95$ | $3.0 \pm 0.9$ |
| D364H recombinant fibrinogen   | $10.2 \pm 0.85$ | $2.2 \pm 0.05$ |
| D364A recombinant fibrinogen   | $10.8 \pm 0.65$ | $1.8 \pm 0.05$ |
| Y363A recombinant fibrinogen   | $11.1 \pm 2.85$ | $2.5 \pm 0.4$ |

Table I: Specificity constants, $k_{cat}/K_m$, for FpA and FpB release. Values are $\times 10^6$ M$^{-1}$ s$^{-1}$ (18).

Thrombin-catalyzed Fibrin Polymerization—Thrombin-catalyzed polymerization was monitored as the change in turbidity at 350 nm, as described (22). Representative turbidity curves are shown in Fig. 2, and quantitative data averaged from several experiments are summarized in Table II. We measured the lag period, which reflects the rate of protofibril formation, and the maximum slope, which reflects the rate of protofibril assembly into fibers. As shown in Fig. 2A, under experimental conditions where normal fibrinogen has a lag period of about 3 min and a maximum slope of $37 \times 10^{-8}$ sec$^{-1}$, all three variant fibrinogens showed markedly impaired polymerization. At 60 min, the Y363A and D364A variants showed only a slight increase in turbidity. As presented in Table II, for the Y363A variant the lag period was 8-fold longer, and the maximum rate of polymerization was 16-fold slower than normal fibrinogen. The consequences were larger for the D364A variant, with a 13-fold longer lag period and a 31-fold slower rate of polymerization. With the D364H variant, there was no change in tur-
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**Table II**

| Recombinant fibrinogen | Experiment 1 | Experiment 2 | Experiment 3 |
|-------------------------|--------------|--------------|--------------|
|                         | $V_{\text{max}}$ | Lag period  | $V_{\text{max}}$ | Lag period  | $V_{\text{max}}$ | Lag period  |
| Normal                  | 37 ± 3       | 3.1 ± 0.1   | 170 ± 12      | 2.3 ± 0.2   | 130 ± 4       | 0.56 ± 0.06   |
| γ-Y363A                 | 2.3 ± 0.3    | 24 ± 2      | 62 ± 0.5      | 6.2 ± 0.1  | 6.3 ± 1.1    | 7.5 ± 0.3     |
| γ-D364A                 | 1.2 ± 0.5    | 41 ± 4      | 34 ± 6        | 8.7 ± 1.4  | 2.3 ± 0.4    | 14 ± 2        |
| γ-D364H                 | No change$^{a}$ | No change$^{a}$ | 1.4 ± 0.1 | 150 ± 15   | No change$^{a}$ | No change$^{a}$ |

$^{a}$ The experiments were monitored for at least 48 h.

Turbidity within 1 h, as shown in Fig. 2A. Moreover, we continued to monitor the D364H sample for 48 h and still saw no change in turbidity (data not shown).

To determine the factor limiting the polymerization of these variants, we examined polymerization at higher fibrinogen or higher thrombin concentration. As shown in Fig. 2B, when the fibrinogen concentration was increased 5-fold, to 0.45 mg/ml, changes in turbidity were clearly evident within 60 min for both the Y363A and D364A variants. With the D364H variant, no increase in turbidity was seen at 2 h, and slight increase was evident within 3 h. The turbidity continued to rise over the 10 h of observation with the D364H variant but did not reach a plateau (data not shown). Thus, increasing the fibrinogen concentration did enhance polymerization for all variants, although polymerization was clearly abnormal. The results presented in Table II, Experiment 2, show that at this higher fibrinogen concentration, polymerization of the D364A variant was again more delayed than the Y363A variant. At the higher fibrinogen concentration, the shapes of the polymerization curves for both Ala variants were more like normal fibrinogen. At this higher concentration, we were able to measure an increase in turbidity with the D364H variant; the lag period was 65-fold longer, and the maximum rate of polymerization was 120-fold slower than normal fibrinogen. Observation of the material in the cuvette after prolonged incubation showed that the D364H variant did not produce a normal clot but rather appeared like a suspension of white precipitate.

When the concentration of thrombin was increased 10-fold (with fibrinogen at 0.09 mg/ml), we saw a 5.5-fold decrease in the lag period and a 3.5-fold increase in the maximum rate with normal fibrinogen (Table II, compare Experiment 1 with Experiment 3). Both of the Ala substitution variants showed similar changes, a 3-fold decrease in the lag period and a 2–3-fold increase in the maximum rate. These data indicate that an increase in thrombin concentration influenced polymerization for the two Ala variants in a manner analogous to that of normal fibrinogen (9). At 0.09 mg/ml D364H fibrinogen, a 10-fold increase in thrombin had no effect; we saw no change in turbidity even after 48 h with thrombin.

**Clottability Determinations**—As the turbidity measurements indicated that polymerization was impaired, we decided to measure the clottability of these fibrinogens to test whether fibrin polymers were formed. Fibrinogen was incubated with thrombin, and the reaction was terminated by centrifugation. Clottable fibrin was determined as the percent of protein that remained in the supernatant by SDS-PAGE and stained with Coomassie Blue (lanes 1–3) or immunoblotted and developed with a monoclonal antibody (9C3) to A chain (lanes 4–6) or a polyclonal antibody to human fibrinogen (lanes 7 and 8). Lanes 1 and 4, variant fibrinogen D364H; lanes 2, 5, and 7, nonclottable D364H after 18-h incubation with thrombin; lanes 3, 6, and 8, nonclottable D364H after 48-h incubation with thrombin.

FIG. 3. Fibrinogen clottability. A, clottability was expressed as the percent protein removed by centrifugation after incubation with thrombin for 18 h (black bars) or 48 h (gray bar). Fibrinogens were normal recombinant (N) and γ chain variants D364A, Y363A and D364H. B, analysis of the nonclottable material (sample supernatants) from γ chain variant D364H. Samples were run under reducing conditions on 9% SDS-PAGE and stained with Coomassie Blue (lanes 1–3) or immunoblotted and developed with a monoclonal antibody (9C3) to A chain (lanes 4–6) or a polyclonal antibody to human fibrinogen (lanes 7 and 8). Lanes 1 and 4, variant fibrinogen D364H; lanes 2, 5, and 7, nonclottable D364H after 18-h incubation with thrombin; lanes 3, 6, and 8, nonclottable D364H after 48-h incubation with thrombin.

Clottability Determinations—As the turbidity measurements indicated that polymerization was impaired, we decided to measure the clottability of these fibrinogens to test whether fibrin polymers were formed. Fibrinogen was incubated with thrombin, and the reaction was terminated by centrifugation. Clottable fibrin was determined as the percent of protein that was removed from solution by centrifugation. Samples were prepared using the same conditions as for the turbidity measurements shown in Fig. 2B, and clottable fibrin was determined after 18 or 48 h. The results are shown in Fig. 3A. As is typical, we found that normal recombinant fibrinogen was 97% clottable. The two Ala substitutions were only slightly less clottable, about 92%. In sharp contrast, the D364H variant did not form a fibrin gel. During the 18-h incubation, we observed the formation of a white, amorphous precipitate. After centrifugation, we found that only 56% D364H was clottable. When the incubation time was extended to 48 h, this number increased to 63% clottable. We examined the nonclottable material that remained in the supernatant by SDS-PAGE and immunoblots, as shown in Fig. 3B. The Coomassie stained gel showed additional bands with higher mobilities than the α, β, and γ chains, and the immunoblots showed that these additional bands are fibrin. The immunoblot developed with the monoclonal antibody 9C3 (lanes 4–6, Fig. 3B), which reacts with the α chain, showed that most of the higher mobility bands were degraded α chains; after 48 h, no intact α chain band was evident. These results are a clear indication that the protein that remained in the supernatant was mostly degraded D364H fibrin. In control experiments, all four fibrinogens (normal and the three variants) were incubated for 48 h under identical conditions but without thrombin; analysis of these samples by SDS-PAGE showed no indication of protein degradation (data not shown). These results indicate that the prolonged incubation with thrombin generated the degraded D364H fibrin.
DISCUSSION

The data presented here confirmed that the C-terminal γ chain residues 363 and 364 are important participants in polymerization. We found that Ala substitutions at either position 363 or 364 dramatically delayed polymerization. We measured turbidity, which with normal fibrin polymerization gauges the rate of protofibril formation from the lag period, and the rate of fiber formation from the slope of the turbidity change. With both Ala substitutions, the lag periods were lengthened and the slopes were less steep. The 363 Ala substitution was the most normal, and even with this mutant the shortest lag period and the most rapid increase in turbidity were about 3-fold delayed relative to normal fibrinogen under the most favorable conditions. These results suggest that both protofibril formation and fiber formation were affected by the single Ala substitutions. Alternatively, the Ala substitutions may change only one of the polymerization steps and still alter both the lag period and the slope. This possibility has been carefully described by Weisel and Nagaswami (9), who showed with simulated data that changing the rate of protofibril formation or the rate of lateral aggregation can change both the lag and the slope of a turbidity curve. For example, if the rate of protofibril formation is slowed, then the lag period is lengthened, and the slope of the turbidity increase is decreased. Similarly, if the rate of fiber formation is slowed, then normal protofibrils form in the normal time frame, but the lateral aggregation of these protofibrils is less efficient, which is seen as both a longer lag period and a reduced slope. Thus, further studies using assays that distinguish these two steps are needed to determine whether one or both steps of polymerization is impaired in these Ala variants. Even though polymerization was delayed with both of the Ala variants, the clottability data and clot appearance, which was similar to the normal clot, indicate that large, insoluble polymers were finally assembled from these molecules.

The turbidity curves obtained with the Asp-364 to His substitution were more remarkable. We were able to measure changes in turbidity only at 0.45 mg/ml fibrinogen. At lower fibrinogen concentrations (0.09 mg/ml), where normal polymerization is complete within a few hours, polymers with D364H were not detected even after days of incubation. A delay in D364H fibrin polymerization was predicted from studies with fibrinogen Matsumoto I, which contains normal and variant γ chains, but the striking loss of function found with the homozygous recombinant variant was unanticipated. The clottability data showed that normal polymers were not formed; no fibrin gel was seen and only half the protein was incorporated into precipitated material. This result was observed even though fibrinopeptide release indicated normal fibrin formation. Analysis of the nonclottable protein suggests that the soluble fibrin molecules were degraded, probably by prolonged exposure to thrombin. Thrombin cleavage of the C-terminal region of α chains has been previously reported (25). This extensive proteolysis most likely occurred because the fibrin monomers were not readily incorporated into fibrin polymers, but the proteolysis may subsequently have contributed to the lowered clottability.

The normal kinetics for FpB release from these three variants, which show delayed polymerization, was unanticipated. It is generally accepted that the conversion of fibrinogen to fibrin proceeds in an ordered fashion such that FpA is released, desA-fibrin polymers are formed, and FpB is released from these polymers (7, 21). Consequently, the kinetics of thrombin-catalyzed FpB release depend on polymerization, such that FpB release is delayed when fibrin polymerization is limited. For example, both EDTA and the peptide Gly-Pro-Arg-Pro inhibit polymerization, and both decrease the rate of FpB release from normal fibrinogen (21, 26, 27). Further, two abnormal fibrinogen variants, London I and Ashford, show impaired polymerization of fibrin monomers and exhibit a decreased rate of FpB release. Thus, the normal kinetics for FpB release from the variants studied here (Table I) indicate that an intermolecular structure, either the normal D:E structure found in protofibrils or something that mimics the function of the protofibril structure, was formed at a normal rate. This result is surprising because the residues γ363 and γ364 are thought to support D:E interactions, such that a significant change at these residues would be reflected in the rate of protofibril formation. Our results showed that changes at residues γ363 and γ364 did in fact significantly alter polymerization, so we anticipated a change in D:E interactions and the consequential change in FpB kinetics. The observed normal release of FpB therefore implies that intermolecular structures are formed at a normal rate. Of course, our data are also consistent with the conclusion that these intermolecular structures are not necessary for normal FpB kinetics, but this would be contrary to previous reports.

Our results are similar to those seen with several dysfibrinogens with known changes in the C-terminal domain of the γ chain, impaired polymerization associated with normal rate of FpB release (28). The kinetics of FpB release from these dysfibrinogens must be considered with caution, however, as these variants, like Matsumoto I, were identified in individuals with heterozygous genotypes. Kinetic analysis of a mixture of normal and variant proteins is ambiguous, especially at concentrations of fibrinogen and thrombin that are higher than those used to obtain the data in Table I. Nevertheless, the reports on these dysfibrinogens lend support to the possibility that changes in the C-terminal domain of the γ chain impair polymerization but do not prevent intermolecular interactions needed to support normal FpB release.

Recently, high resolution structures have been published for the isolated 30-kDa C-terminal fragment of the human fibrinogen γ chain alone and in a complex with the peptide GPRP (29, 30). As microcalorimetry data show that the C-terminal domain of the γ chain is folded independently (31), it is likely that this isolated fragment, which was expressed in the yeast Pichia pastoris, preserves the structure it has in the fibrinogen molecule. These structures, therefore, allow us to speculate on the molecular basis for the functional changes we have observed. A deep pocket, called the polymerization pocket, is defined by four loops, P-1 through P-4. Tyr-363 and Asp-364 lie within loop P-3. As shown in Fig. 4, the side chain for Tyr-363 faces into the pocket such that it can participate in D:E interactions. Thus, exchanging the large, aromatic, hydrogen bond-forming side chain of Tyr for the methyl group of Ala is likely to eliminate significant aspects of this intermolecular interaction and thereby abate polymerization. It is reasonable to assume that multiple D-domain residues participate in the D:E interactions, such that Tyr-363 is not the only critical residue. As shown in Fig. 2 and Table II, polymerization with the γ363A variant is abated but not eliminated, and fibrin clots do form. Nevertheless, the extensive delay in polymerization of γ363A demonstrates that this single residue has a consequential role in fibrin clot formation.

As shown in Fig. 4, Asp-364 is oriented such that it forms a salt bridge with Arg-375 in loop P4. This electrostatic interaction could anchor the relative orientation of loops P-3 and P-4. As both loops contribute residues that line the polymerization pocket, it is reasonable to expect that changes in the orientation of these loops would alter polymerization. A critical role for Arg-375 is supported by the observation of abnormal fibrin...
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polymerization with the substitution of Gly for Arg-375 in the heterozygous dysfibrinogen Osaka V. Furthermore, the structure obtained in the presence of GPRP showed that Asp-364 forms a salt bridge with the N terminus of the GPRP. Thus, Asp-364 likely participates in two ways: forming an intermolecular salt bridge that is critical to fibrinogen structure and supporting the intermolecular D:E interactions that are critical to fibrin polymerization. Therefore, the substitution of Ala for Asp would be expected to significantly delay polymerization. Our data are also consistent with the recent report by Cote et al. (32) who synthesized and characterized the 30-kDa recombinant fragment with the D364A substitution. They found that the peptide GPRP did not bind to this variant and that this variant fragment was unable to inhibit polymerization of normal fibrin.

Interpretation of the results from the substitution of His for Asp-364 is more difficult, because the changes in function were so dramatic. In contrast to the two Ala substitutions, this mutant did not form a fibrin gel under the conditions described here. After long incubations with thrombin, turbidity increases were apparent, but these were not associated with fibrin clot formation. Both the lag period and the rate of turbidity change with this mutant were 10-fold or more longer that than observed with the substitution of Ala at this position. Obviously, the size and potential charge of the histidine side chain differ significantly from alanine, and these characteristics likely contribute to the extreme loss of function, but the rationale for the extensive differences is unclear. The data suggest that the overall structure of the polymerization pocket is changed by this substitution such that no reasonable polymerization site is present. That is, the presence of histidine not only disrupts the salt bridge with Arg-375 but also disrupts the overall conformations of loops P-3 and P-4, with the consequent loss of the polymerization pocket. This conclusion that large structural changes accompany the histidine substitution is supported by our finding that a high Ca$^{2+}$ concentration was required for binding to the conformation-sensitive IP-1 antibody used for immunopurification of D364H fibrinogen.

We interpret our data as indicating that the C-terminal γ chain domain participates in both protofibril formation and fiber formation. Many previous experiments show that protofibrils form when the N terminus of the α chain of one fibrin molecule (exposed after Fpα is cleaved from fibrinogen) binds in the polymerization pocket in the C-terminal γ chain domain of a second fibrin molecule. Our data are consistent with this conclusion. In addition, our data indicate that changing the polymerization pocket also changes lateral aggregation. This would be the case if the first interactions, between the polymerization pocket and the N terminus of the α chain, induced a conformational change such that the C-terminal domain of the γ chain takes on a role in the lateral aggregation of protofibrils. This conclusion emanates from a comparison of the data with the two 364 variants. Because the Ala variant was able to form a continuous fibrin gel and the His variant was not, we conclude that sites for both protofibril formation and lateral aggregation are missing in the His variant. As stated above, our results with the Ala variants are also consistent with the conclusion that both protofibril formation and fiber formation are altered by these substitutions. We have initiated experiments that are designed to test whether these single-amino acid substitutions alter more than one step in fibrin clot formation.

**REFERENCES**

1. Blomback, B., Hessle, B., Hogg, D., and Therkildsen L (1978) Nature 275, 501–505
2. Oleza, S. A., and Budzynski, A. Z. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1374–1378
3. Weisel, J. W., Vekich, Y., and Gorkun, O. (1993) J. Mol. Biol. 232, 285–297
4. Shainoff, J. R., and Dardik, B. N. (1983) Ann. N. Y. Acad. Sci. 408, 254–268
5. Okada, M., and Blomback, B. (1983) Thromb. Res. 29, 269–280
6. Carr, M., Gabriel, D., and McDonagh, J. (1986) Biochem. J. 239, 513–516
7. Mihalyi, E. (1988) Biochemistry 27, 976–982
8. Gorkun, O. V., Vekich, Y. I., Medved, L. V., Henschel, A. H., and Weisel, J. W. (1994) Biochemistry 33, 6996–6997
9. Weisel, J. W., and Nagaswami, C. (1992) Biochem. J. 283, 111–128
10. Cierpinski C. S., and Budzynski, A. Z. (1992) Biochemistry 31, 4248–4253
11. Hasegawa, N., and Sasaki, S. (1990) Thromb. Res. 57, 183–185
12. Yamazumi, K., and Doolittle, R. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2893–2896
13. Okumura, N., Furuhata, K., Terasawa, F., Nakagoshi, R., Ueno, I., and Matsuyama T (1996) Thromb. Haemostasis 75, 887–891
14. Gorkun, O. V., Vekich, Y. I., Weisel, J. W., and Lord, S. T. (1997) Blood 89, 4407–4414
15. Birnie, C. G., Hettsch, J. M., Strickland, E., and Lord, S. T. (1993) Biochemistry 32, 107–110
16. Rooney, M. M., Parise, L. V., and Lord, S. T. (1996) J. Biol. Chem. 271, 8553–8555
17. Nickoll, J. A., Deng, W. P., Miller, E. M., and Ray, F. A. (1996) Methods Mol. Biol. 58, 455–468
18. Lord, S. T., Strickland, E., and Jayjock, E. (1996) Biochemistry 35, 2342–2346
19. Takebe, M., Sue G., Kobuo, I., Suga, T., and Matsuura M. (1995) Thromb. Haemostasis 73, 662–667
20. Mihalyi, E. (1968) Biochemistry 7, 208–223
21. Higgins, D. L., Lewis, S. D., and Shafer, J. A. (1983) J. Biol. Chem. 258, 9276–9283
22. Furlan, M., Rupp, C., and Beck, E. A. (1983) Biochem. Biophys. Acta 742, 25–32
23. Birken, S., Wilner, G., and Consofield, R. (1975) Thromb. Res. 7, 599–611
24. A. S., Lewis, S. D., and Shafter, A. J. (1995) Methods Enzymol. 222, 341–358
25. Yoshida, N., Wada, H., Morita, K., Hirata, H., Matsuura, M., Yamazumi, K., Asakura, K., and Shirahara, S. (1991) Blood 77, 1958–1963
26. Rul, W., Bender, A., Lane, D. A., Freissner, K. T., Selmayr, E., and Muller-Bergmann, G. (1988) Biochim. Biophys. Acta 965, 169–175
27. Lewis, S. D., Shields, P. P., and Shafer, J. A. (1985) J. Biol. Chem. 260, 10192–10199
28. Ebert, R. F. (1994) Index of Variant Human Fibrinogens 1994 Edition, CRC Press, Inc., Boca Raton, FL
29. Yee, V. C., Pratt, K. P., Cote, H. C., Trong, I. L., Chung, D. W., Davie, E. W., Stenkamp, R. E., and Teller, D. C. (1991) Structure (London) 1, 125–138
30. Pratt, K., Cote, H., Chung, D., Stenkamp, R., and Davie, E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7176–7178
31. Litvinovich, S. V., Henschel, A. H., Kriegstein, K. G., Ingham, K. C., and Medved, L. V. (1995) Eur. J. Biochem. 229, 605–614
32. Cote, H., Pratt, K., Chung, D., and Davie, E. (1997) Thromb. Haemostasis 78, suppl., 757