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# The Relationship Between the Fibrinogen D Domain Self-Association/Cross-Linking Site (gammaXL) and the Fibrinogen Dusart Abnormality (Aalpha R554C-albumin): Clues to Thrombophilia in the "Dusart Syndrome"

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# The Relationship between the Fibrinogen D Domain Self-Association/Cross-linking Site (γXL) and the Fibrinogen Dusart Abnormality (Aα R554C-Albumin)

## Clues to Thrombophilia in the "Dusart Syndrome"

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## Abstract

**Cross-linking of fibrinogen at its COOH-terminal γ chain cross-linking site occurs in the presence of factor XIIIa due to self-association at a constitutive D domain site ("γXL"). We investigated the contribution of COOH-terminal regions of fibrinogen Aα chains to the γXL site by comparing the γ chain cross-linking rate of intact fibrinogen (fraction I-2) with that of plasma fraction I-9, plasmic fraction I-9D, and plasmic fragment $D_1$, which lack COOH-terminal Aα chain regions comprising ~ 100 , ~ 390, and 413 residues, respectively. The cross-linking rates were I-2 > I-9 > I-9D = $D_1$, and indicated that the terminal 100 or more Aα chain residues enhance γXL site association. Fibrinogen Dusart, whose structural abnormality is in the COOH-terminal "αC" region of its Aα chain (Aα R554C-albumin), is associated with thrombophilia ("Dusart Syndrome"), and is characterized functionally by defective fibrin polymerization and clot structure, and reduced plasminogen binding and tPA-induced fibrinolysis. In the presence of XIIIa, the Dusart fibrinogen γ chain cross-linking rate was about twice that of normal, but was normalized in proteolytic fibrinogen derivatives lacking the Aα chain abnormality, as was reduced plasminogen binding. Electron microscopy showed that albumin-bound Dusart fibrinogen "αC" regions were located in the vicinity of D domains, rather than at their expected tethered location near the fibrinogen E domain. In addition, there was considerable fibrinogen aggregation that was attributable to increased intermolecular COOH-terminal Aα chain associations promoted by untethered Dusart fibrinogen aC domains. We conclude that enhanced Dusart fibrinogen self-assembly is mediated through its abnormal αC domains, leads to increased γXL self-association and γ chain cross-linking potential, and contributes to the thrombophilia that characterizes the "Dusart Syndrome." (*J. Clin. Invest.* 1996. 97:2342–2350.) Key words: fibrinogen • fibrin • factor XIII • plasminogen • thrombophilia**

## Introduction

Congenital abnormal fibrinogens provide useful tools for correlating abnormal structure with functional aspects of the fibrinogen molecule, and they present an important opportunity to gain insight into the relationship between the molecular abnormality and associated clinical problems, such as thrombophilia. More than 260 familial cases of dysfibrinogenemia had been catalogued as of 1994 (1) and of these, 51 were associated with thrombophilia (2). Fibrinogen Dusart (Aα R554C-albumin) is one of them (3), and the thrombophilia with which it is associated has been termed the "Dusart Syndrome" (4). A second Aα R554C family with the Dusart Syndrome, fibrinogen Chapel Hill III (5), has been described.

Dusart fibrinogen molecules contain disulfide-linked albumin molecules, most of which are bound in the carboxy-terminal region of the Aα chain at Aα554 (6). Functional defects include reduced plasminogen binding (4), impaired fibrin-dependent plasminogen activation by tPA (7), and abnormal fibrin polymerization (4, 6) and clot structure (3, 6, 8). The abnormal polymerization and fibrin clot structure have been correlated directly with the Aα chain defect, in that proteolytic removal of the abnormal region of the Aα chain normalizes both defects (6). The observation that Dusart thin fiber clot structure and tPA-induced fibrinolysis is normalized in the presence of dextran, led to the suggestion that thrombophilia is attributable to hypofibrinolysis caused by the abnormal clot structure itself (8).

During the course of recent studies on factor XIIIa-mediated fibrinogen and fibrin γ chain cross-linking (9), we identified two constitutive self-association sites in fibrinogen D domains, termed "D:D" and "γXL", respectively. These sites participate in the process of fibrin(ogen) polymerization (9, 10), and are independent of the well-known constitutive D domain "a" polymerization site ("Da") that participates in fibrin "D:E" assembly by reacting with a thrombin-exposed E domain "A" site ("EA") (11–13) (see Fig. 1). The D:D site promotes end-to-end molecular alignment in assembling fibrils, whereas the γXL:γXL interaction results in intermolecular carboxy-terminal γ chain association that facilitates XIIIa-mediated cross-linking.

In the studies presented here, we explored the structures in normal fibrinogen that contribute to γXL site association and cross-linking, and report that carboxy-terminal regions of Aα chains enhance formation of that site. We also investigated the contribution of the Dusart Aα chains to γXL site function, and found that the abnormality promotes "pre-assembly" of fibri-

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nogen molecules and consequent increased cross-linking potential, a phenomenon that probably plays a causal role in the thrombophilia that is associated with the abnormality.

## Methods

All chemicals and reagents were the highest purity available from commercial sources. Glycerol was from Fisher Chemical (Fair Lawn, NJ). PMSF, Coomassie Brilliant Blue R250 and Dextran (average mol wt, 35,600) were purchased from Sigma Chemical Co. (St. Louis, MO). Trasylol (aprotinin) was obtained from Miles Inc. (Kankakee, IL). Human α-thrombin (specific activity, 3.4 U/μg), glu-plasminogen, and lys-plasminogen were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). The plasminogens were labeled with $^{125}$I by the iodine monochloride method (14). Plasmin (12.3 U/mg) was prepared by the method of Robbins and Summaria (15). Factor XIII (2460 Loewy U/mg; reference 16) was prepared from human plasma (17). Human serum albumin was obtained from Armour Pharmaceutical Co., Kankakee, IL.

Normal and Dusart fibrinogen fractions I-2 and I-9 were isolated from citrated plasma by glycine precipitation (18) and then further purified (19). Fibrinogen concentrations were determined spectrophotometrically at 280 nm using an absorbance coefficient $\left(A\frac{1\%}{1\text{ cm}}\right)$ of 15.1 (20). Normal or Dusart fraction I-9D fibrinogen was prepared from fraction I-2 by incubating fibrinogen (5 mg/ml) with 0.1 U/ml plasmin in 50 mM Tris, 100 mM NaCl, pH 7.5, for 1.5 min at 37°C. The brief digestion was terminated by adding aprotinin (10 U/ml) followed by gel sieving over Sepharose 4B to separate cleaved fragments from core material. The subunit structure of the core fragments was determined by SDS-PAGE (21) on 1.5 mm polyacrylamide slab gels (5% gels, nonreduced samples; 9% gels, reduced samples) (see Fig. 2). As assessed by immunoblot assay, normal and Dusart fraction I-9D fibrinogen each contained only 0.06 mole of intact Aα chains per mole fibrinogen (6).

Fragment $D_1$ was prepared by digestion of normal fibrinogen (10 mg/ml) for 6 h at 37°C with plasmin, 0.1 U/ml, in 0.10 M NaCl, 0.05 M Tris, pH 8.6 buffer containing 10 mM $CaCl_2$ and 5 mM *N*-ethylmaleimide (NEM). Fragments $D_1$ and E were separated by anion exchange chromatography on DE-52 (Whatman) (22). Fragment $D_1$ concentrations were determined spectrophotometrically at 280 nm using an absorbance coefficient $\left(A\frac{1\%}{1\text{ cm}}\right)$ of 20.8 (23).

Fibrin polymerization was initiated by the addition of thrombin (0.1 U/ml) and was monitored at room temperature at 350 nm in a Gilford Response recording spectrophotometer. Reaction mixtures contained fibrinogen (normal or Dusart fraction I-2 or I-9D) at 0.5 mg/ml in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4 buffer.

Plasminogen binding experiments were carried out on normal or Dusart fractions I-2, I-9, and I-9D using $^{125}$I-labeled glu- or lys-plasminogen as follows: duplicate solutions containing 25 μg (0.074 nMol) to 200 μg (0.58 nMol) fibrinogen in 50 mM Tris, 50 mM NaCl, pH 7.4 buffer containing aprotinin (5 KIU/ml) (final volume, 100 μl), were clotted by adding thrombin (0.5 U/ml, final), incubated for 1 h at room temperature, and then overnight at 4°C. $^{125}$I-labeled glu- or lys-plasminogen in the Tris-NaCl buffer (final volume, 100 μl) was added at a final molar ratio to fibrinogen of 10:1, and incubated overnight at 4°C. The resulting clots were synerized, washed four times with buffer, and radioactivity in the clot and clot liquor was determined. The coagulability of normal and Dusart fibrinogen fractions under these conditions was $> 90\%$.

Plasma factor XIII at a concentration of 500 Loewy U/ml in 0.1 M NaCl, 20 mM Hepes, pH 7 buffer containing 0.1–0.5 mM DTT, was activated to XIIIa by adding human α-thrombin (5 U/ml, final concentration), and incubating at 37°C for 30 min. (In comparison experiments in which the DTT concentration in the XIII activation mixture was varied between 0.1 and 0.5 mM, there were no significant differences in subsequent fibrinogen or fibrin cross-linking behavior attributable to the concentration of DTT.) For preparing XIIIa–cross-linked fibrinogen, thrombin in the activation mixture was inactivated by adding a 10-fold excess of hirudin (Sigma Chemical Co.) (50 U/ml, final concentration). Hirudin-treated XIIIa had no measureable thrombin activity as assessed by its failure to release detectable FPA from fibrinogen (24) at a final XIIIa concentration of 100 U/ml over a 24-h incubation period, or to cleave the fibrinogen Aα chain as assessed by SDS-PAGE of reduced fibrinogen specimens (9).

For SDS-PAGE analyses of fibrinogen or fibrin cross-linking, XIIIa (10 to 100 U/ml, final concentration) containing hirudin-inactivated thrombin or active thrombin (not hirudin treated), was added to a fragment $D_1$ or fibrinogen solution (1.5 mg/ml, final) in 0.1 M NaCl, 20 mM Hepes, pH 7 buffer containing 10 mM $CaCl_2$ and incubated at room temperature for up to 24 h. In some experiments human serum albumin was included in the cross-linking mixture at a final concentration of 0.5 mg/ml. At selected intervals, the cross-linking reaction was terminated by adding an equal volume of 5% SDS, 10 mM Tris, 1 mM EDTA, 10% β-mercaptoethanol, pH 8 solution, and the samples subjected to electrophoresis on 8–25% gradient gels in a Phast gel apparatus (Pharmacia/LKB). Densitometric scanning of Coomassie blue–stained gels was carried out at 540 nm in a Gilford Response UV-VIS spectrophotometer. The initial rate of γ chain cross-linking of fibrinogen or fibrin was determined from the ratio of γ dimers to the total γ chain population $\left(\frac{\gamma\gamma}{\gamma+\gamma\gamma}\right)$ at the earliest sampling time. In the case of fragment $D_1$, the /β and /γ subunit bands were not resolved from one another. Therefore, in calculating the /γ chain cross-linking rate, the scanned density of the /β+/γ position was corrected for the /β band contribution by assuming that the specific absorptivity for each component was the same.

Samples of fibrin for transmission electron microscopy (TEM) were prepared from fibrinogen (normal or Dusart; fraction I-2 or I-9D) as 50-μl drops (50 μg/ml in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4 buffer). After thrombin addition (0.1 U/ml) and incubation overnight at room temperature, a fibrin clot specimen was picked up on a carbon-coated 200-mesh grid, fixed with glutaraldehyde/tannic acid, stained with uranyl acetate, dehydrated, and critical point dried (24). Samples of fibrin for negative staining were clotted and picked up on grids as described above and negatively contrasted with 2% (wt/vol) uranyl acetate. Electron microscopy was carried out in a Philips 400-T electron microscope at 80 or 120 kV.

Fresh, partially cross-linked fibrinogen for scanning transmission electron microscopy (STEM) was prepared as described (9). Briefly, fibrinogen (1 mg/ml)-XIIIa (25 U/ml or 100 U/ml) mixtures were deposited as 50 μl drops on a parafilm surface and incubated at room temperature for 5–20 min. At a selected time interval, the specimen was diluted to a concentration of 5 to 10 μg/ml in 0.1 M NaCl, 20 mM Hepes, pH 7 buffer, 3 μl injected into a 3 μl droplet of buffer on a microscope grid, and the specimen allowed to attach to the grid surface for one minute. Fluid on the grid surface was then exchanged 8–10 times with 150 mM ammonium acetate solution, the specimen frozen in liquid nitrogen, freeze-dried, transferred under vacuum to the microscope stage, and imaged at the Brookhaven STEM facility using a 40-kv probe focused at 0.25 nm. Fibrinogen specimens for STEM were prepared from room temperature stock solutions that had been diluted with the Hepes buffer to a final concentration of 2 μg/ml, applied directly to grids, and processed as described above. Background filtering of digitized STEM images was optimized for contrast and brightness offset by an image processing program (Adobe Photoshop), and the adjusted images downloaded to a GCC Technologies ColorFast film recorder.

## Results

*Characterizing the fibrinogen constituents that contribute to γXL self-association.* In recent studies concerned with the location of carboxy-terminal γ chain cross-linking sites in assembled fibrin polymers (9), we identified two constitutive D domain self-association sites, γXL and D:D, respectively, that

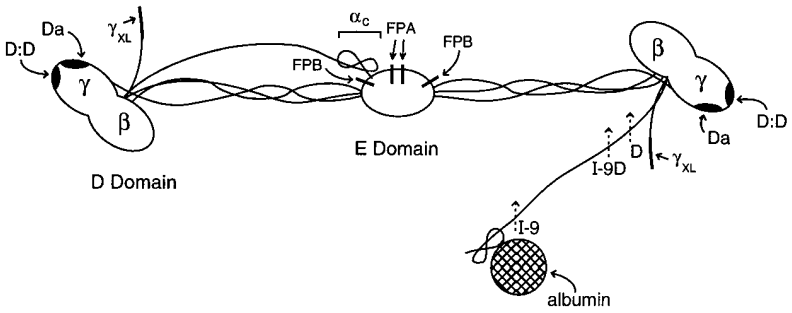


*Figure 1.* Schematic diagram of the fibrinogen molecule illustrating the location of the D domain association sites Da, D:D, and γXL, and the Aα chain carboxy-terminal αC domain. One αC domain is depicted as associating with the E domain. The αC domain in the other Aα chain is not associated with the E domain, and has an albumin molecule bound in a position corresponding to Dusart Aα R554C. The approximate cleavage site on the Aα chain resulting in the α/ chain remnant found in fractions I-9, I-9D, or fragment D, respectively, is shown by a dashed arrow.

participate in the assembly of cross-linked fibrinogen and fibrin (Fig. 1). The sites are distinguishable from one another and from the Da site that participates in fibrin fibril assembly through the fibrin D:E interaction. γXL contains the γ chain cross-linking site (γ398-406), and is recognized from the observation that in XIIIa-mediated fibrinogen cross-linking, γ chains become cross-linked first to form γ dimers, just as they do in fibrin, albeit at a slower rate. The D:D interaction promotes end-to-end alignment of fibrin(ogen) molecules within a fibril strand, and is distinct from γXL on several grounds (9), but most notably from the observation that fibrinogen Tokyo II (γ R275C) undergoes XIIIa-mediated fibrinogen and fibrin γ chain cross-linking at the same respective rates as normal (i.e., normal D:E and γXL:γXL), yet displays abnormal end-to-end molecular associations (i.e., abnormal D:D site) (10).

To investigate the constituents of the fibrinogen molecule that comprise the γXL site, we evaluated the γ chain cross-linking rate of fibrinogen and fibrinogen derivatives and fragments that contain the γ chain cross-linking site (Fig. 2). Plasma fraction I-9 fibrinogen, whose Aα chains lack carboxy-terminal segments comprising ~ 100 or more residues (25–27), underwent γ chain cross-linking at 68% of the rate of fraction I-2 (0.046/min vs 0.068/min), which contains a very high proportion of intact Aα chains (~ 67 kD). Fragment $D_1$, lacking the 413 residues beyond Aα 197 (28, 29), yet retaining the carboxy-terminal γ chain sequence and thus the capability of γ chain cross-linking (30) and of binding to the platelet fibrinogen receptor (31), showed an initial rate of cross-linking (0.018/min) amounting to 26% of that for I-2. Plasmic fraction I-9D, which contains a smaller residual Aα chain population than I-9 (26), mainly lacking segments of ~ 390 residues (28, 32), showed the same initial cross-linking rate as fragment $D_1$. Overall, these experiments show a progressive reduction of the γ chain cross-linking rate as a function of the Aα chain composition, indicating that the carboxy-terminal 100+ residues of Aα chains enhance association at the γXL site.

*Normalizing the clotting and plasminogen binding functions of fibrinogen Dusart by removing carboxy-terminal Aα chain segments.* We previously reported on the polymerization and ultrastructure of Dusart fractions I-2 and I-9D fibrin (6), and we briefly summarize these results here to provide a description of the structure of Dusart fibrin from which carboxy-terminal segments of its Aα chains had been removed. As assessed by turbidity measurements, Dusart I-2 fibrin polymerized with a longer lag time and at a much reduced turbidity rate compared to normal I-2 fibrin, confirming an earlier report (4). Electron microscopy of a critical point dried Dusart I-2 fibrin clot showed a branched network of fibers that were thinner than those in normal fibrin. These network structures were similar to those reported by Collet et al. (8), except they did not branch as extensively. By contrast, Dusart I-9D fibrin polymerized faster than the original I-2 fibrin, but at nearly the same rate as normal I-9D fibrin, which itself polymerized more slowly than the I-2 fibrin from which it had been derived. Clot matrices formed from normal and Dusart fraction I-9D fibrinogen formed networks that were virtually indistinguishable from one another, and were composed of thick fibers showing typical 22.5 nm periodicity.

Previous experiments demonstrated that fibrinogen Dusart bound less plasminogen than normal fibrinogen (4), and possibly as a consequence, showed reduced fibrin-dependent tPA-mediated plasminogen activation (7). In these present experiments, we evaluated plasminogen binding to Dusart plasma fractions I-2 and I-9, and plasmic fraction I-9D (Table I), and confirmed that there was reduced binding of both glu- and lys-plasminogen to intact fibrin. Plasminogen binding was normalized in Dusart I-9 or I-9D fibrin, both of which lack that portion of the Aα chain containing the Dusart structural abnormality.

*Cross-linking of Dusart fibrinogen and fibrin.* Fibrinogen Dusart γ chains became cross-linked in the presence of XIIIa about twofold more rapidly than did normal fibrinogen γ chains (Figs. 3 and 4). Including albumin (0.5 mg/ml) in the normal fibrinogen cross-linking mixture did not significantly change its

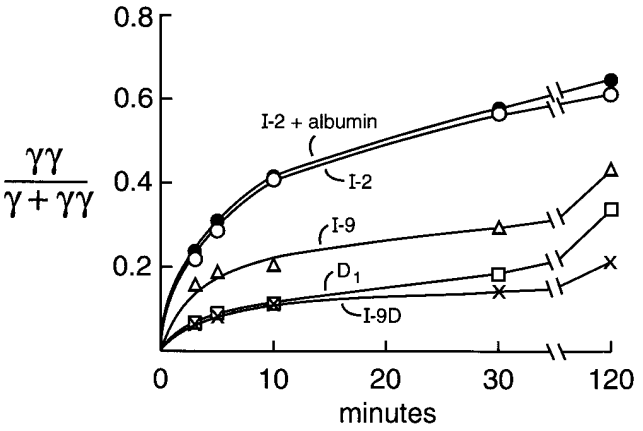


*Figure 2.* The cross-linking of normal fibrinogen fractions and fragment $D_1$ by factor XIIIa (100 U/ml). The data shown for I-2, I-9, and fragment $D_1$ are the mean values of triplicate determinations, and duplicate determinations for I-9D. The γ chain cross-linking rate (ordinate) is plotted as the ratio of γ dimer or /γ dimer (fragment $D_1$) over the total γ or /γ chain population $\left(\frac{\gamma\gamma}{\gamma+\gamma\gamma}\right)$ versus time (min). ○, I-2; ●, I-2 + albumin; △, I-9; X, I-9D; □, fragment $D_1$.

*Table I. Plasminogen Binding to Fibrin (mol/mol)*

| Fibrin | *n* | glu-plasminogen/fibrin | lys-plasminogen/fibrin |
|---|---|---|---|
| Fraction I-2 | | | |
| Normal | 8 | 1.6±0.6 | 3.3±0.7 |
| Dusart | 8 | 0.87±0.2 | 1.4±0.5 |
| | | ($P < 0.001$) | ($P < 0.0001$) |
| Fraction I-9 | | | |
| Normal | 2 | 1.5 (1.51,1.52) | 3.4 (3.3, 3.5) |
| Dusart | 2 | 1.6 (1.61, 1.62) | 3.1 (3.06, 3.14) |
| Fraction I-9D | | | |
| Normal | 2 | 1.6 (1.55, 1.62) | 3.2 (3.18, 3.21) |
| Dusart | 2 | 2.1 (2.05, 2.07) | 3.1 (3.01, 3.23) |

cross-linking rate (Fig. 2), suggesting that the presence of albumin, per se, does not cause a change in the γ chain cross-linking rate. As is the case for normal fibrinogen, the Aα chain cross-linking rate of Dusart fibrinogen was considerably slower than that of the γ chains (9, 33) and showed little, if any, tendency to form Aα polymers more rapidly than its normal counterparts. Thus, accelerated XIIIa-mediated cross-linking of Dusart fibrinogen is evidently confined to its γ chain population. In contrast to the accelerated cross-linking rate observed for intact Dusart fibrinogen, Dusart I-9D became cross-linked at about half the rate of normal fibrinogen, but at virtually the same rate as I-9D from normal fibrinogen (Fig. 4).

Fibrin γ chains become cross-linked much more rapidly than those of fibrinogen (9, 10, 33), a rate enhancement that directly reflects D:E driven fibrin fibril assembly. At this level of XIIIa (100 U/ml) and thrombin (1 U/ml), both Dusart and normal fibrin γ chains were completely cross-linked at the earliest sampling time (Fig. 4, *lower panel*; 1 min), suggesting that the Dusart fibrin D:E interaction is normal. Further support for this conclusion was obtained from a fibrin cross-linking experiment that was carried out at a lower XIIIa (10 U/ml) and thrombin (0.1 U/ml) concentration. Under these conditions of slower fibrin assembly, γ chain cross-linking of intact Dusart and normal fibrin was nearly the same at the 1-min sampling time (80 vs 74%, respectively). The Dusart I-9D fibrin γ chain cross-linking rate was greater than that of Dusart I-9D fibrinogen, no doubt reflecting the effect of the D:E interaction, but the cross-linking rate of Dusart I-9D fibrin did not differ significantly from that of normal I-9D fibrin. Thus, as had been the case for fibrin clot assembly, normalization of the fibrinogen Dusart cross-linking rate can be achieved by removing the regions of its Aα chains containing the structural abnormality.

*STEM of Dusart fibrinogen and cross-linked fibrinogen.* A possible explanation for the acceleratory effect of Dusart fibrinogen on γXL:γXL association and cross-linking could lie with the fact that Dusart molecules have an increased self-association tendency due to the Aα chain defect. We examined that possibility directly by assessing the structure and distribution of normal or Dusart fibrinogen molecules that had been deposited at a concentration of 2 μg/ml on carbon films and then imaged by STEM. At this concentration, the particle density of Dusart fibrinogen molecules deposited on the grid surface was robust (Fig. 5 *A*), and exhibited a clear tendency for molecular clustering. In contrast, normal fibrinogen molecules were relatively sparse and more evenly distributed (Fig. 5 *B*), and showed only a modest tendency for intermolecular aggre-

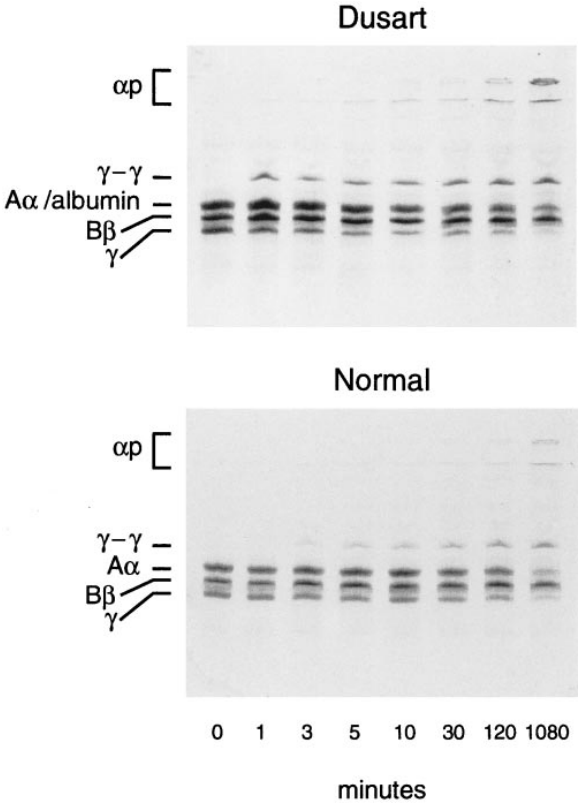


*Figure 3.* SDS-PAGE gels of the cross-linking of Dusart versus normal I-2 fibrinogen in the presence of XIIIa (100 U/ml). The positions of the various reduced fibrinogen subunit chains are indicated; albumin from Dusart fibrinogen molecules migrates in the Aα position. Time of cross-linking in minutes.

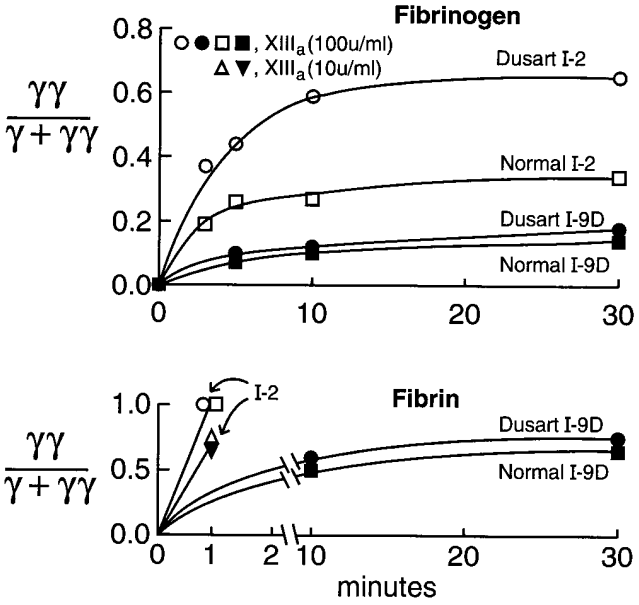


*Figure 4.* The γ chain cross-linking rate of Dusart and normal fractions I-2 and I-9D plotted as $\left(\frac{\gamma\gamma}{\gamma+\gamma\gamma}\right)$ versus time (min). (*Upper panel*) Fibrinogen, (*lower panel*) Fibrin. ○, Dusart I-2; □, normal I -2; ●, Dusart I-9D; ■, normal I-9D ( XIIIa, 100 U/ml). △, Dusart I-2; ▼, normal I-2 (XIIIa, 10 U/ml).

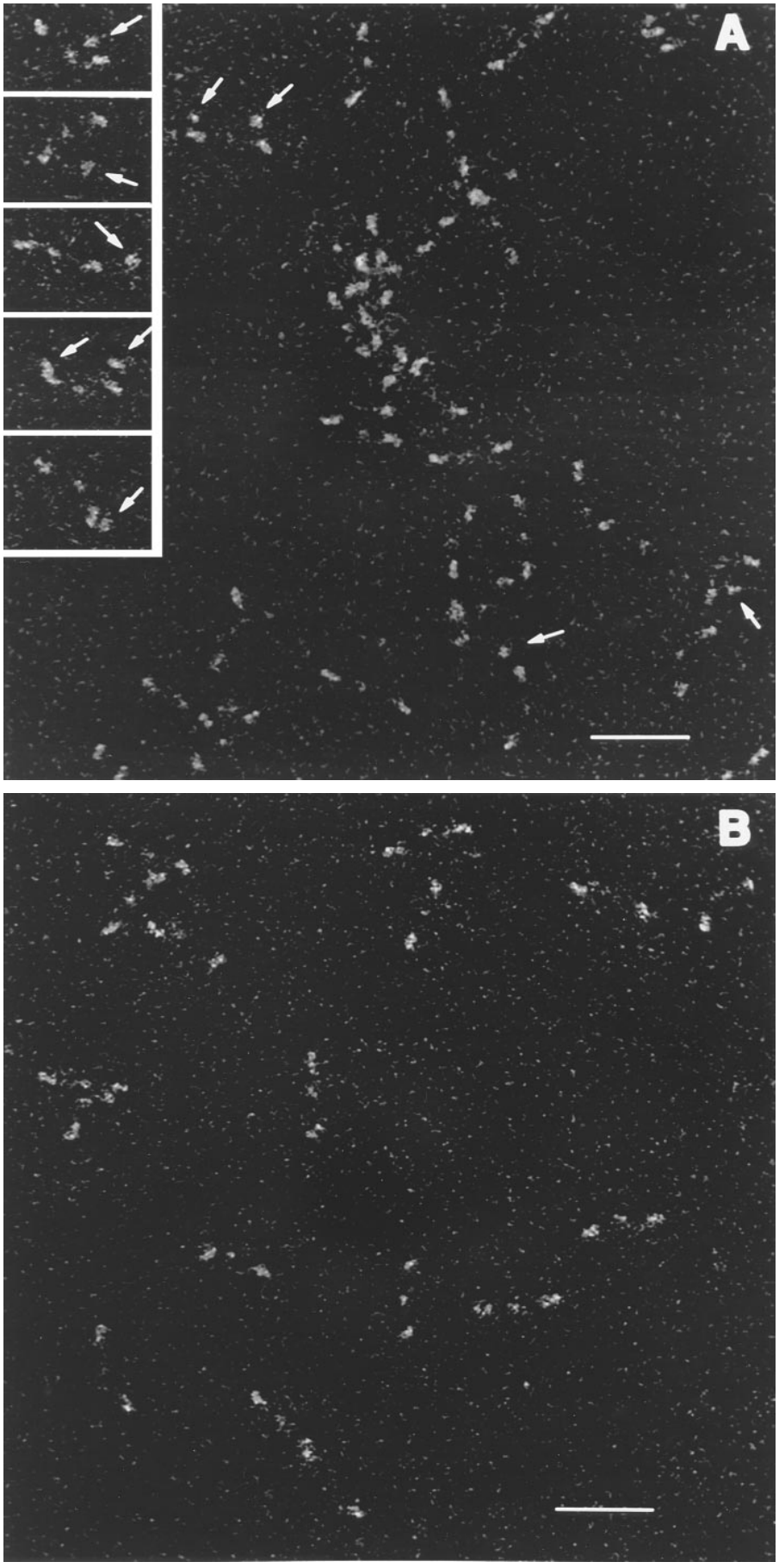


*Figure 5.* STEM images of Dusart (*A*) and normal fibrinogen (*B*) deposited at a concentration of 2 μg/ml. The particle density of normal fibrinogen molecules in these fields was 42.7±26.3 per $\mu m^2$ ($n$ = 18 fields), whereas the density of Dusart fibrinogen molecules was 93.5±24.8 per $\mu m^2$ ($n$ = 17 fields; $P$, < 0.0001). Selected examples of heterodimeric or homodimeric Dusart fibrinogen molecules are shown in the insets to *A*. Albumin molecules bound to fibrinogen are indicated by arrows. Bar, 50 nm.

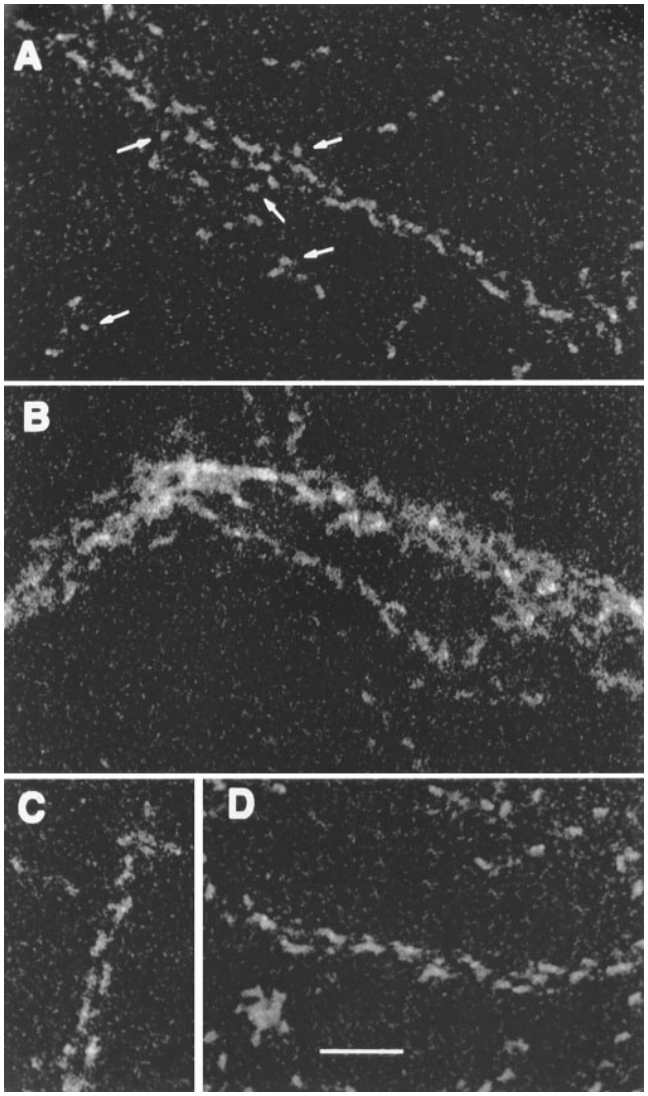


*Figure 6.* STEM images of cross-linked Dusart fibrils (*A* and *B*) and normal fibrinogen fibrils (*C* and *D*). Dusart fibrinogen and normal fibrinogen were cross-linked at XIIIa concentrations of 25 U/ml and 100 U/ml, respectively. Albumin bound to fibrinogen Dusart molecules are indicated by arrows. Bar, 50 nm.

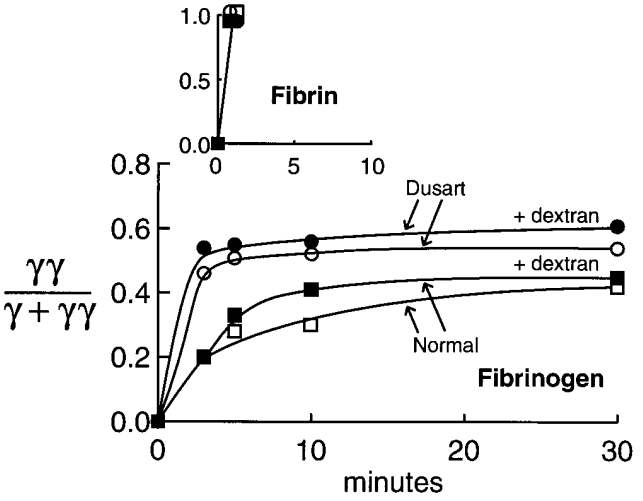


*Figure 7.* The cross-linking rate $\left(\frac{\gamma\gamma}{\gamma+\gamma\gamma}\right)$ of Dusart versus normal fraction I-2 fibrinogen ±30% dextran in the presence of XIIIa (100 U/ml). (*Inset*), Fibrin; ○, Dusart I-2; □, normal I-2; ●, Dusart I-2 + dextran; ■, normal I-2 + dextran.

gation (e.g., occasional end-to-end dimers), as previously described (9). Albumin molecules bound to Dusart fibrinogen were usually easy to identify (arrows, Fig. 5 *A*) and were most often found in the vicinity of D domains rather than in close proximity to E domains, a finding that extends previous observations on the position of fibrinogen-bound albumin molecules that had been deposited from glycerol-containing solutions (6).

In view of the accelerated γ chain cross-linking of Dusart fibrinogen, we carried out cross-linking of Dusart fibrinogen at lower than usual XIIIa concentrations (25 U/ml versus 100 U/ml). This lower enzyme concentration allowed us to obtain cross-linked samples of sufficiently low viscosity to permit sample dilution and deposition on the microscope grid (Fig. 6). Like normal fibrinogen (*C* and *D*), cross-linked Dusart fibrinogen formed elongated double-stranded fibrils (*A*) and did not show a tendency to branch. (Cross-linked normal fibrinogen fibrils were rarely found in cross-linking mixtures at XIIIa concentrations of 25 U/ml-not shown.) Formation of double-stranded linear fibril structures indicates that D:D interactions in Dusart fibrinogen molecules are normal, as would be expected. In addition, Dusart cross-linked fibrinogen fibrils showed a tendency to associate laterally to form multi-stranded fibers (Fig. 6 *B*) to a much greater extent than is seen in normal fibrinogen cross-linking mixtures. However, Dusart fibrinogen fibrils differed in subtle ways from their normal counterparts, in that Aα chain-bound albumin molecules could sometimes be identified (*arrows*) protruding from the fibril strands.

*The effect of dextran and glycerol on Dusart fibrinogen cross-linking.* Collet et al. (8) reported that dextran normalized the structure of the Dusart fibrin clot from a thin fiber matrix to a more coarse and permeable structure containing thicker fibers, concomitant with normalization of the impaired tPA-induced fibrinolysis, an effect that is consistent with the known property of dextran to produce a more coarse fibrin network structure (34–36). To determine whether dextran had a normalizing effect on the cross-linking of Dusart fibrinogen, we evaluated XIIIa-mediated cross-linking in the presence of 30% dextran (Fig. 7). At this level, at which normalization of the abnormal Dusart clot structure was reported to have occurred (8), dextran not only did not reduce the accelerated γ chain cross-linking rate of Dusart fibrinogen, but very likely caused a modest increase in the cross-linking rate (∼ 10%). Dextran had no significant negative effect on the fibrin cross-linking rate (Fig. 7, *inset*) inasmuch as γ chain cross-linking was complete at the first sampling time (1 min) in each case. Thus, whatever action dextran has on Dusart fibrin, it does not appear to be mediated through modification of γXL:γXL associations.

Unlike dextran, which promotes coarse matrix formation, polyhydroxyl compounds like glycerol impair fibrin polymerization and promote formation of thin fiber networks (37, 38). To contrast these opposite effects, we evaluated the effect of 20% glycerol on fibrinogen and fibrin cross-linking (Fig. 8),

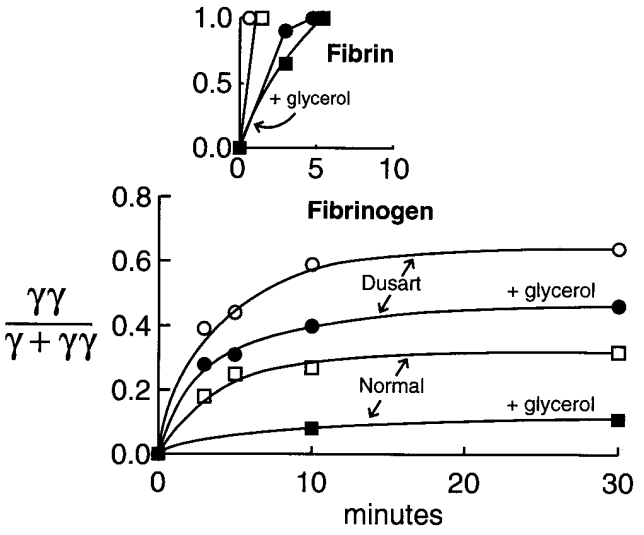


*Figure 8.* The cross-linking rate $\left(\frac{\gamma\gamma}{\gamma+\gamma\gamma}\right)$ of Dusart versus normal fraction I-2 fibrinogen ±20% glycerol in the presence of XIIIa (100 U/ml). (*Inset*), Fibrin; ○, Dusart I-2; □, normal I-2; ●, Dusart I-2 + glycerol; ■, normal I-2 + glycerol.

and found a marked effect in reducing the γ chain cross-linking rate of both Dusart and normal fibrinogen, and a similar effect in reducing their fibrin γ chain cross-linking rates. These observations indicate that glycerol reduces the γXL:γXL association rate, as well as D:E assembly.

## Discussion

Fibrin molecules lacking carboxy terminal regions of Aα chains form less turbid clots and contain thinner fibers than those formed from intact fibrin (19, 39, 40). Recent studies (32, 41) indicated that a carboxy-terminal Aα chain segment (termed "aC") that is associated with the fibrinogen E domain in the native molecule, became untethered as a result of FPB cleavage, thereby becoming available for association with other αC domains in promoting lateral fibrin fiber associations. To explore the Aα chain αC self-association phenomenon in relation to the D domain self-association sites, particularly γXL, we investigated the structural constituents in fibrinogen that contribute to forming the γXL association site. Normal fibrinogen having intact Aα chains showed the fastest rate of γ chain cross-linking, whereas fraction I-9 which lacks carboxy-terminal Aα chain segments of ~ 100 residues, showed a reduced γ chain cross-linking rate. Fragment $D_1$, lacking 413 carboxy-terminal Aα chain residues, showed a lower rate of cross-linking than I-9, but the same rate as that for I-9D. These findings indicate that the carboxy-terminal ~ 100 residues of the fibrinogen Aα chains contribute to association and cross-linking at γXL sites, and that the contributory effect extends over an even longer stretch of the Aα chains. The effect may be even greater when the "αC" domain is not associated with the E domain, as discussed below with respect to fibrinogen Dusart.

In view of the finding that carboxy-terminal segments of normal Aα chains participate in γXL site function, we evaluated fibrinogen Dusart from this standpoint since its structural abnormality resides in this region. Dusart γ chains were more rapidly cross-linked in the presence of XIIIa than was normal fibrinogen, but the enhanced activity was normalized in Dusart fractions lacking the carboxy-terminal Aα chain abnormality. This indicates that the Dusart Aα chain has a significant effect in promoting an increased intermolecular association and potential cross-linking rate at the γXL site. Furthermore, the Dusart fibrin γ chain cross-linking rate, although more rapid than that of fibrinogen, was the same as that of normal fibrin, thus indicating that the fibrin D:E interaction was not impaired.

STEM images of cross-linked Dusart fibrinogen fibrils confirmed the increased γXL:γXL self-association tendency, in that XIIIa-cross-linked Dusart fibrinogen formed longer and more numerous double-stranded fibrils than did normal fibrinogen at a XIIIa level only 25% of that used for normal. The normal structure of Dusart fibrinogen fibrils indicated that the D:D interaction, per se, was normal. Increased self-association was also supported by STEM images of Dusart fibrinogen molecules, which showed a considerable tendency to aggregate compared with normal. Of collateral interest was the finding that albumin bound to Dusart fibrinogen molecules were situated in the vicinity of its D domains, indicating that the carboxy-terminal Aα chain was located in this region rather than with the E domain. In contrast to this observation, there is ultrastructural evidence derived from observations of fibrinogen compared with fibrin (32, 41), and data from STEM mass measurements on fibrinogen molecules (42), that the carboxy-terminal αC domain of normal fibrinogen tends to be situated at or near the E domain. Of note in this regard is that release of the αC region from the E domain after thrombin cleavage of FPB, is associated with increased lateral fibrin fibril association (41). Conversely, the absence of this domain (19, 32, 39-41) or competitive inhibition of its binding function (32), results in decreased lateral fibrin fibril association. Dissociation of αC domains in Dusart fibrinogen evidently involves at least the abnormal aC domains in affected heterozygotic individuals (~ 50%), an amount that far exceeds the level of αC dissociation (< 10%) observed in normal fibrinogen molecules (32). We interpret these findings as suggesting that increased αC domain dissociation in Dusart fibrinogen molecules leads to increased intermolecular association, and concomitantly or consequently, to enhanced γXL:γXL association. The tendency for molecular "pre-assembly" due to demodulation of the E domain binding activity of αC may be a most important factor contributing to the thrombophilia that accompanies the "Dusart Syndrome" (4, 5). Although there is no evidence that premature dissociation of αC domains occurs in any other abnormal fibrinogen, it is very likely that investigation of existing "thrombophilic" dysfibrinogens along the lines developed in this study will reveal other examples of this phenomenon.

In fibrinogen Dusart, the Aα 554 Arg to Cys substitution, the concomitantly disulfide-bound albumin, or a combination of the two has an effect on fibril assembly that results in low turbidity clots containing thinner than normal fibers (3, 6, 8). It is not clear on experimental grounds how these abnormalities contribute to thin fibrin fiber formation. It appears to us that this may be directly related to the tendency for increased Dusart fibrinogen self-association before its conversion to fibrin, an event that may lead to enhanced linear fibrinogen fibril formation and concomitant reduction in "trimolecular" fibrin network branch junctions (43, 44). Dextran, which is known to induce a thick fiber network structure (34–36), normalized the abnormal Dusart thin fiber structure (8), but ac-

celerated Dusart fibrinogen γ chain cross-linking was not normalized (Fig. 7), and dextran had no measureable effect on fibrin γ chain cross-linking. Thus, whatever action dextran has on Dusart fibrin fiber formation, it does not appear to be mediated through modification of the γXL:γXL or D:E association. Glycerol on the other hand, which impairs fibrin polymerization and promotes formation of thin fiber networks (37, 38), had a marked effect in reducing the γ chain cross-linking rate of both Dusart and normal fibrinogen and fibrin, suggesting that unlike dextran, glycerol impairs both γXL:γXL and D:E interactions.

Hypofibrinolysis has been proposed as the main causal factor for thrombophilia in the Dusart Syndrome (8), an effect that may be related to its thin 'fiber' clot structure, a type of network structure that reportedly lyses at a slower rate than 'coarse' clots containing thick fibers (45, 46). Enhanced thin fiber formation resulting directly from increased self-association of Dusart fibrinogen molecules and enhanced linear fibril formation, could account for this effect. Another factor, however, contributing to reduced fibrinolysis may be that Dusart fibrinogen binds less plasminogen than normal (reference 4, Table I) and consequently displays reduced fibrin-enhanced plasminogen activation (7). Since the abnormal functions of fibrinogen Dusart are normalized when the abnormal Aα chain region is removed, it would seem that therapy directed at enhancing fibrinolysis, by promoting proteolytic release of carboxy-terminal regions of Aα chains, might ameliorate the thrombophilia that accompanies the abnormality.

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