

FOR THE RECORD

## Crystallization of fragment D from human fibrinogen

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**Abstract:** Fragment D from human fibrinogen has been crystallized. The fragment, which is composed of three disulfide-linked chains ( $\alpha'$   $\beta'$   $\gamma'$  = 88,000), was generated with either plasmin or mild trypsin digestion. The crystals diffracted out to 3.5 Å; the space group is  $P2_1$ , unit cell dimensions  $a = 108$  Å,  $b = 48$  Å,  $c = 167$  Å,  $\beta = 106^\circ$ . Fragment D was also co-crystallized with the ligand GPRP-amide, in which case the space group is consistent with  $P2_12_12_1$ , unit cell dimensions  $a = 476$  Å,  $b = 82$  Å,  $c = 432$  Å.

**Keywords:** crystallization; fibrinogen; fragment D; GPRP-amide; X-ray crystallography

Fibrinogen is a large glycoprotein found in the blood plasma of all vertebrates; it is the precursor of fibrin, the backbone of blood clots. In humans, the molecule has a molecular weight of 340,000 Da and is a multi-domained protein formed from three pairs of disulfide-linked, nonidentical polypeptide chains, ( $\alpha\beta\gamma$ )<sub>2</sub>.

An early model of fibrinogen was proposed by Hall and Slayter (1959) on the basis of shadow-cast electron microscope images that revealed three linearly arranged globules, the terminal ones of which appeared to be of similar size, the central one being slightly smaller. In the intervening 35 years, a host of biochemical studies have been conducted that support the triglobular structure. Thus, when fibrinogen is digested with plasmin, two core fragments are produced: fragment E, which corresponds roughly to the central globule, and two equivalents of fragment D, which correspond to the two terminal domains (Nussenzweig et al., 1961). Similar products can be generated by mild digestion with trypsin (Mihalyi & Godfrey, 1963).

The transformation of fibrinogen to fibrin occurs when thrombin cleaves arginyl-glycine bonds near the amino termini of the  $\alpha$  and  $\beta$  chains, releasing small fibrinopeptides (Bailey et al., 1951). Polymerization ensues when the newly exposed

amino termini of those chains interact with sites on the terminal domains of other fibrin(ogen) molecules. In this regard, Laudano and Doolittle (1978) found that peptides patterned on the newly formed  $\alpha$  chain amino terminus could prevent polymerization. These same Gly-Pro-Arg peptide derivatives were found to bind to fibrinogen or to fragment D (Laudano & Doolittle, 1980). Suitably derivatized, they can be used also for affinity purification of fibrinogen or fragment D (Kuyas et al., 1990), photoaffinity labeling (Shimizu et al., 1992; Yamazumi & Doolittle, 1992a), and in the protection of fragment D against proteolysis (Yamazumi & Doolittle, 1992b).

Over the years, there have been numerous efforts directed toward obtaining a genuine three-dimensional structure of fibrinogen (Tooney & Cohen, 1972, 1977; Cohen & Tooney, 1974; Cohen et al., 1983; Weisel et al., 1985; Gollwitzer & Bode, 1986). The formidable difficulties associated with the crystallization of this molecule are attested to by the best structure reported at this point only having a resolution of 18 Å (Rao et al., 1991). Accordingly, efforts in our own laboratory have concentrated on the crystallization of fragments of fibrinogen and fibrin. Our primary emphasis has been on fragment D (MW  $\approx$  88,000), a moiety composed of three disulfide-linked polypeptide chains ( $\alpha'$ ,  $\beta'$ , and  $\gamma'$ ) and a carbohydrate cluster. Here we report the successful crystallization of fragment D from human fibrinogen. We have also co-crystallized the fragment in the presence of a Gly-Pro-Arg-type peptide. Preliminary data have been collected and several promising heavy metal isomorphous derivatives obtained.

Human fibrinogen was prepared from outdated plasma (San Diego Blood Bank) by the cold ethanol precipitation procedure described in Doolittle et al. (1967). The precipitated fibrinogen was stored as a paste at  $-20^\circ\text{C}$ . This material ( $\approx$ 400 mg) was dissolved in 0.15 M NaCl, 0.05 M imidazole, pH 7.2, to a concentration of 5 mg/mL. Two different forms of fragment D were generated—one (FD-P) the result of plasmin digestion, the other (FD-T) from trypsin digestion. Plasmin (human; KabiVitrum) or trypsin (bovine; Sigma) was added to a final concentration of 0.0016 or 0.01 mg/mg fibrinogen, respectively. The fibrinogen was pretreated for 10 min with 5 mM iodoacetamide (to inhibit Factor XIII crosslinking) prior to the addition of either enzyme;  $\text{CaCl}_2$  was present at a final concentration of 5 mM. In either case, the digestion was allowed to progress for 4 h at

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room temperature (22 °C). Plasmin digestions were stopped by the addition of 20  $\mu$ L Trasylol (Miles, Inc.) per milliliter of digest; trypsin digestions were terminated by the addition of 0.03 mg soybean trypsin inhibitor (Sigma) per milligram of fibrinogen. FD-P and FD-T were isolated with a Gly-Pro-Arg affinity column (18 mL) equilibrated with 0.15 M NaCl, 0.005 M CaCl<sub>2</sub>, 0.05 M imidazole, pH 7.0. Elution was obtained with 1 M NaBr, 0.05 M NaAc, pH 5.3; the elutant was pooled and stored as an ammonium sulfate paste at -20 °C. The material (typical yield = ca. 160 mg) appears homogeneous on SDS gels (Fig. 1A).

Amino terminal sequence analysis revealed the positions at which plasmin and trypsin cut the fibrinogen molecule. In the case of plasmin, the amino termini of the FD-P were  $\alpha$  Asp-105,  $\beta$  Asp-134, and  $\gamma$  Ala-63 and, in a lesser amount,  $\gamma$  Ser-86. Trypsin cut at  $\alpha$  Val-111,  $\beta$  Asp-134, and  $\gamma$  Met-89, a trace of  $\gamma$  Lys-88 also being apparent. (These analyses were conducted in the laboratory of Dr. Takashi Takagi, Tohoku University, Sendai,

Japan.) The observed cut points were consistent with the trypsin preparations being slightly more compact than the plasmin preparations (Fig. 1A).

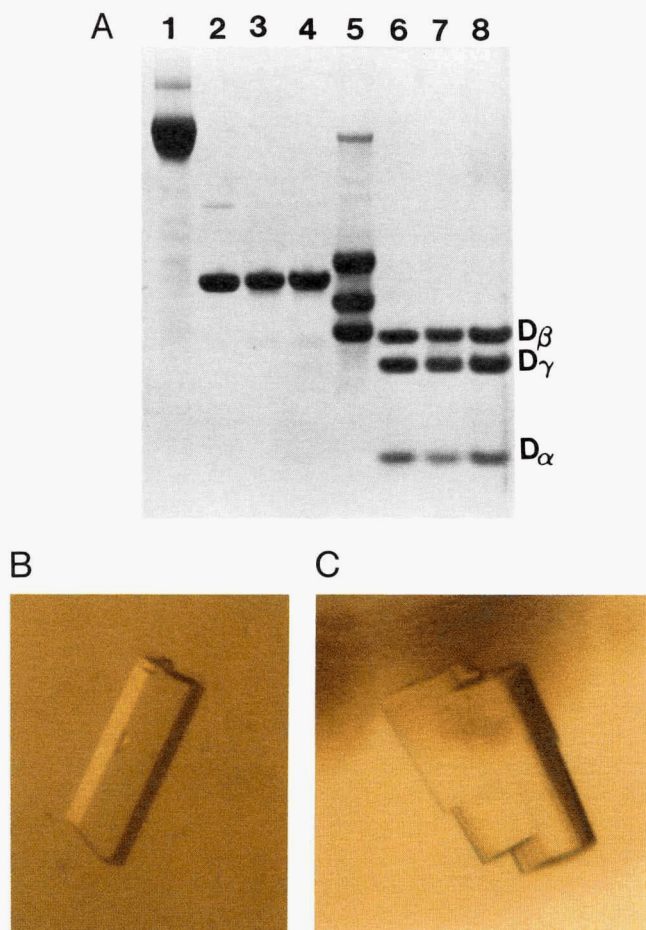
Some preparations of FD-T were desialylated with neuraminidase. To this end, FD-T was dissolved in 0.1 M NaAc, pH 5.3, buffer and the concentration adjusted to 20 mg/mL. Trasylol was added to a final concentration of 1  $\mu$ L/mg FD-T to inhibit any proteases. Neuraminidase (Type X from *Clostridium perfringens*; Sigma) was added to a final concentration of 0.01 unit/mg FD-T, and the solution was incubated at 30 °C for 2 h. The FD-T was then dialyzed overnight against the starting buffer. To ensure a complete digestion, the procedure was repeated. Desialylated FD-T (dFD) was dialyzed against 0.15 M NaCl, 0.005 M CaCl<sub>2</sub>, 0.05 M imidazole, pH 7.0, and repurified by the Gly-Pro-Arg affinity column. The Warren test (Warren, 1959) confirmed the absence of sialic acid in dFD preparations.

Crystals of FD-P, FD-T, or dFD were all grown under similar conditions by sitting-drop vapor diffusion techniques. Typically, wells contained 2 mM NaN<sub>3</sub>, 70 mM CaCl<sub>2</sub>, 50 mM Tris, pH 8.5, and 16–19% PEG 3350, although crystals also grew when the CaCl<sub>2</sub> was varied from 35 to 133 mM or the pH from 7.5 to 8.5. The protein was dissolved in 50 mM Tris, pH 7.0, and the final concentration adjusted to between 15 and 30 mg/mL. Equal volumes (10  $\mu$ L) of protein and well-solution were mixed and the sealed trays incubated at room temperature (22 °C). Usually, clusters of crystals appeared in 7–10 days. Ordinarily, 8–12 seeds would be broken free from a cluster and washed three times in the initial well-solution. Individual seeds (Fig. 1B) were then placed into sitting drops containing 3–5 mg/mL (final protein concentration) as described above. Seeds grew to diffraction size (0.4  $\times$  0.8  $\times$  0.2 mm) in 5–7 days, most often as a large crystal with small crystals attached; occasionally, individual crystals were obtained without seeding.

Crystals of FD-T or dFD were also grown in the presence of the ligand GPRP-amide. In this case, well-solutions contained 2 mM NaN<sub>3</sub>, 10 mM CaCl<sub>2</sub>, 50 mM Tris, pH 8.5, and 14–15% PEG 3350; the starting protein solution drop (12 mg/mL) also contained 5 mM GPRP-amide. Sitting drops were made from equal volumes as described above. Better results were obtained when D<sub>2</sub>O replaced H<sub>2</sub>O in all solutions. Diffraction-size crystals (0.3  $\times$  0.5  $\times$  0.7 mm) could be obtained in 4 weeks without seeding (Fig. 1C).

Diffraction data were collected at room temperature with a Rigaku RU200 rotating anode generator and two area detectors from San Diego Multiwire Systems. FD-T and dFD crystals diffracted equally well, extending out to 3.5 Å (Fig. 2; Table 1) Symmetry considerations and systematic absences revealed the space group as P2<sub>1</sub>. The density of these crystals was found to be 1.20  $\pm$  0.01 g/cm<sup>3</sup>, as determined in bromobenzene-toluene gradients. The Matthews coefficient of 2.36 Å<sup>3</sup>/Da indicated two molecules in the asymmetric unit, with a solvent content of 50% (Matthews, 1968). Several putative isomorphous derivatives have been identified, and attempts to derive proper phases are under way.

Crystals grown in the presence of the peptide GPRP-amide were orthorhombic (space group consistent with P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>), with unit cell dimensions  $a = 47$  Å,  $b = 82$  Å,  $c = 432$  Å. The density of these crystals was also 1.20  $\pm$  0.01 g/cm<sup>3</sup>. The Matthews coefficient was calculated to be 2.31 Å<sup>3</sup>/Da, indicating two molecules in the asymmetric unit and a solvent content of 49%. Efforts to collect data with the multiwire detectors were ham-



**Fig. 1.** **A:** Sodium dodecyl sulfate–polyacrylamide (5%) gel electrophoresis of fibrinogen and the fragment D preparations used in this study. Lanes 1–4, unreduced; lanes 5–8, reduced with  $\beta$ -mercaptoethanol. Lanes 1 and 5, human fibrinogen; lanes 2 and 6, fragment D plasmin (FD-P); lanes 3 and 7, fragment D trypsin (FD-T); lanes 4 and 8, desialylated FD-T (dFD). The three chains of reduced fragment D preparations are denoted on the right. **B:** Seeded FD-T crystal. **C:** Co-crystal of FD-T and the ligand GPRP-amide.

**Table 1.** Fragment D diffraction statistics

| Data set | $d_{min}$ (Å) | $I/\sigma^a$ | Total observations/unique reflections | Completeness (%) | $R_{sym}^b$ | $a$    | $b$   | $c$    | $\beta$ |
|----------|---------------|--------------|---------------------------------------|------------------|-------------|--------|-------|--------|---------|
| fd1      | 4.1           | 1.8          | 24,578/11,414                         | 88               | 0.108       | 107.87 | 48.28 | 167.63 | 105.87  |
| fd2      | 4.3           | 2.0          | 18,288/11,564                         | 58               | 0.094       | 107.73 | 48.33 | 167.55 | 105.79  |
| fd3      | 3.9           | 1.9          | 36,661/18,240                         | 91.5             | 0.093       | 107.63 | 48.33 | 167.79 | 105.70  |
| fd4      | 3.7           | 2.0          | 37,476/18,086                         | 91.3             | 0.081       | 107.72 | 48.08 | 167.56 | 105.69  |
| fd5      | 4.0           | 2.0          | 25,413/12,450                         | 86.8             | 0.100       | 106.93 | 48.29 | 166.06 | 106.22  |
| dfd1     | 4.0           | 1.8          | 43,774/17,057                         | 87               | 0.161       | 107.30 | 48.09 | 167.07 | 105.59  |
| dfd2     | 4.0           | 1.8          | 33,218/17,908                         | 90               | 0.145       | 107.72 | 48.22 | 167.24 | 105.76  |
| dfd3     | 3.9           | 2.0          | 30,062/16,723                         | 85.5             | 0.113       | 107.89 | 48.09 | 167.48 | 105.85  |
| dfd4     | 3.5           | 1.9          | 47,378/20,215                         | 94.5             | 0.078       | 107.42 | 48.01 | 167.00 | 105.59  |
| dfd5     | 3.6           | 2.0          | 9,135/7,582                           | 35.8             | 0.095       | 107.32 | 47.88 | 166.13 | 105.36  |

<sup>a</sup> Average ratio of observed intensity to  $\sigma$  in the highest resolution shell of reflections.

$$^b R_{sym} = \frac{\sum |I_{obs} - I_{avg}|}{\sum I_{avg}}$$

pered by the large unit cell, but a preliminary set of data has been collected on an imaging plate system (these data were collected in the laboratory of Dr. Nguyen-Huu Xuong, University of California at San Diego). The change in space group and unit cell dimensions of the co-crystallized derivative suggest a significant conformational change upon binding of the ligand.

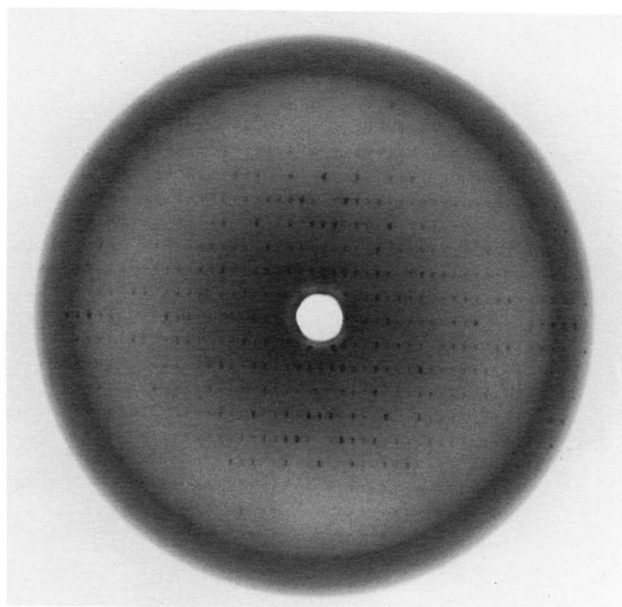
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**Fig. 2.** Eight-hour 10° precession photograph of FD-T along the reciprocal lattice plane  $c^* = 0$ .

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