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# BIOSYNTHESIS OF CANINE FIBRINOGEN: IN VITRO SYNTHESIS OF Aα, Bβ AND γ PRECURSOR CHAINS

Sharon Yu, C.M. Redman, J. Goldstein and B. Blombäck

The Lindsley F. Kimball Research Institute
of the New York Blood Center
New York, N.Y. 10021

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## SUMMARY

An mRNA fraction from dog liver translated with a rabbit reticulocyte protein synthesizing system in the presence of [$^{35}$S]-methionine produces fibrinogen-related proteins which are immunoprecipitated with rabbit antiserum to dog fibrinogen. Analyses of these radioactive proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography indicate that the three fibrinogen chains (Aα, Bβ and γ) are synthesized separately as larger precursors. The putative pre Aα and pre Bβ chains were characterized by their susceptibility to treatment with thrombin and batroxobin. Thrombin degraded the pre Aα and pre Bβ chains, while batroxobin only acted on the pre Aα chain. The pre γ chain was not degraded by these enzymes.

## INTRODUCTION

Fibrinogen is a dimeric molecule with each unit consisting of three non-identical polypeptide chains held together by disulfide bonds. In human fibrinogen the three chains (Aα, Bβ and γ) have molecular weights of 65,000, 55,000 and 47,000, respectively. Dog fibrinogen is similar to human fibrinogen in size, amino acid composition of the individual chains and in the amino-terminal sequences (1). Fibrinogen is synthesized by the liver and, prior to secretion, it is thought to follow the conventional hepatic intracellular route described for other plasma proteins, since immunocytochemical studies have localized rat hepatic fibrinogen in the endoplasmic reticulum and the Golgi apparatus (2). Studies in our labora-

Abbreviation: SDS, sodium dodecyl sulfate

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tory have shown that within the rough endoplasmic reticulum, nascent fibrinogen exists as a dimeric molecule with all three chains disulfide bonded (3). In this study we analyze the translation products of dog liver mRNA and show that the fibrinogen chains are synthesized separately and that each polypetide chain is probably synthesized as a larger precursor with a 'signal' extension.

## MATERIALS AND METHODS

Materials: L[$^{35}$S]-Methionine was purchased from New England Nuclear Corp., Boston, Mass.; Protein-A Sepharose (CL-4B) was obtained from Pharmacia, Piscataway, N.J.; and Trasylol was from Mobay Chemical Co., New York. Oligo-(dT)-cellulose was purchased from Collaborative Research, Waltham, Mass. Batroxobin (Defibrase), 200 units/mg, is a product of Pentapharm, Basel, Switzerland and thrombin (207 NIH units/mg) is a bovine preparation (4).

Preparation of mRNA fraction. RNA was extracted from dog liver by the 8 M guanidine-HCl, alcohol precipitation method of Deeley et al. (5) and an mRNA fraction was obtained by oligo-(dT)-cellulose column chromatography (6).

In vitro synthesis and immunoprecipitation of fibrinogen. An mRNA dependent cell-free protein-synthesizing system was prepared from rabbit reticulocytes by the method of Pelham and Jackson (7) and used to translate liver mRNA. [$^{35}$S]-Methionine was used as the radioactive tracer in all experiments. Fibrinogen synthesized in vitro was immunoprecipitated from the incubation mixture by a modification of the method described by Chang et al. (8). The incubation mixtures (usually 250 µl) were adjusted first to 0.3% SDS and then to 150 mM NaCl, 33 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1.7% Triton X-100 and 100 units/ml Trasylol. To this mixture 10 µl of rabbit antiserum to dog fibrinogen was added and it was then incubated overnight at 4°C. The antibody-antigen complexes which formed were precipitated by the addition of 100 µl of packed protein A-Sepharose and the mixture was further incubated at room temperature for one hour. The protein A-Sepharose containing the bound antigen-antibody complexes was sedimented by centrifugation and washed 4 times with buffer (0.1% SDS, 150 mM NaCl, 150mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 100 units/ml of Trasylol). The immuno-complexes were dissociated from protein A-Sepharose by boiling for 5 min in a solution containing 6 M urea, 4% SDS, 0.05 M Tris-glycine, pH 8.4 and 2% dithiothreitol and were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Treatment of nascent fibrinogen with thrombin and batroxobin. For the characterization of the fibrinogen chains synthesized in vitro, immunoprecipitation was carried out without use of protein A-Sepharose as previously described (9). [$^{35}$S]-Methionine labeled fibrinogen chains and 100 µg of dog fibrinogen carrier were dissolved in 0.1 ml of 0.3 M Tris-HCl pH 7.6, 0.15 M NaCl, 6 M urea and 300 units/ml Trasylol. The solution was then diluted two-fold and incubated for 4 hours at 37° with either 42 units of thrombin or 20 units of batroxobin. After incubation the solutions were adjusted to 6 M urea, 4% SDS, 2% dithiothreitol and were boiled for 3 min prior to analysis by SDS-polyacrylamide gel electrophoresis and autoradiography.

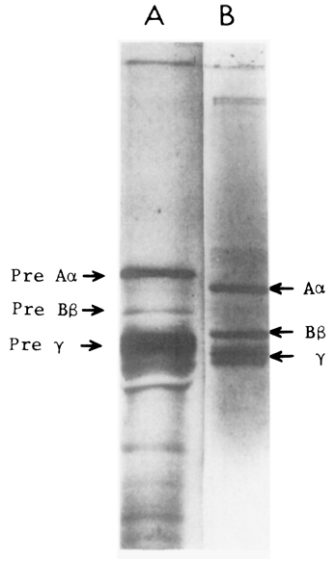


Figure 1. SDS-polyacrylamide gel analyses of fibrinogen synthesized in vitro. Dog liver mRNA was translated with a reticulocyte lysate system and the radioactive fibrinogen products were immunoprecipitated with rabbit antiserum to dog fibrinogen. The immunoprecipitate was analyzed on 7.5% SDS-polyacrylamide gels. In Lane A an autoradiogram is shown and the location of Pre Aα, Pre Bβ and Pre γ Chains are indicated. Lane B shows the profile of Coomassie-blue stained reduced dog fibrinogen. The location of Aα, Bβ and γ chains are indicated.

## RESULTS AND DISCUSSION

Dog liver mRNA directs the synthesis of proteins that are immunoprecipitated by rabbit antiserum to dog fibrinogen. About 0.5% of the radioactivity in total trichloroacetic acid precipitable proteins was immunoprecipitated with anti-serum to fibrinogen. The mobilities on SDS-polyacrylamide gel electrophoresis of the radioactive proteins in the immunoprecipitate were compared to those of the three polypetide chains from reduced dog fibrinogen (Fig. 1). Reduced dog fibrinogen yields single Aα and Bβ bands of approximately 65,000 and 55,000 daltons, respectively and doublet γ bands of approximately 47,000 daltons. Heterogeneity in the γ chains has been noticed in other species (10). Several radioactive fibrinogen related proteins were present in the immunoprecipitate, including some proteins which migrated more slowly on SDS-polyacrylamide gel

electrophoresis than authentic Aα, Bβ and γ chains. These, apparently larger proteins (termed pre Aα, pre Bβ and pre γ), have estimated molecular weights of 68,000, 58,000 and 52,000. Also present in the anti-fibrinogen immunoprecipitate were radioactive proteins with lower molecular weights than the γ chain, which may represent either incompleted fibrinogen chains or degradative products of the nascent fibrinogen chains.

Fibrinogen is a glycoprotein with both the Bβ and γ chains containing sugar residues (11). Since the nascent chains produced in vitro are probably not glycosylated and since glycosylation is known to influence the migration of glycoproteins on SDS-polyacrylamide gel electrophoresis it is difficult to estimate the difference in size of the Bβ and γ chains obtained from plasma fibrinogen with that of the primary translation products. The Aα chain of fibrinogen is not glycosylated, however, and we estimate from the relative mobilities on SDS-polyacrylamide gel electrophoresis that pre Aα is about 3000 daltons larger than Aα which indicates the additional presence of about 20 amino acid residues in pre Aα.

The radioactive proteins which correspond to pre Aα, pre Bβ and pre γ chains (Fig. 1) were eluted from the polyacrylamide gels and aminoterminal analyses indicated the presence of N-terminal methionine which is consonant with these proteins being primary translation products of mRNA. These data indicate that the three chains of fibrinogen are probably synthesized separately as larger 'signal' precursors and that fibrinogen is not first synthesized as a large polypeptide, with the three polypeptides in tandem, which is later cleaved into three separate chains.

Specific enzymatic cleavages were used to identify the putative pre Aα pre Bβ and pre γ chains. Under controlled conditions thrombin specifically removes small aminoterminal portions (fibrinopeptides A and B) of the Aα and Bβ chains but not of the γ chain. Further treatment with thrombin also cleaves other portions of the Aα and Bβ chains. The nascent radioactive fibrinogen, obtained by immunoprecipitation, was treated with thrombin and

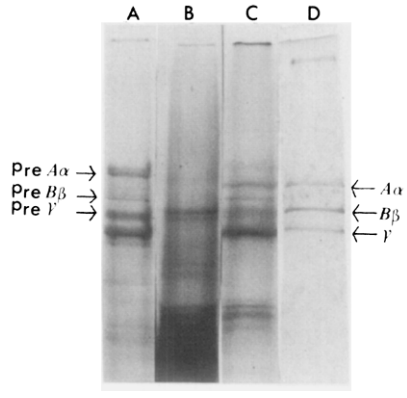


Figure 2. Treatment of nascent fibrinogen chains with thrombin and batroxobin. In vitro synthesized fibrinogen was isolated by immunoprecipitation, digested with either thrombin or batroxobin as described in Methods and analyzed by SDS-polyacrylamide gel electrophoresis. Lane A shows an autoradiogram of untreated nascent fibrinogen. The locations of pre Aα , pre Bβ and pre γ chains are indicated. Lane B shows the thrombin-treated fraction and lane C batroxobin-treated nascent fibrinogen. Lane D contains reduced dog fibrinogen, stained with Coomassie blue, to indicate the positions of the Aα, Bβ and α chains.

the mobilities on SDS-polyacrylamide gel electrophoresis of the thrombin-treated fibrinogen was compared to that of the untreated nascent fibrinogen and also to that of authentic plasma fibrinogen chains. Thrombin caused the disappearance of pre Aα and pre Bβ chains but did not affect the pre γ chain. Several low molecular weight proteins were produced by thrombin treatment and faint bands were seen at the positions of authentic Aα and Bβ chains (Fig. 2). On 7.5% SDS-polyacrylamide gels it is sometimes difficult to distinguish between the Aα and α or Bβ and β chains. We expected greater radioactivity, on thrombin treatment, in the Aα (α) position but the result obtained may be due to extended degradation of the pre Aα chain since high concentrations of thrombin had to be used for digestion in urea solutions.

Batroxobin, in contrast to thrombin, is an enzyme which specifically removes fibrinopeptide A from the Aα chain but does not affect the Bβ nor the γ chains of fibrinogen. Treatment of nascent fibrinogen with this enzyme clearly showed the disappearance of the pre Aα, and the appearance of a new radioactive protein with the same mobility as the α chain (Fig.

2B). The mobilities of pre Bβ and pre γ chains on SDS-polyacrylamide gel electrophoresis were not affected by batroxobin. Treatment of nascent fibrinogen with batroxobin, as with thrombin, also caused the disappearance of some of the radioactive proteins in the region near the pre γ chain, which we suspected of containing incompleted fibrinogen polypetide chains. The putative pre γ chain was not, however, affected by treatment with batroxobin or thrombin.

The sensitivity of the pre Aα band to both batroxobin and thrombin and its apparent conversion to a protein with a similar mobility to the α chain of fibrinogen suggests that this is a larger precursor of the Aα chain. Likewise, the sensitivity of pre Bβ to thrombin but not to batroxobin suggests that it is a precursor of the Bβ chain. Characterization of the pre γ chain is dependent upon its immunoprecipitation with antiserum to dog fibrinogen and its insensitivity to thrombin and batroxobin. Additionally, using an antiserum specific for the γ chain, which does not cross react with the Aα and Bβ chains, we have shown that only the radioactive pre γ chain and a small amount of low molecular weight proteins, but not pre Aα or pre Bβ, are immunoprecipitated from the translation products of liver mRNA when this antiserum is used. (Data not shown).

These studies indicate that fibrinogen is not first synthesized as a single large protein which is then processed into its three component chains. Each of the three chains are synthesized separately with each individual chain probably containing its own "signal" leader extension. Our in vivo studies have shown that the separate chains of fibrinogen are quickly assembled in the rough endoplasmic reticulum into a dimeric fibrinogen molecule without prior accumulation of free fibrinogen chains (3). This therefore suggests that fibrinogen chains may be disulfide bonded during translation of the chains before they are discharged into the lumen of the endoplasmic reticulum or that assembly occurs immediately afterwards. Alternatively, fibrinogen assembly in the rough endoplasmic reti-

culum may occur in a stepwise fashion with some chains being released first into the lumen of the endoplasmic reticulum and then these early released chains may quickly interact with the other fibrinogen chains which may still be attached to the membrane-attached polysomes. The exact intracellular mechanisms by which the separate chains are vectorically discharged and assembled in the rough endoplasmic reticulum remain to be elucidated. However, our studies show that fibrinogen chains are synthesized separately and this, coupled with the fact that fibrinogen is quickly assembled in the rough endoplasmic reticulum, suggests that synthesis of the separate chains occurs in a coordinated and/or synchronous manner.

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