The role of fibrinogen αC-domains in the fibrin assembly process

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Turbidity development registration and electron microscopic observation of the assembly process of the fibrin monomer and its derivative lacking in intact αC-domains (monomeric X_1 fragment) have shown that these domains participate in fibrin polymerization, not as structural components, but as a factor promoting the ordered process of fibrin assembly.

Fibrinogen *X_1 fragment* *αC-domain* *Electron microscopy* *Turbidity curve* *Fibrin assembly*

1. INTRODUCTION

Due to thrombin action, fibrinogen converts to a fibrin monomer, which polymerizes spontaneously to form a fibrin clot. A two-step mechanism of fibrin assembly has been proposed [1–3]. The first step is the polymerization of fibrin molecules to form long protofibrils two molecules wide. This process is provided by the interaction between the active sites situated in the central and terminal domains of the molecules [4]. At the second step the protofibrils associate laterally producing ordered cross-striated fibers, which may comprise more than a hundred protofibrils. The fibers join together forming a 3-dimensional network-solid clot.

C-terminal parts of both A α-chains of the fibrinogen molecule are split at the early stage of its proteolytic degradation [5]. The rest of the molecule, called fragment X [6] or fibrinogen fraction I-8 [7], is characterized by lowered clottability, retarded polymerization and prolonged clotting time [6,8,9]. It was shown that in the native molecule these parts of A α-chains form two compact structures – αC-domains – which strongly interact with each other [10–12]. αC-domains are connected with the terminal parts of the molecule through extended flexible polypeptide junctions [11,12], which probably endow them with some freedom of mobility about the bulk of the molecule. The question arises as to the role which these unique domains may fulfil in the fibrin assembly mechanism.

We have prepared a fibrin monomer which was lacking in intact αC-domains (X_1 monomer) and studied its polymerization in comparison with that of native monomeric fibrin. The results obtained clearly show that αC-domains take part in the polymerization process, not as structural components, but as those which raise the affinity between fibrin monomer molecules, accelerating essentially the rate of its ordered assembly.

2. MATERIALS AND METHODS

Bovine fibrinogen (clottability, 98%) was prepared from oxolate plasma by salting out with sodium sulphate [13] and subsequent purification [9]. The X_1 fragment (clottability, 92%) has been obtained from plasmic hydrolysate of fibrinogen as in [11]. The fibrin monomer (f_m) was prepared by dissolving the fibrin clot at low temperature with acetic acid as in [14]. The X_1 monomer (X_{1m}) was prepared from the X_1 fragment in the same manner as the fibrin monomer. To remove nonclotting contaminations, the fibrin monomer and

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X_1 monomer were subjected to two further reclottings, after which both proteins were practically 100% clottable. The purity of preparations was checked by SDS-electrophoresis (fig.1a). No detectable contaminations were found in both proteins. Fig.1b demonstrates the absence of intact Aα-chains in the X_1 monomer. The polymerization process was initiated by 10-fold dilution of concentrated (c = 0.5–2.0 mg/ml) X_{1m} or f_m solution with 0.063 M sodium phosphate buffer (pH 6.8) at 37°C. The solution contained 0.1 M NaCl and 5×10^{-4} M $CaCl_2$. Samples for electron microscopy were prepared at various times after initiation of polymerization in the following way. A drop of mixture was placed on the grid covered with carbon film, washed twice with 0.02 M Tris–maleate buffer (pH 6.8) and negatively stained with 1% uranyl acetate (pH 4.5). Samples were examined in a JEM 100 CX electron microscope (Jeol, Japan) operating at 80 kV high tension and a magnification of 48000×. The absorbance (turbidity) changing at X_{1m} and f_m polymerization was recorded on a Specord M 40 Spectrophotometer (Carl Zeiss, Jena, GDR) at 350 nm. The delay of turbidity growth (lag-time, t_{lag}) and maximum rate of turbidity growth (v_{max}) were estimated from turbidity curves as in [15]. A tangent line was drawn through the maximum slope of the curve. Its intercept on the time axis was denoted as t_{lag} and the slope of this line, expressed as tangent $\alpha = \Delta E_{350}/t$ (s), was denoted as v_{max}.

Clotting time (t_{clot}) was determined visually.

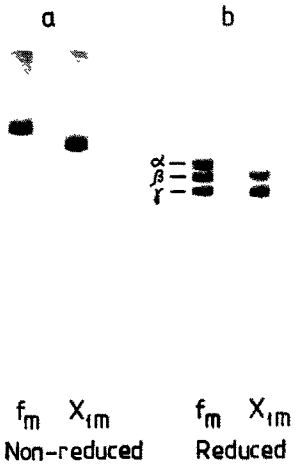


Fig.1. SDS-polyacrylamide gel electrophoretic patterns of fibrin monomer (f_m) and X_1 monomer (X_{1m}), non-reduced (a) and reduced (by 0.2% β-mercaptoethanol) (b); α, β, γ are the bands of the corresponding polypeptide chains of fibrin [9]. Electrophoresis was performed in 9% polyacrylamide gels as in [16].

3. RESULTS AND DISCUSSION

Fig.2 presents electron micrographs of the fiber formation process by the X_1 monomer (lacking intact αC-domains) and the fibrin monomer. It is evident that these processes are identical. In both cases the appearance of small islands of stretched protofibrils was the first sign of ordering (fig.2a,d). Then such a friable bunch of protofibrils becomes thicker and more compact (fig.2b,e) and at last forms mature compact fibers with typical cross-striations (fig.2c,f). It is known that cross-banding of fibers is a result of the strictly ordered packing of protofibrils, accompanied by the distribution of protein material in a definite way giving cross-striation at negative staining. Our electron microscope data presented in fig.2 clearly show that αC-domain damaging does not cause a noticeable disturbance of the ordered packing of protofibrils inside the fiber. The gels of both proteins were homogeneous and quite similar in the concentration range studied (c = 0.05–0.2 mg/ml). In this connection it is worthwhile to note that the rigidity of fibrin gels prepared from fibrinogen fraction I-8, which has somewhat shorter Aα-chains than intact fibrinogen is the same as that of the intact fibrin clot [17]. Since the X_1 fragment has a similar damage of αC-domains as the fibrinogen fraction I-8, we can expect that it forms gel with the same rigidity as intact fibrin does. It was also found that the normal fibrin network does not lose its rigidity when C-terminal parts of α-chains are removed proteolytically [17]. Thus the facts presented above allow us to conclude that the absence of intact αC-domains in the X_1 monomer does not influence noticeably the structure of fibers and the rigidity of gel.

Fig.3 presents the curves of turbidity changes at the X_1 monomer or fibrin monomer polymerization. As was shown in [2,3] such curves reflect the assembly course of these proteins. The delay of turbidity growth (lag-period) corresponds to the first polymerization step during which presumably

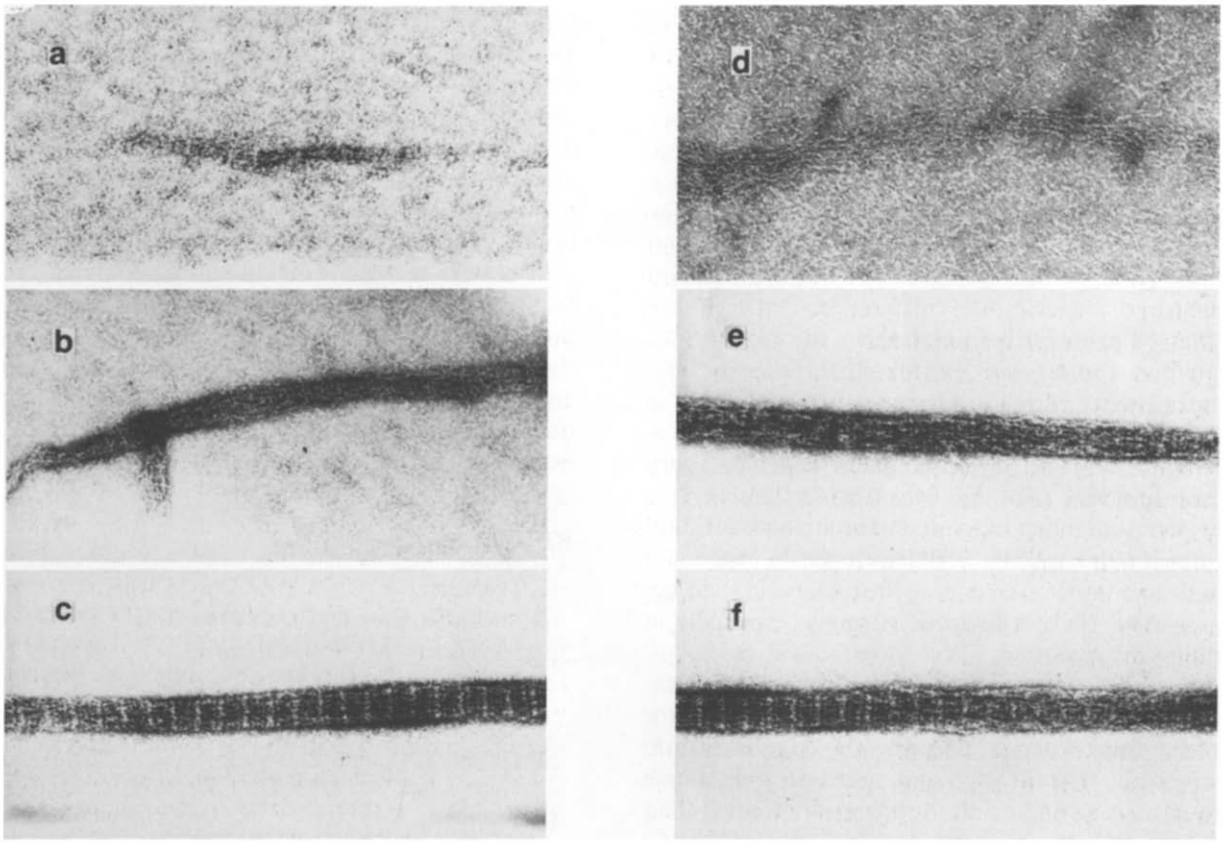
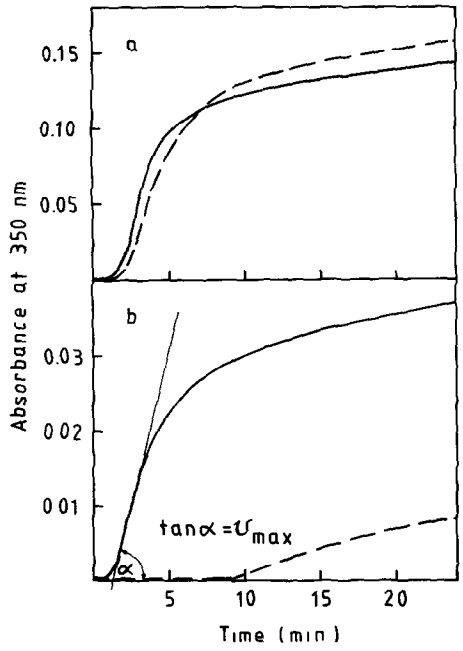


Fig.2. Consecutive stages of X_1 monomer (a–c) and fibrin monomer (d–f) polymerization. Negative staining with uranyl acetate. Protein concentration, 0.05 mg/ml. Magnification, 140000 ×.



fibrin monomers polymerize into thin soluble protofibrils. A sharp turbidity increase corresponds to the second step of fibrin assembly when the lateral aggregation of protofibrils into fibers occurs. It can be seen that at a higher concentration (c = 0.2 mg/ml) the curves differ only slightly (fig.3a). But these differences are most pronounced at lower concentrations (0.05 mg/ml) (fig.3b). We have studied in some detail the dependence of the rates of the first and second reaction steps as well as the clotting time (t_{clot}) from the degree of dilution of the mixture. The delay of turbidity growth (t_{lag}) and maximum rate turbidity growth (v_{max}) were chosen to describe the rates of the first and second polymerization step correspondingly. These

Fig.3. Polymerization curves of the X_1 monomer (– – –) and the fibrin monomer (——). Protein concentrations: 0.2 mg/ml (a); 0.05 mg/ml (b). Absorbance at 350 nm (ordinate) is plotted against time (abscissa).

parameters were evaluated from turbidity curves as described in section 2. The ratios of t_{lag} values of X_{1m} and f_m, v_{max} values of f_m and X_{1m} and t_{clot} values of X_{1m} and f_m plotted against dilution are presented in fig.4. Such plots allow us to present the differences between the X_1 monomer and the fibrin monomer assembly demonstratively. The assembly of X_{1m} is far more susceptible to dilution than f_m. A 4-fold dilution of the original reaction mixtures increases the differences between the rates of protofibril formation in the f_m and X_{1m} solution about 6-fold and the differences of fiber and network formation between f_m and X_{1m} up to 9–10-fold (fig.4). This clearly shows that proteolytic damage of αC-domains leads to sharp retarding with dilution of the fibrin assembly. It is evident that intact αC-domains contribute substantially to the specific affinity of fibrin molecules with each other accelerating the process of their aggregation. This is revealed strongly, especially in dilute solutions.

Thus αC-domains take part in fibrin clot formation not as structural components but as the factor promoting ordered assembly of complex fibrin structure. The αC-domains seem to help fibrin molecules in finding and properly orientating their twin partners. Two structural features of αC-domains found earlier allow us to propose the following mechanism of this process. Firstly, αC-domains should have been a very mobile system due to the extended flexible junctions connecting their compact parts with the bulk of the molecule

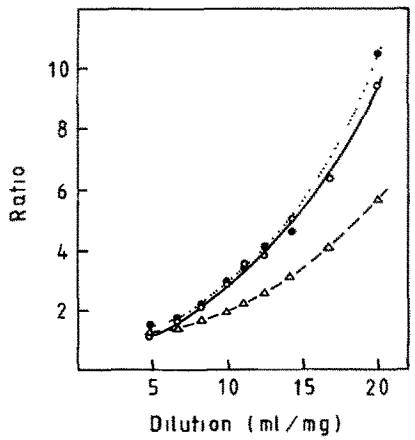


Fig.4. Plot of the ratio of t_{lag} values of X_{1m} and f_m (Δ---Δ), v_{max} values of f_m and X_{1m} (○—○) and t_{clot} values of X_{1m} and f_m (●···●) *vs* dilution (the inverse proportion of concentration).

[11,12]. Secondly, in the fibrinogen molecule, αC-domains are in direct contact, strongly interacting with each other [11], evidently by means of some complementary surface sites. If this interaction is decreased in the fibrin molecule, mobile αC-domains may dissociate and reassociate in a new manner, intermolecularly, forming intermolecular bonds. This allows the major active polymerization centers to react quickly and accurately. The facilitating role of αC-domains becomes more important on dilution of the reaction mixture when the distances between the molecules are large enough to decrease the interaction between the major active centers. This hypothesis needs further experimental checking.

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