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The cleavage of β-chain in bovine fibrinogen D_H fragment (95 kDa) leads to a significant increase in its anticlotting activity

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It is shown that in the presence of Ca^{2+} plasmin converts bovine fibrinogen fragment D_H (95 kDa) into D_{LA} fragment by the cleavage of its β-chain Arg_{372}-Thr_{373} bond. D_{LA} fragment consists of two components (82 and 12 kDa) held together by non-covalent bonds and has 3.5-fold higher anticlotting activity than D_H fragment. The D_H to D_{LA} fragment conversion leads to the destabilization of thermolabile domains of the latter without the loss of their compact structure. The results obtained show that the activation of D_H fragment by the cleavage of its Arg_{372}-Thr_{373} bond bears some resemblance to the general activation of proenzyme into enzyme.

Fragment D_H; Anticlotting activity; Proteolysis; Activation; Sequence homology

1. INTRODUCTION

The plasma protein fibrinogen is converted enzymatically into fibrin to form a fibrin clot. In vivo the clot is broken down by the enzyme plasmin resulting in various soluble fragments, some of which inhibit fibrin assembly. Among them is $D_{(cate)}$ or D_H (95 kDa) fragment. This fragment contains a number of polymerization sites by which it interacts with growing fibrin polymer thus terminating further polymerization. Further proteolysis of D_H fragment without Ca^{2+} leads to splitting of its C-terminal 13 kDa portion of γ-chain, which forms one of the active sites [1,2]. This leads to the appearance of D_{EGTA} or light D_L (82 kDa) fragment, which has no anticlotting properties. Trypsin produces similar fragments, but on prolonged trypsin digestion of bovine fibrinogen in the presence of Ca^{2+} along with D_H and D_L fragments the D_{L2} fragment with unknown structure appears [3]. The latter (D_{L2}) is a more potent inhibitor of the fibrin assembly process than D_H fragment [3]. The question arises as to the cause of the greater extent of the anticlotting properties of the active D_{L2} fragment (D_{L2}).

Here we present results demonstrating that D_H fragment can be converted into the more active D_{LA} fragment by plasmin action. The results obtained allow clarification of the mechanism of increasing of D_H fragment anticlotting activity by limited plasminolysis.

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2. MATERIALS AND METHODS

Bovine fibrinogen and fibrin monomer were prepared as described [4]. Fibrin-Sepharose was prepared as described [5]. D_H fragment obtained from a 2 h plasmin hydrolysate of fibrinogen and purified by the method described [6]. Hydrolysis was performed in 0.1 M Tris-HCl, pH 7.4, with 0.15 M NaCl and 5×10^{-3} M $CaCl_2$, at 25°C; protein was 20 mg/ml and plasmin activity was 0.2 caseinolytic units/ml.

Clotting time was determined visually at 37°C in samples containing 0.05 M Tris-HCl buffer, pH 7.4, with 0.15 M NaCl, 1×10^{-4} M $CaCl_2$ and 0.3 mg/ml fibrin monomer. Anticlotting effect was presented as the $(t - t_0)/t_0$ ratio, where t and t_0 are the clotting times of fibrin monomer in the presence and absence of inhibitor, respectively. The inhibitor activity of fragments was expressed in special units proposed by Belitser et al. [7].

The M_r values of the studied protein fragments were deter-

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mined by high-speed equilibrium ultracentrifugation as described [8] and by SDS-polyacrylamide gel electrophoresis.

Amino-terminal amino acid sequence was determined by the Dansyl chloride method [9].

Calorimetric studies were carried out on a scanning microcalorimeter (DASM-1M) as in [6].

3. RESULTS AND DISCUSSION

Prolonged hydrolysis of D_H fragment (specific anticlotting activity 13.3 units) by plasmin in the presence of Ca^{2+} leads to the formation of new products of 82 and 12 kDa (fig.1). The accumulation of these products is accompanied by the increase of anticlotting activity of hydrolysate up to 33.4 units (fig.2). We inhibited 48 h hydrolysate by contrykal and passed it through the column with Lys-Sepharose to remove plasmin. Then hydrolysate (30 mg) was applied to a column (3.6 × 15 cm) with fibrin-Sepharose and fractionated as described [3]. As seen from fig.3, hydrolysate was separated into three fractions. Peak 2 contained D_H fragment (95 kDa) with anticlotting activity of 13.3 units and peak 3 contained the fraction with higher affinity and anticlotting activity of 45.4 units. The latter consisted of two bands of 82 kDa (as D_L fragment) and 12 kDa as revealed by SDS-electrophoresis (fig.4a). We have denoted it as active D_L fragment (D_{LA} fragment). It is obvious that D_{LA} fragment derives from D_H fragment and its appearance causes the increase of hydrolysate anticlotting activity.

We have analyzed the structural organization of D_{LA} fragment. As mentioned above, the SDS-electrophoretogram of this fragment shows two bands of 82 and 12 kDa (fig.4a). At the same time the molecular mass of the D_{LA} fragment determined by equilibrium ultracentrifugation is 94 ± 2 kDa, i.e., it corresponds to the sum of molecular masses of the two components revealed by SDS-electrophoresis. These components are not separated on a column (2.5 × 60 cm) of Sephadex G-75 SF in 0.1 M Tris-HCl buffer, pH 7.4, with 0.15 or 2 M NaCl. Since the M_r determination by SDS-electrophoresis was performed in denaturing conditions (0.1% SDS) and by equilibrium ultracentrifugation in non-denaturing conditions, one can conclude that native D_{LA} fragment consists of two unequal components of 82 and 12 kDa linked together by non-covalent bonds.

We have separated these components in denaturing conditions (0.1 M Tris-HCl, pH 7.4, with 6 M urea) and analyzed their polypeptide chain composition by SDS-electrophoresis with 2% β-mercaptoethanol to reduce their S-S bonds. As seen from fig.4b, the 82 kDa component of D_{LA} fragment, like D_H fragment, consists of three chains while the 12 kDa component is a one-chain fragment. An 82 kDa component reveals two bands of 38 and 12 kDa, which correspond to the γ and α chain of D_H fragment, and double band β' of 33 and 31.5 kDa. The double β'-band corresponds to the N-terminal remnants of D_H-fragment β-chain because they are linked with α and γ-chains by disulphide bonds in the non-reduced 82 kDa component and contains a carbohydrate as revealed by Schiff's reagent staining of reduced patterns. It is likely that the 31.5 kDa component of the β'-band is distinguished from the 33 kDa component by the absence of a small N-terminal polypeptide region. It is known that carbohydrate is attached to Asn_{371} of bovine

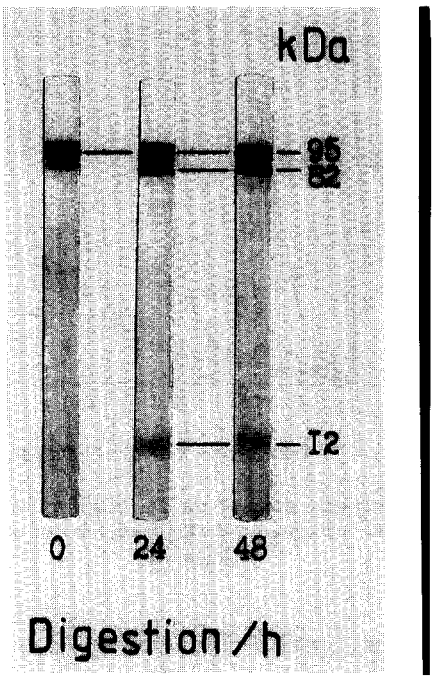

Fig.1. SDS-polyacrylamide gel electrophoretic patterns of timed plasmin digest of bovine D_H fragment. Hydrolysis was performed in 0.1 M Tris-HCl, pH 7.4, with 0.15 M NaCl and 5×10^{-3} M $CaCl_2$ at 37°C; fragment was 2.5 mg/ml; plasmin activity was 1.0 caseinolytic units/ml. Electrophoresis was performed in 7% polyacrylamide gel as described [10].

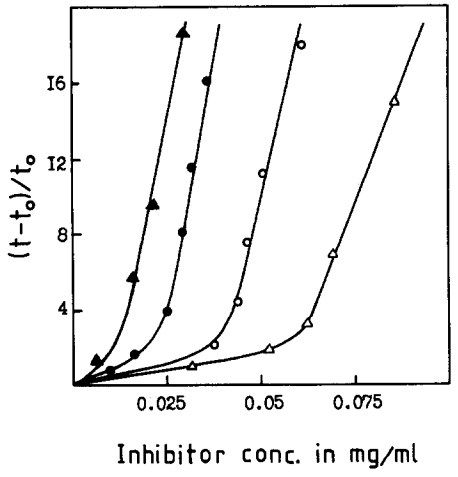

Fig.2. Anticlotting effect of the D_H fragment (△), 24 h (○) and 48 h (●) hydrolysates of D_H fragments and purified D_{LA} fragment (▲).

fibrinogen β-chain [11], therefore β-chain (β') of the 82 kDa component includes this amino acid residue. The 12 kDa component is a C-terminal remnant of D_H fragment β-chain because it has a typical tryptophan-like light absorption spectrum, while the N-terminal portion of this chain with the same molecular mass contains no tryptophan [11]. We have denoted it as βC-peptide. The N-terminal amino acids of βC-peptide found by us in two degradation cycles were Thr-Met. The amino acid

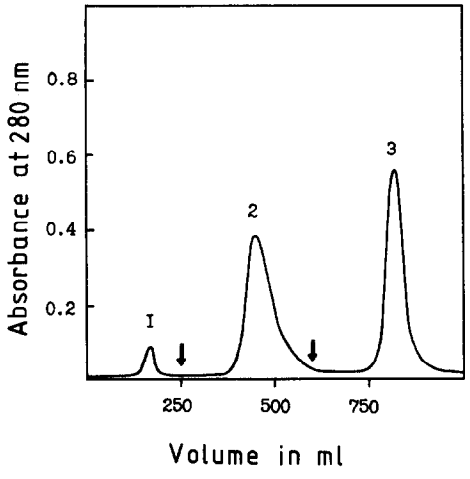

Fig.3. Affinity chromatography of a 48 h D_H fragment hydrolysate on fibrin-Sepharose as described [3]. The replacement of elution buffers is indicated by arrows.

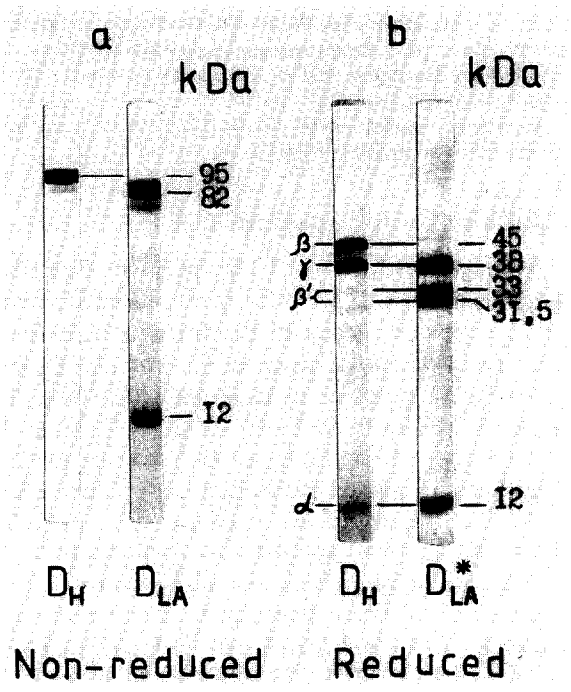

Fig.4. SDS-polyacrylamide gel electrophoretic patterns of fraction 2 (D_H fragment) and fraction 3 (D_{LA} fragment) (a) and D_H fragment (D_H) and 82 kDa component of D_{LA} fragment (D_{LA}) with reduction of S-S bonds by 2% β-mercaptoethanol (b). Electrophoresis was performed in 7% (a) and 10% (b) polyacrylamide gel as described [10].

sequence analysis of bovine D_H-fragment β-chain shows that only cleavage of its Arg_{372}-Thr_{373} peptide bond may produce a C-terminal fragment of 12 kDa with N-terminal Thr-Met. It follows from the above that D_H fragment is converted into D_{LA} fragment by the cleavage of its β-chain at the position of Arg_{372}-Thr_{373}. This conversion leads to a 3.5-fold increase in D-fragment anticlotting activity. One can consider this process as the activation of D_H fragment by the proteolytic cleavage of the polypeptide chain.

As was shown earlier [12], D_H fragment consists of five domains (fig.5b). The Arg_{372}-Thr_{373} bond is situated between two interacting domains [13]. It is clear that after cleavage of this bond βC-peptide forming a single domain does not separate from the D_{LA} fragment due to strong interaction with another domain. The question arises as to the structural changes on D_H to D_{LA} fragment conversion, i.e. on activation of D_H fragment. This question was clarified partly by the study of

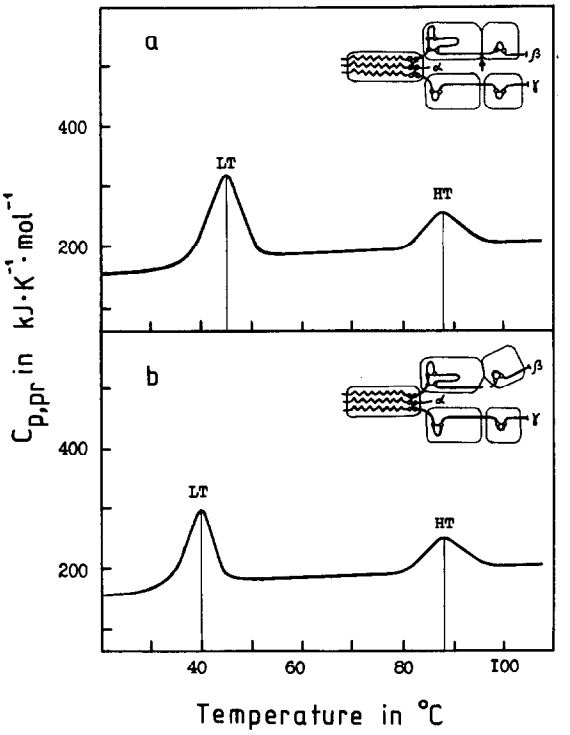

Fig.5. Temperature dependence of the partial molar heat capacity function of the D_H (a) and D_{LA} fragment (b) in 0.05 M glycine (pH 3.5). Structure of D_H fragment as described [12] and D_{LA} fragment to be studied are given schematically in the upper right corner of both panels; the arrow indicates site of cleavage at D_H to D_{LA} conversion.

denaturation processes of both these fragments.

Fig.5a and table 1 present calorimetrically obtained melting curves and thermodynamic characteristics of melting of D_{LA} and D_H fragments. As seen, both fragments have high temperature (HT) and low temperature (LT) transitions which correspond to the melting of their N-terminal coiled-coil and C-terminal globular regions, respectively [12]. The temperature and enthalpies of HT transitions in both fragments are similar as are the ratios $\Delta H_m^{cal}/\Delta H_m^{vh}$ reflecting the number of melting domains. At the same time LT-domains in D_{LA} fragment melt at 5°C lower temperature and with lower enthalpy. However, extrapolation of the enthalpy value for the D_{LA} fragment to 45°C via the equation:

$$\Delta H_m(T) = \Delta H_m(T_m) + \int_{T_m}^{T} \Delta_m C_p dT$$

where $\Delta_m C_p = 22 \pm 2\ kJ \cdot K^{-1} \cdot mol^{-1}$ is the experimental value, gives a value for $\Delta H_m(T_m = 45°C) = 1113\ kJ \cdot K^{-1} \cdot mol^{-1}$, which is very close to the value for the ΔH_m of D_H fragment (table 1). It follows that D_H to D_{LA} fragment conversion is accompanied by the destabilization of thermolabile (LT) domains of the latter without loss of their compact structure.

It should be noted that Lys-plasminogen activation, which consists of cleavage of only the Arg_{560}-Val_{561} peptide bond, leads to a similar destabilization of the domain, forming an active site [14]. Pepsinogen to pepsin conversion is also accompanied by the destabilization of the pepsin structure [15]. It was tempting to suppose that the activation of D_H fragment described in this paper is similar to proenzyme activation into enzyme. In this connection we have analyzed the activation process of some factors of the blood clotting system. It was found that on prothrombin activation one of the bonds to be cleaved is Arg_{271}-Thr_{272} [16]. This bond is in the region Asp_{261}–Phe_{281},

Table 1

Thermodynamic characteristics of melting of the D_H and D_{LA} fragments

Protein fragments	Peak code							
	LT				HT			
	T_m	ΔH_m^{cal}	ΔH_m^{vh}	$\Delta H_m^{cal}/\Delta H_m^{vh}$	T_m	ΔH_m^{cal}	ΔH_m^{vh}	$\Delta H_m^{cal}/\Delta H_m^{vh}$
D_H (95 kDa)	45.0	1104	451	2.4	89.0	481	464	1.0
D_{LA} (82 + 12 kDa)	40.0	1003	422	2.4	89.0	468	460	1.0

T_m, temperature of peak maximum in °C; ΔH_m^{cal} and ΔH_m^{vh}, calorimetric and van 't Hoff enthalpies in $kJ \cdot mol^{-1}$. The experimental error does not exceed 5%

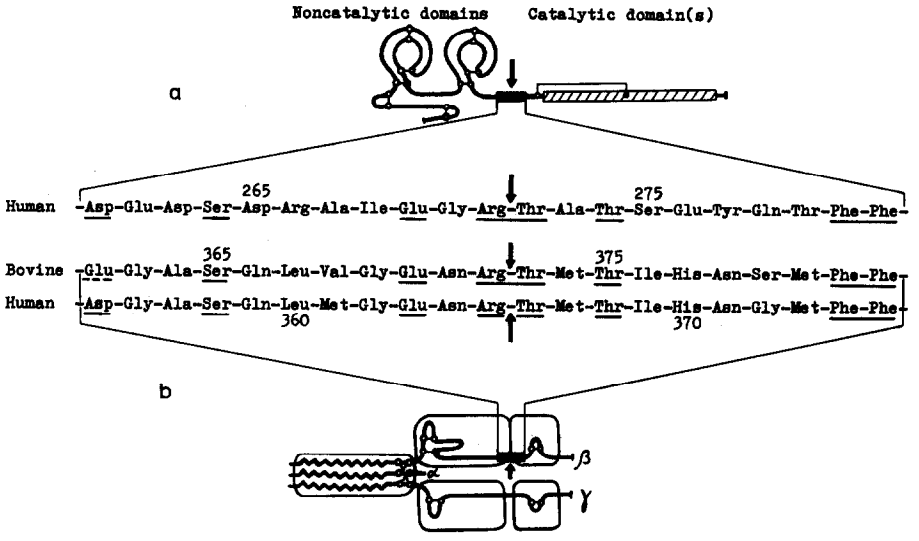

Fig.6. Diagram demonstrating homology between sequences in human prothrombin (a) and human and bovine fibrinogen D_H fragment β-chains (b) in the 21-residue segment containing peptide bond Arg-Thr to be cleaved. Arrows indicate sites of cleavage by plasmin.

which is highly homologous to the Glu_{362}–Phe_{382} region of bovine fibrinogen β-chain, containing the bond Arg_{372}-Thr_{373} to be cleaved (fig.6). In both proteins these regions are situated between domains. Apparently these polypeptide chain segments evolved from the common protein module which was inserted into prothrombin and fibrinogen to ensure the realization of some of their similar properties, namely, activation by limited proteolysis.

The data presented above show that the significant increase of the anticlotting activity of the D_H fragment after the cleavage of its Arg_{372}-Thr_{373} bond is not a random phenomenon, but probably a physiologically important mechanism involved in the regulation of the fibrin clot formation process.

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