Domain organization of the terminal parts in the fibrinogen molecule

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Using limited proteolysis and scanning microcalorimetry it was shown that each terminal part of the fibrinogen molecule is constituted by four co-operative domains. Among these domains two strongly interacting domains are formed by the C-terminal part of the β-chain, while the two other domains are formed by the C-terminal part of the γ-chain.

Fibrinogen *Fragment D_L* *Proteolysis* *Scanning calorimetry* *Domain organization*

1. INTRODUCTION

For a long period, among fibrinogen models the most popular was the three-nodular one based on electron microscopic and proteolytic studies of this molecule [1]. It was shown later, using scanning microcalorimetry [2,3] and high-resolution electron microscopy [4–6], that the arrangement of the fibrinogen molecule is much more complicated and includes at least 12 domains. This was supported by the fact that proteolysis of the terminal parts of fibrinogen, D_H fragments (95 kDa), proceeds in discrete steps: the splitting of a 13 kDa C-terminal fragment of its γ-chain leads to the formation of a D_L fragment (82 kDa), which then degrades to the final product – the TSD fragment (28 kDa) [3,7]. The latter consists of three N-terminal regions of α-, β- and γ-chains held by disulfide cross-links and is likely to be a part of the coiled-coil connector between the terminal and central parts in fibrinogen [7].

Here, we present the results of a detailed study of the degradation of D_H fragment to the TSD fragment, which has revealed all intermediate products and has permitted clarification of the domain organization of the terminal parts in fibrinogen.

2. MATERIALS AND METHODS

Bovine fibrinogen (clottability 98%) was prepared from oxalate plasma by salting out with sodium sulphate [9]. The D_H (95 kDa) and D_L (82 kDa) fragments were prepared as described in [3].

The D_L fragment was hydrolyzed by trypsin or chymotrypsin in 0.1 M sodium phosphate buffer (pH 7.0), 0.1 M NaCl, at 37°C and a fragment concentration of 10 mg/ml and enzyme-substrate ratio of 1:50 (w/w). Elastase digestion was performed in 0.05 M Tris-HCl buffer (pH 8.4), 0.1 M NaCl, 0.02% NaN_3, at 30°C (fragment concentration 10 mg/ml, enzyme-substrate ratio 1:100, w/w). Pepsin digestion was performed as in [3].

The M_r values of the studied protein fragments were determined by high-speed equilibrium ultracentrifugation according to [10] and by SDS-polyacrylamide gel electrophoresis.

The concentration of the fragments in solution was determined spectrophotometrically using the following values of $E_{280}^{1\%}$: 20.0 for the D_H and D_L fragments [3] and 16.0 for the D_Y (63 kDa) fragments. The latter was determined by measuring the concentration of solution by means of the nitrogen content according to [11].

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Calorimetric measurements were made on a DASM-1M scanning microcalorimeter [12] at a heating rate of 1 K/min and a solution concentration of 1–2 mg/ml. The partial molar heat capacity and enthalpy of protein thermal transition were determined as in [3]. The deconvolution analysis of the observed heat absorption has been carried out using a recurrent procedure described in [13].

3. RESULTS AND DISCUSSION

Fig.1 presents the time course of the D_L fragment hydrolysis by different proteases illustrating that at the early stages of proteolysis in all cases two discrete fragments appear designated as D_X (70 kDa) and D_Y (63 kDa). Since the D_L fragment, according to calorimetric studies, consists of several domains [2,3], one can suppose that the discrete character of its proteolysis reveals the discreteness in its structure, i.e. the appearance of new discrete fragments is a result of splitting of the pre-existing co-operative domains. To substantiate this assumption, we separated and studied one of these fragments.

As seen from fig.1, fragment D_Y is the only stable intermediate product accumulated in large amounts during chymotrypsin and elastase digestion of the D_L fragment. The D_Y fragment was isolated by gel filtration of a 10 h chymotrypsin digest (fig.1a) on a column (2.5 × 90 cm) of Sephadex G-100 superfine in 0.05 M sodium phosphate (pH 7.5), 0.1 M NaCl. Both D_L and D_Y fragments show a single band on the SDS electrophoretic pattern corresponding to 82 and 63 kDa, respectively (fig.2a). The same value has been obtained by equilibrium ultracentrifugation. It is known that the D_L fragment consists of the remnants of three polypeptide α-, β- and γ-chains, held together by a 'disulfide ring' [1]. Correspondingly, the SDS electropherogram of this fragment with the reduced S-S bonds shows three bands of 45, 25 and 12 kDa (fig.2b) identified as the β-, γ- and α-chains, respectively [3,8]. As for the D_Y fragment, it reveals only two bands of 45 and 9 kDa, i.e. its γ-chain is degraded greatly. Since the TSD (28 kDa) fragment consists of 3 equal (9 kDa) N-terminal parts of α-, β- and γ-chains [7], one can conclude that chymotrypsin splits mainly a large piece from the C-terminal part of the γ-chain in the D_L fragment, producing an intermediate fragment D_Y which consists of a β-chain (45 kDa) and shortened α- and γ-chains of equal size (9 kDa). Indeed, the sum of the molecular masses of β (45 kDa), α (9 kDa) and γ (9 kDa) chains amounts to 63 kDa which was found experimentally for the non-reduced D_L fragment. This is also confirmed by the results of the scanning calorimetric study.

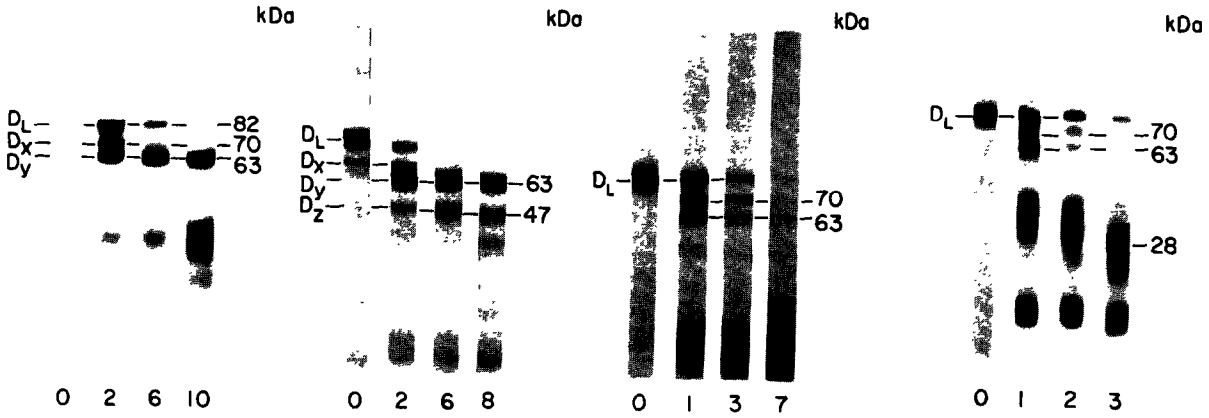

Fig.1. SDS-polyacrylamide gel electrophoresis of timed chymotrypsin (a), elastase (b), trypsin (c) and pepsin (d) digests of the D_L (82 kDa) fragment. Electrophoresis was performed in 7% polyacrylamide gel as in [14]. The molecular masses of proteolytic fragments were determined using protein markers.

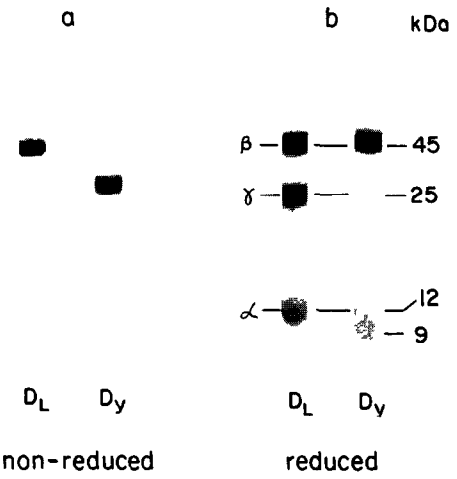

Fig.2. SDS-polyacrylamide gel electrophoresis patterns of the D_L (82 kDa) and D_Y (63 kDa) fragments, non-reduced (a) and reduced by 2% β-mercaptoethanol (b). Electrophoresis was performed in 7% (a) and 9% (b) polyacrylamide gel as in [14].

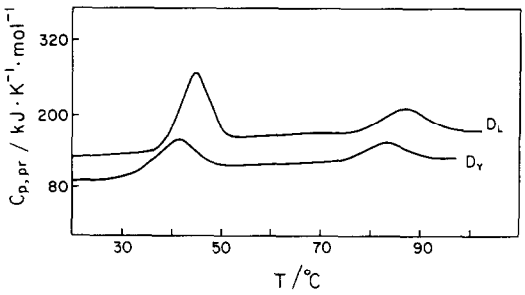

Fig.3. Temperature dependence of the partial molar heat capacity of the D_L (82 kDa) and D_Y (63 kDa) fragments.

Fig.3 presents the calorimetrically obtained melting profiles of the D_L and D_Y fragments. As shown, 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 [3]. The calorimetrically obtained enthalpies (ΔH_m^{cal}) for the HT transition of both fragments are similar but for the LT transition the value for the D_Y fragment is half of that for the D_L fragment (see table 1). This enthalpy decrease cannot be explained by the 4.3 K melting temperature shift but only by the decrease in the amount of ordered structure which melts in this temperature region. This fact provides us with an additional argument that the D_Y fragment is formed from the D_L one by splitting of a large piece from the C-terminal part of the γ-chain.

From the sharpness of the heat absorption peak upon protein melting one can calculate the van 't Hoff enthalpy of the denaturation process. The comparison of this enthalpy with the calorimetric enthalpy obtained from the area of the peak gives an idea as to the number of co-operative domains in protein [15]. As seen in table 1, the ratio ΔH_m^{cal} for the HT transition for both D_L and D_Y fragments is close to 1.0 which means that only one co-operative domain melts in this temperature region. Previously, we discussed all difficulties in analyzing the LT transition in fragments D_H and D_L [3]. The smaller D_Y fragment has a simpler structure and consequently a simpler melting curve which can be analyzed with higher accuracy. For

Table 1

Thermodynamic characteristics of melting of the D_L and D_Y fragments

Protein fragments	Peak code							
	LT				HT			
	T_m	ΔH_m^{cal}	ΔH_m^{vh}	$\frac{\Delta H_m^{cal}}{\Delta H_m^{vh}}$	T_m	ΔH_m^{cal}	ΔH_m^{vh}	$\frac{\Delta H_m^{cal}}{\Delta H_m^{vh}}$
D_L (82 kDa)	45.0	916	468	2.0	86.1	451	431	1.0
D_Y (63 kDa)	41.3	477	376	1.3	83.5	359	376	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·mol^{-1}. The experimental error does not exceed 5%

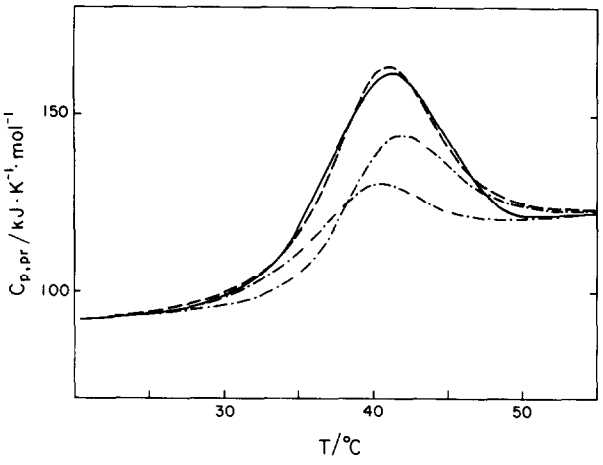

Fig.4. Deconvolution of the LT peak of the D_Y (63 kDa) fragment. (——) Experimental curve, (– · – · –) composite transitions obtained by deconvolution of the experimental curve, (– – –) the line synthesized from composite transitions.

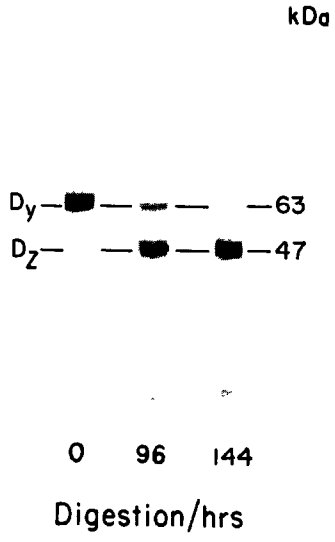

Fig.5. SDS-polyacrylamide gel electrophoresis of timed elastase digest of the purified D_Y (63 kDa) fragment. Electrophoresis was performed in 7% polyacrylamide gel as in [14].

the LT transition in the D_Y fragment, the ratio $\Delta H_m^{cal}/\Delta H_m^{vh}$ is significantly larger than 1.0 but less than 2.0 (see table 1). This means that the structure which melts here does not represent a single co-operative unit but consists of at least two strongly interacting co-operative domains. This suggestion is supported by a deconvolution analysis of the heat capacity function which shows that the melting process consists of two two-state transitions (fig.4).

The most convincing evidence for the existence of two thermolabile domains in the D_Y fragment was obtained from the study of its proteolysis by elastase. Elastolysis of the purified D_Y fragment leads to accumulation of the D_Z (47 kDa) fragment (fig.5), which is likely to be the result of splitting of one of the two thermolabile co-operative domains in the D_Y fragment. The splitting of the second thermolabile domain leads to the formation of the TSD (28 kDa) fragment. Therefore, the D_Y fragment includes two thermolabile strongly interacting co-operative domains formed by the C-terminal part of the β-chain. It should be noted that the C-terminal part of the γ-chain also consists of two thermolabile co-operative domains. The first is split proteolytically upon the conversion of the D_H to the D_L fragment [3] and the second upon conversion of the D_L to the D_Y fragment (fig.6).

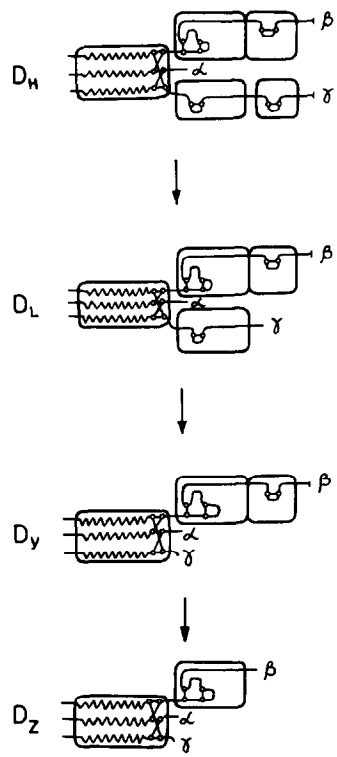

Fig.6. Diagram of the arrangement of calorimetric domains in the D_H (95 kDa), D_L (82 kDa) and D_Y (63 kDa) fragments. α, β, γ – polypeptide chains. (≈≈) Structure which corresponds to the N-terminal thermostable coiled-coil connector according to [1,3].

Thus we conclude that in the fibrinogen molecule the C-terminal parts of the β-chain and of the γ-chain individually form two co-operative domains of the globular type.

It should be noted that recent electron microscopic studies of modified fibrinogen microcrystals have revealed four high protein density regions in each terminal part of fibrinogen molecule [16]. One can suggest that these regions correspond to the co-operative domains described above.

REFERENCES

[1] Doolittle, R.F. (1975) in: The Plasma Proteins (Putnam, F.W. ed.) vol.2, pp.109–161, Academic Press, London.
[2] Medved', L.V., Tiktopulo, E.I., Privalov, P.L. and Varetskaya, T.V. (1980) Mol. Biol. (USSR) 14, 835–842.
[3] Privalov, P.L. and Medved', L.V. (1982) J. Mol. Biol. 159, 665–683.
[4] Williams, R.C. (1981) J. Mol. Biol. 150, 399–408.
[5] Mosesson, M.W., Hainfeld, J., Wall, J. and Haschemeyer, R.H. (1981) J. Mol. Biol. 153, 695–718.
[6] Erickson, H.P. and Fowler, W.E. (1983) Ann. NY Acad. Sci. 408, 146–163.
[7] Medved', L.V., Privalov, P.L. and Ugarova, T.P. (1982) FEBS Lett. 146, 339–342.
[8] Nieuwnhuizen, W. and Haverkate, F. (1983) Ann. NY Acad. Sci. 408, 92–96.
[9] Varetskaya, T.V. (1960) Ukr. Biokhim. Zh. 32, 13–24.
[10] Yphantis, D.A. (1964) Biochemistry 3, 297–317.
[11] Jaenicke, L. (1974) Anal. Biochem. 61, 623–627.
[12] Privalov, P.L. (1980) Pure Appl. Chem. 52, 479–497.
[13] Filimonov, V.V., Potekhin, S.A., Matveev, S.V. and Privalov, P.L. (1982) Mol. Biol. (USSR) 16, 551–562.
[14] Cummins, P. and Perry, S.V. (1973) Biochem. J. 133, 765–777.
[15] Privalov, P.L. (1982) Adv. Protein Chem. 35, 1–104.
[16] Hewat, E.A., Tranqui, L. and Wade, R.H. (1982) J. Mol. Biol. 161, 459–477.