Plasminogen-binding site of the thermostable region of fibrinogen fragment D

T.I. Lezhen, S.A. Kudinov and L.V. Medved'

Institute of Biochemistry, Academy of Sciences of the Ukrainian SSR, Kiev, USSR

Received 24 October 1985; revised version received 20 December 1985

Affinity chromatography of plasminogen and its proteolytic fragments on immobilized fibrinogen TSD fragment has shown that the latter contains a plasminogen-binding site which is complementary to the lysine-binding site(s) of plasminogen molecule 1–3 kringle structures.

Plasminogen *Plasmin* *Plasmin fragment* *Miniplasminogen* *Fibrinogen fragment* *Affinity chromatography*

1. INTRODUCTION

The main biological function of plasmin is the lysis of fibrin clots. Specific binding of the plasmin precursor (plasminogen) and its activator to fibrin is the basis of the molecular mechanism of fibrinolysis. Fibrin considerably stimulates the activation of plasminogen into plasmin as well as protecting the latter from inactivation by inhibitors. At present the localization of plasminogen-binding sites on fibrin is under active investigation [1–5]. Nevertheless, the mechanism of interaction of plasminogen with fibrin remains unclear.

Convenient objects for studying different types of interaction between these proteins are their proteolytic fragments. These fragments retain structural and functional properties which are inherent to the entire molecule. Fibrinogen E fragment, originating from the central part of the molecule, was shown to contain a Lys-plasminogen-binding site. Therefore the central fibrin domain was supposed to be the site of plasminogen binding [2,3]. It has recently been demonstrated that fibrinogen fragment D is also able to bind to Lys-plasminogen [2]. On the other hand, there are some data suggesting that fibrinogen D fragment does not possess plasminogen-binding properties [1,5]. This discrepancy may be accounted for by the non-identical character of the fibrinogen fragment D preparations studied by different authors since it is known that the properties of the D fragment, in particular its anti-coagulation activity, are strongly dependent on the method of preparation [6,7].

Early D_I or D_H fragments (M_r 95 000) are split from the terminal parts of the fibrinogen molecule during restricted proteolysis [7,8]. A further proteolysis of D_I or D_H fragments in the absence of Ca^{2+} results in splitting of the C-terminal part of the γ-chain and formation of the so-called 'light' D fragment (D_L) or D_{EDTA} with an M_r of 82 000 [8,9]. It has been shown that the association constant of the light fragment D_{EDTA} with Lys-plasminogen-Sepharose is about 11-fold higher than that of fragment D_I [4]. Using special conditions it is possible to isolate the thermostable region of the D_L fragment as the TSD fragment with an M_r of 28 000 [10]. This fragment has a compact structure of the coiled-coil type as well as the terminal parts of the E fragment [10,11]. This structure has been shown to be necessary for retaining the plasminogen-binding site on fragment E [4]. Supposing structural homology of the plasminogen-binding sites of fibrinogen fragments D and E we can expect the plasminogen-binding site of the D_L fragment to be situated on its ther-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

mostable part. Therefore we studied the interaction of the TSD fragment of fibrinogen with plasminogen and its proteolytic fragments. The results obtained show that the TSD fragment of fibrinogen contains a plasminogen-binding site which is complementary to the lysine-binding site of plasminogen molecule 1-3 kringle structures.

2. MATERIALS AND METHODS

Human Lys-plasminogen was obtained by the method in [12]. Miniplasminogen, K1-3 and K4 fragments were prepared by elastolysis of Lys-plasminogen as described in [13,14]. Plasmin heavy (A) and light (B) chains were prepared by selective reduction of its interchain disulphide links as in [15]. All these preparations retained their functional activity: Lys-plasminogen and miniplasminogen were able to be activated with streptokinase to plasmin and miniplasmin, respectively, and plasmin light chain in combination with streptokinase was able to form the activation complex [16]. The heavy chain of plasmin as well as the K1-3 and K4 fragments possessed lysine-binding properties. D_H and D_L fragments were prepared by the method described in [8] from bovine fibrinogen isolated according to [17]. The TSD fragment was prepared by pepsin hydrolysis of the D_H fragment [10]. Purity of the obtained preparations was checked by SDS electrophoresis in 7.5% polyacrylamide gel as in [18]. Fig.1 shows that none of the employed preparations contained perceptible impurities.

The TSD fragment as well as the D_L fragment were immobilized on CNBr-activated Sepharose. The concentration of the immobilized protein was determined using three different ways: (i) the method of Lowry et al. [19]; (ii) amino acid analysis of immobilized protein after 24 h hydrolysis in 6 M HCl at 105°C; (iii) calculation of the difference between the amount of immobilized protein and that of unbound protein. The concentration of the immobilized TSD fragment was 20 μM. Immobilized D_L fragment was prepared at the same concentration. The interaction of the immobilized TSD fragment with plasminogen and its derivatives was studied by affinity chromatography. Protein in 100 mM sodium phosphate, pH 7.4, was applied to a column of 5 ml volume. Unbound protein was washed out by the same buffer. Then specifically sorbed protein was eluted with a 3 mM solution of ϵ-aminocaproic acid (ϵ-ACA). All experiments on affinity binding of protein were performed at 20°C.

3. RESULTS AND DISCUSSIONS

Fig.2 presents an elution profile of plasminogen from the immobilized TSD fragment. It is obvious that plasminogen possesses a strong selective affinity for the immobilized TSD fragment which

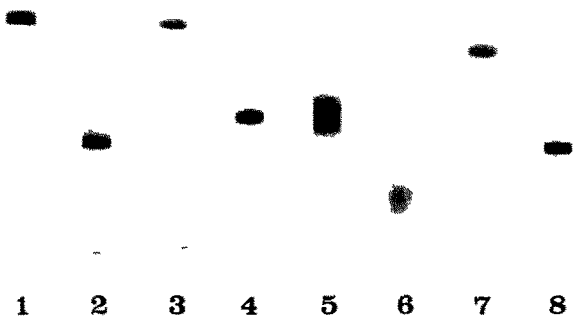

Fig.1. SDS-polyacrylamide gel electrophoretic patterns of fibrinogen D_L (1) and TSD (2) fragments, Lys-plasminogen (3), miniplasminogen (4), plasminogen fragments K1-3 and K4 (5 and 6, respectively) and plasmin heavy (7) and light (8) chains.

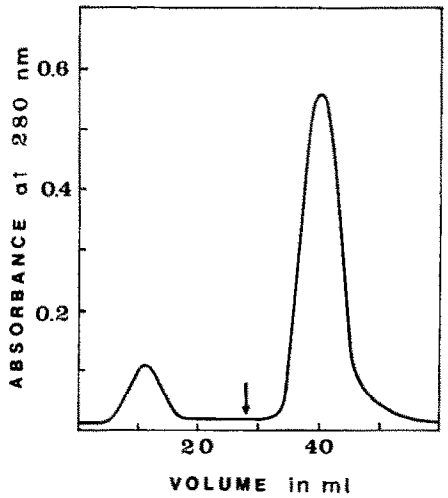

Fig.2. Lys-plasminogen affinity chromatography on immobilized TSD fragment. The arrow indicates elution with 3 mM ϵ-ACA.

demonstrates the presence of plasminogen-binding sites in its structure. The results from the affinity chromatography were quantitatively analyzed. Using immobilized TSD fragment we were able to determine equilibrium concentrations of Lys-plasminogen both bound and unbound with TSD fragment. It was determined that the time necessary to establish equilibrium did not exceed 5 min and thus the protein applied to the column was exposed for about 10 min. Unbound protein was rapidly eluted by 100 mM phosphate buffer to detect the equilibrium concentration of free ligand in the incubation mixture. The concentration of the complex was determined from the amount of protein specifically bound to the affinity column. Specifically bound plasminogen presents a fraction eluted by 3 mM ϵ-ACA. Fig.3a shows that the quantity of bound plasminogen increases with the amount of protein applied to the column and reaches saturation asymptotically approaching a maximal value. This dependence is a typical adsorption isotherm for affinity chromatography [20], which permits presentation of the obtained results on Scatchard coordinates and determination of the dissociation constant and quantity of binding centres for Lys-plasminogen. Fig.3b reveals that the apparent dissociation constant amounts to 0.44 μM and that there is one plasminogen-binding site in each molecule of the TSD fragment. The interaction of the immobilized D_L fragment with Lys-plasminogen was also

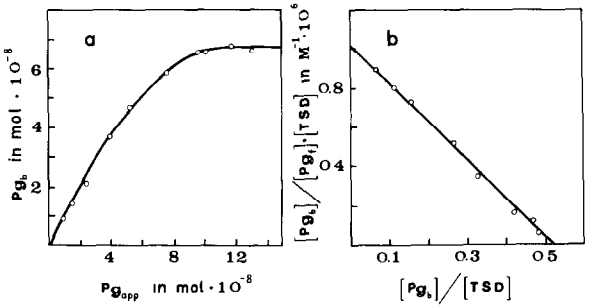

Fig.3.(a) Isotherm of affinity sorption of Lys-plasminogen on the immobilized TSD fragment. Pg_{app} and Pg_b, applied and bound Lys-plasminogen, respectively. (b) Scatchard plot for the specific binding of Lys-plasminogen to TSD fragment. [TSD], $[Pg_b]$ and $[Pg_f]$, the molar concentration of the TSD fragment, specifically bound and free Lys-plasminogen, respectively.

studied using this method. We found that there is one site in the structure of the D_L fragment for binding Lys-plasminogen with a dissociation constant of 0.43 μM which in fact equals the dissociation constant of the complex formed between immobilized TSD fragment and Lys-plasminogen. Thus, it can be concluded that the plasminogen-binding site of D_L fragment is located in its thermostable part. The low and equal values of the dissociation constants provide evidence of the high affinity of the interacting molecules and of preservation of binding site structure during immobilization.

Fig.2 shows that all plasminogen bound to the affinity column was completely eluted by ϵ-ACA. Subsequent elution with 100 mM L-arginine revealed no protein in the eluate. It follows that the immobilized TSD fragment interacts with Lys-plasminogen via the lysine-binding site(s) of the latter. Benzamidine-binding sites of plasminogen, which have been localized in miniplasminogen and the plasmin light chain [21], are apparently excluded from this interaction. Varadi and Patthy [4] have found that the E fragment plasminogen-binding site is located in its coiled-coil region. As fragment D also carries a large region of the coiled-coil connector rod, they supposed that structural homology between plasminogen-binding sites of the E and D fragments was possible. Since the TSD fragment includes a coiled-coil region of the D fragment, our data strongly support their assumption.

To localize on the plasminogen molecule the binding sites complementary to that of the TSD fragment we have studied the sorption of miniplasminogen, K1-3, K4 fragments, and plasmin heavy and light chains on the immobilized TSD fragment. Fig.4a shows that miniplasminogen and plasmin light chain were not bound by the affinity column, because both were completely eluted by 100 mM phosphate buffer. At the same time the plasmin heavy chain consisting of 5 kringle structures (K1-5) is specifically sorbed on the immobilized TSD fragment and completely eluted by ϵ-ACA (fig.4b). Thus one may conclude that the interaction of Lys-plasminogen with the TSD fragment is mediated by lysine-binding sites localized in the heavy chain region. K1-3 and K4 fragments are parts of the plasmin heavy chain and both contain lysine-binding sites [22], however,

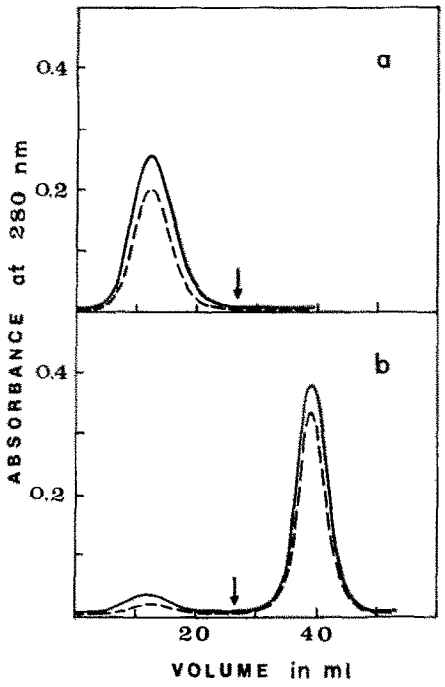

Fig.4. Affinity chromatography of (a) miniplasminogen (—) and plasmin light chain (– –), (b) plasmin heavy chain (—) and K1-3 fragment (– –) on the immobilized TSD fragment of fibrinogen. Elution with 3 mM ϵ-ACA is indicated by the arrow.

K1-3 is bound by the immobilized TSD fragment whereas K4 is not. Therefore, the lysine-binding site(s) of the Lys-plasminogen molecule, which is (are) complementary to the binding site on the TSD fragment, is (are) on its 1-3 kringle structures and differ structurally from the 4 kringle binding site.

ACKNOWLEDGEMENTS

The authors are thankful to Dr V.V. Novokhatny and to S.V. Litvinovitch for help in this work.

REFERENCES

[1] Cederholm-Williams, S.A. and Swain, A. (1979) Thromb. Res. 16, 705-713.
[2] Varadi, A. and Patthy, L. (1983) Biochemistry 22, 2440-2446.
[3] Kudinov, S.A. and Lezhen, T.I. (1984) Biokhimia (USSR) 49, 2003-2007.
[4] Varadi, A. and Patthy, L. (1984) Biochemistry 23, 2108-2112.
[5] Lucas, M.A., Fretto, L.J. and McKee, P.A. (1983) J. Biol. Chem. 258, 4249-4256.
[6] Belitser, V.A., Pozdnjakova, T.M. and Ugarova, T.P. (1980) Thromb. Res. 19, 807-814.
[7] Doolittle, R.F. (1984) Annu. Rev. Biochem. 53, 195-229.
[8] Privalov, P.L. and Medved', L.V. (1982) J. Mol. Biol. 159, 665-683.
[9] Haverkate, F. and Timan, G. (1977) Thromb. Res. 10, 803-812.
[10] Medved', L.V., Ugarova, T.P. and Privalov, P.L. (1982) FEBS Lett. 146, 339-342.
[11] Doolittle, R.F., Goldbaum, D.M. and Doolittle, L.R. (1978) J. Mol. Biol. 120, 311-325.
[12] Deutsch, D.G. and Mertz, E.T. (1970) Science 170, 1095-1096.
[13] Powel, J.R. and Castellino, F.J. (1980) J. Biol. Chem. 255, 5329-5335.
[14] Novokhatny, V.V., Kudinov, S.A. and Privalov, P.L. (1984) J. Mol. Biol. 179, 215-232.
[15] Gonzalez-Gronov, M., Violand, B.N. and Castellino, F.T. (1977) J. Biol. Chem. 252, 2175-2177.
[16] Summaria, L. and Robbins, K.C. (1976) J. Biol. Chem. 251, 5810-5813.
[17] Varetskaja, T.V. (1960) Ukr. Biokhim. Zh. 32, 13-24.
[18] Cummins, P. and Perry, S.V. (1973) Biochem. J. 133, 765-777.
[19] Lowry, O.H., Rosenbrough, N.J., Farr, A.Z. and Randall, R. (1951) J. Biol. Chem. 193, 265-275.
[20] Graves, D.I. and Wu, Y. (1974) Methods Enzymol. 34, 140-163.
[21] Varadi, A. and Patthy, L. (1981) Biochem. Biophys. Res. Commun. 103, 97-102.
[22] Winn, E.S., Hu, S.-P., Hochschwender, S.M. and Laursen, R.A. (1980) Eur. J. Biochem. 104, 579-586.