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CROSS-LINKING OF α_2-PLASMIN INHIBITOR AND FIBRONECTIN TO FIBRIN BY FIBRIN-STABILIZING FACTOR

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Two plasma proteins, α_2-plasmin inhibitor and plasma fibronectin, are cross-linked to fibrin by plasma transglutaminase (R-glutaminyl-peptide : amine γ-glutamyl-yltransferase, EC 2.3.2.13, fibrin stabilizing factor) when blood coagulation takes place. The cross-linking reactions of these proteins were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) using these radioactively labeled proteins. Both proteins were cross-linked exclusively to the α-chain of fibrin, and each of these cross-linking reactions proceeded independently without being influenced by the other cross-linking reaction. The cross-linking of fibronectin to the α-chain proceeded steadily at a rate similar to that of the cross-linked polymerization of the α-chain. In contrast, the cross-linking reaction of α_2-plasmin inhibitor to fibrin proceeded markedly faster than that of fibrin polymerization but did not proceed further after reaching a certain relatively low level of cross-linking. Most of the cross-linked α_2-plasmin inhibitor molecules at this stage of the fibrin cross-linking process were in the form of complex with the α-chain monomer. The complex with the α-chain monomer was gradually transformed to a complex with the α-chain polymer as the cross-linking polymerization of the α-chain proceeded. The rate of the transformation was the same as that for the disappearance of the α-chain monomer, indicating that whether the α-chain was cross-linked to α_2-plasmin inhibitor or not, the α-chain underwent cross-linking polymerization at the same rate.

Introduction

α_2-Proteinase inhibitor or α_2-plasmin inhibitor (α_2PI) is a primary inhibitor of plasmin-catalyzed fibrinolysis [1,2]. Its congenital deficiency results in a life-long hemorrhagic tendency [3]. α_2PI exerts its inhibitory activity in three ways [2,4]: (1) rapid inactivation of plasmin; (2) interference with adsorption of plasminogen to fibrin; and (3) by cross-linking to fibrin. Fibrin cross-linked to α_2PI is less susceptible to plasmin-catalyzed fibrinolysis than fibrin not cross-linked to α_2PI [4]. The cross-linking of α_2PI to fibrin is catalyzed by the activated fibrin-stabilizing factor (activated FSF, fibrinoligase, blood coagulation Factor XIIIa, plasma transglutaminase), and the amount of α_2PI cross-linked to fibrin is dependent on the concentrations of activated FSF (FSFa), fibrin and α_2PI [4].

Another plasma component called plasma fibronectin or cold insoluble globulin is also cross-linked to fibrin by FSFa when blood is clotted [5]. FSFa was originally discovered as an enzyme responsible for fibrin-to-fibrin cross-linking at a final stage of blood coagulation [6].

The mechanism of FSFa-catalyzed cross-linking is the formation of intermolecular γ-glutamyl-ε-lysine bridges [6]. Therefore, when blood is clotted, the

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γ-glutamyl-ε-lysine bonds are formed intermolecularly to link fibrin to fibrin, fibrin to fibronectin, and fibrin to α_2PI.

The present studies were undertaken to elaborate further on the processes of α_2PI cross-linking to fibrin and fibronectin cross-linking to fibrin in order to determine the interrelationship between these reactions.

Materials and Methods

Proteins. α_2PI was purified from human plasma by the method described previously [1]. The concentration of α_2PI was determined by single radial immunodiffusion using monospecific antiserum to α_2PI and a purified α_2PI preparation with a known concentration as a standard [7]. Plasma fibronectin was purified from human plasma according to the method described by Vuento and Vaheri [8]. The concentration of fibronectin was determined by single radial immunodiffusion using monospecific antiserum to fibronectin and a purified fibronectin with a known concentration as a standard [9]. Human fraction I-4, prepared by the method of Blombäck and Blombäck [10], was used as fibrinogen preparation after removing contaminating plasminogen from the preparation using lysine-Sepharose [11]. The FSF concentration in the solution of fraction I-4 (250 mg protein/100 ml) was 0.25 U/ml as assayed by an antibody neutralization method [12] using the clotting factor XIII-test kit supplied by Behringwerke AG, Marburg, F.R.G. 1 unit of FSF is defined as the amount of FSF present in 1 ml normal pooled standard plasma. Fibronectin was not detected in the fraction I-4 preparation by immunochemical methods and gel electrophoresis. Purified FSF was prepared from human plasma by the method described previously [13]. The concentration of purified FSF was determined by measuring the absorbance of the FSF solution at 280 nm and converting absorbance to protein concentration using $E^{1\%}_{1\,cm}$ = 13.8 for pure FSF [14]. The specific activity of the purified FSF was 38 U/mg protein. All these protein preparations (α_2PI, fibronectin, fibrinogen and FSF) were homogeneous as judged by SDS-polyacrylamide gel electrophoresis [15] (Fig. 1). Purified thrombin was prepared from a bovine thrombin preparation (Parke, Davis and Co., Detroit, MI) according to the method

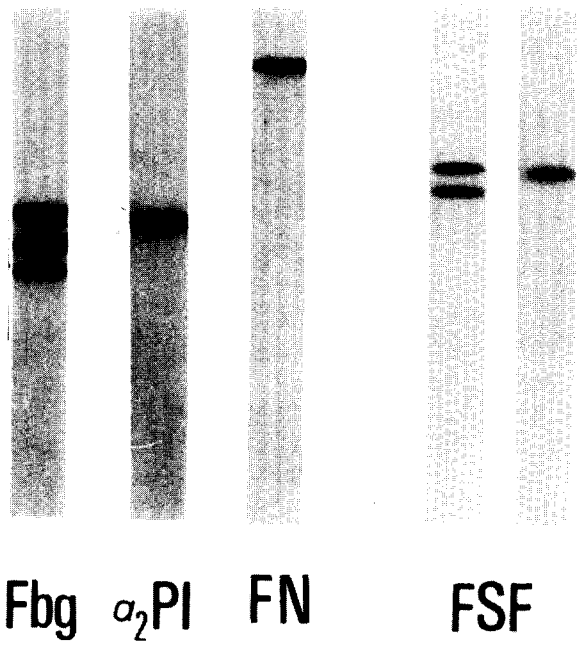

Fig. 1. SDS-polyacrylamide gel electrophoresis of fibrinogen (Fbg), α_2PI, fibronectin (FN) and FSF used in the experiments. Fibrinogen (10 μg), α_2PI (7 μg), fibronectin (6 μg) and FSF (5 μg) were applied after reduction with 10% mercaptoethanol on 5% polyacrylamide gel. One sample of FSF was applied without reduction (the left column) and the result shows two characteristic chains, A and B chains. Three chains of fibrinogen (Aα, Bβ and γ) are distinctly seen without any appreciable degradation. Electrophoresis was run for 3 h with 7 mA/gel.

of Lundblad [16], and was stored as a 600 U/ml solution in 50% glycerol at −20°C.

Radioiodination of proteins. Purified proteins (α_2PI and fibronectin) were radioiodinated by the solid-state lactoperoxidase method [17] using lactoperoxidase (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) and ^{125}I-Na (17 Ci/mg) (New England Nuclear, Boston, MA). The labeled α_2PI preparation had a specific radioactivity of $6.8 \cdot 10^5$ cpm/μg. The labeled fibronectin preparation had a specific radioactivity of $5.05 \cdot 10^5$ cpm/μg. These labeled preparations were diluted with unlabeled proteins before use to obtain a concentration of $5–50 \cdot 10^3$ cpm/μg. The use of preparations with various specific radioactivities differing by 5–10-fold did not produce different results in the cross-linking experiments, indicating that iodination of these proteins did

not significantly alter their capabilities of cross-linking. Radioactivity was counted by the Auto-well gamma system, Aloka ARC-451 (Aloka Co., Tokyo).

Measurement of the cross-linking to fibrin. Fibrinogen, α_2PI and/or fibronectin were clotted and cross-linked at 37°C by thrombin, FSF, CaCl, in Tris-buffered saline (0.05 M Tris-HCl/0.15 M NaCl, pH 7.4) in a counting vial. Radioactively labeled α_2PI or fibronectin was used for estimating the extent of cross-linking of α_2PI or fibronectin, respectively. The volume of each sample was 0.1 ml. After allowing the cross-linking reaction to proceed for various lengths of time, the clotted samples were quickly frozen with dry ice/acetone to stop the reaction. The samples were then lyophilized and to the lyophilized fibrin film formed was added 0.1 ml of 50 mM EDTA containing 1% bovine serum albumin (Miles Lab. Inc., Elkhart, IN). Albumin was used effectively to remove nonspecific binding of α_2PI to fibrin, which amounted to approx. 5% of the total α_2PI when albumin was omitted. After counting the total radioactivity, the fibrin film was washed three times with 0.5-ml aliquots of Tris-buffered saline containing 2 mM EDTA. The fibrin film was subsequently solubilized by incubation for 10 min at 100°C with 0.1 ml of 10 M urea/2% SDS/10% mercaptoethanol. The solubilized sample was counted for radioactivity. The amount of α_2PI cross-linked to fibrin was calculated from the radioactivity remaining in the washed and solubilized fibrin, and was expressed as a percentage of the original total radioactivity. After counting, the sample was mixed with 25 μl of 50% glycerol. 10 μl mixture were then analyzed by SDS-polyacrylamide gel electrophoresis on 3.5% polyacrylamide gel according to the method of Weber and Osborn [15]. Gels were stained with Coomassie brilliant blue and destained by diffusion [15]. Densitometry was performed with a Gilford Spectrophotometer 2400-2. The progression of cross-linking of the α-chain of fibrin or the disappearance rate of the α-chain monomer was estimated by measuring the areas of the recorder traces. After staining, the gel was cut into 1-mm thick slices using a gel slicer (Model HP100 from Hotta Rika, Tokyo). Each of the gel slices was counted for radioactivity. The extent of cross-linking of α_2PI or fibronectin was determined by the counts of the gel slices containing cross-linked protein bands and expressed as percent of the total count.

Testing for cross-linking reactions between α_2PI or fibronectin molecules or between α_2PI and fibronectin. α_2PI or a mixture of α_2PI and fibronectin, FSF, $CaCl_2$ (5 mM) and thrombin (2 U/ml) were mixed and incubated at 37°C for 2 h in Tris-buffered saline in the presence or absence of reduced glutathione (10 or 20 mM). The α_2PI preparation and the fibronectin preparation used contained radioactively labeled α_2PI or fibronectin, respectively. Protein concentrations used in the incubation mixtures were 60 and 600 μg/ml of α_2PI, 0.3 and 3 mg/ml of fibronectin, and 29 and 290 μg/ml of FSF. Various combinations of protein concentrations were used in the incubation mixtures. After the incubation, the sample was mixed with an equal volume of 2% SDS/5% mercaptoethanol, and heated for 10 min at 100°C. 10 μl mixture were then analyzed by SDS-polyacrylamide (3.5%) gel electrophoresis. After the electrophoresis, the gels were stained, sliced, and counted for radioactivity as described in the preceding section.

Results

α_2PI containing radioactively labeled α_2PI, fibronectin containing radioactively labeled fibronectin or a mixture of the α_2PI and fibronectin was incubated with FSFa in various combinations of protein concentrations as described in Materials and Methods, and analyzed by SDS-polyacrylamide gel electrophoresis to determine if there is any cross-linking of α_2PI itself, fibronectin itself or cross-linking between α_2PI and fibronectin. There was no visible band in the gels corresponding to polymerized α_2PI, polymerized fibronectin or an α_2PI-fibronectin complex. Furthermore, there was only a single peak of radioactivity corresponding to the α_2PI monomer or fibronectin monomer and no other peak, indicating that neither self cross-linking between α_2PI or fibronectin molecules themselves, nor between α_2PI and fibronectin occurred.

The rates and extents of cross-linked polymerization of fibrin α-chain and the cross-linking of α_2PI or fibronectin to fibrin were estimated by clotting fibrinogen with thrombin in the presence of FSF, Ca^{2+}, α_2PI and/or fibronectin. The concentrations of the proteins were all normal values found in plasma.

As shown in Fig. 2, the rate and extent of cross-

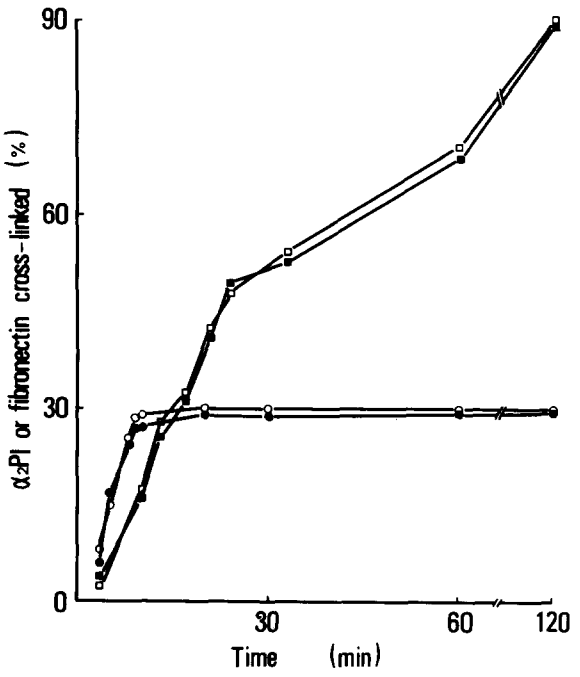

Fig. 2. Time course of the cross-linking reactions of α_2PI and fibronectin to fibrin at 37°C. Fibrinogen (2.5 mg/ml) was clotted by thrombin (2.5 u/ml), Ca^{2+} (5 mM) and α_2PI (60 μg/ml) and/or fibronectin (275 μg/ml). α_2PI and fibronectin preparations contained the respective radioiodinated proteins. FSF contained in fibrinogen preparation was used as a source of the enzyme and its final concentration in the clot was 0.25 u/ml. The amount of α_2PI or fibronectin cross-linked to fibrin is expressed as percent of the total amount of α_2PI or fibronectin present, respectively. α_2PI cross-linking in the absence (○) or the presence (●) of fibronectin. Fibronectin cross-linking in the absence (□) and the presence (■) of α_2PI.

linking of α_2PI to fibrin were not influenced by the presence of fibronectin or vice versa. Even when the concentration of one of these proteins was increased to twice as high as the average concentration in normal plasma, the rate and the extent of cross-linking of the other protein to fibrin were not significantly changed.

Cross-linking of fibronectin increased steadily upon incubation while cross-linking of α_2PI rapidly reached its maximum level and plateaued (Fig. 2). When the concentration of FSF in the reaction mixture was increased by an addition of purified FSF, the cross-linking reaction of α_2PI to fibrin was accelerated but its maximum level at which the reaction plateaued stayed the same unless the concentrations of α_2PI and fibrinogen were changed.

The ratio between the extent of α-chain polymerization and the extent of fibronectin cross-linking to fibrin was nearly constant up to about 50% cross-linking of the α-chain (Fig. 3). The ratio was about 1 : 1.4, indicating that the extent of fibronectin cross-linking continues to be about 40% higher than that of the α-chain during the initial half of the whole process of cross-linking. During the latter half of the cross-linking process, the cross-linking of fibronectin was slowing down and its extent became nearly the same as that of the α-chain: at 120 min incubation, 90% of α-chain and 90% of fibronectin were cross-linked.

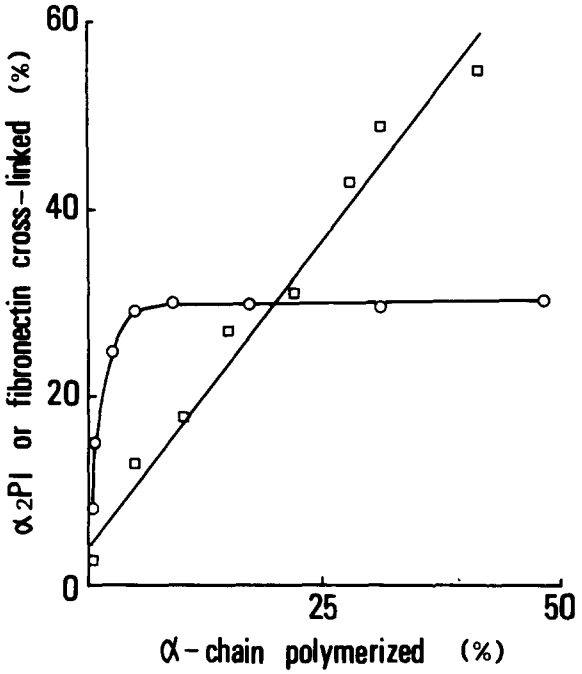

Fig. 3. Relationship between the amount of polymerized (cross-linked) α-chain of fibrin and the amount of α_2PI (○) or fibronectin (□) cross-linked to fibrin. Fibrinogen was clotted by thrombin in the presence of FSF, Ca^{2+}, and α_2PI or fibronectin. The experimental conditions were the same as in Fig. 1. Various degrees of cross-linking were obtained by stopping the reaction after incubation of the clotting mixture for various times. The amount of polymerized α-chain is expressed as percent of the total (monomeric and polymerized) α-chain. The amount of α_2PI or fibronectin cross-linked to fibrin is expressed as percent of the total amount of α_2PI or fibronectin present, respectively.

On the contrary, cross-linking of α_2PI to fibrin rapidly reached its maximum level and the extent of cross-linking thereafter stayed at the same level regardless of the continued process of the α-chain polymerization (Fig. 2).

Although the total amount of α_2PI cross-linked to the α-chain did not change after the initial rapid increase to the plateau level, the relative proportion of α_2PI cross-linked to the α-chain monomer decreased steadily with a reciprocal increase of α_2PI cross-linked to the α-chain polymers (Fig. 4). The decrease of α_2PI cross-linked to the α-chain monomer is fairly parallel to the decrease of the α-chain monomer (Fig. 4). After prolonged incubation, the α_2PI cross-linked to fibrin became totally cross-linked to the highly polymerized α-chain, and no band of radioactivity was found on the SDS-polyacrylamide gel corresponding to the molecular weight of α_2PI-γ-chain complex or α_2PI-γ-chain-dimer complex, indicating no cross-link formation between α_2PI and the γ-chain of fibrin.

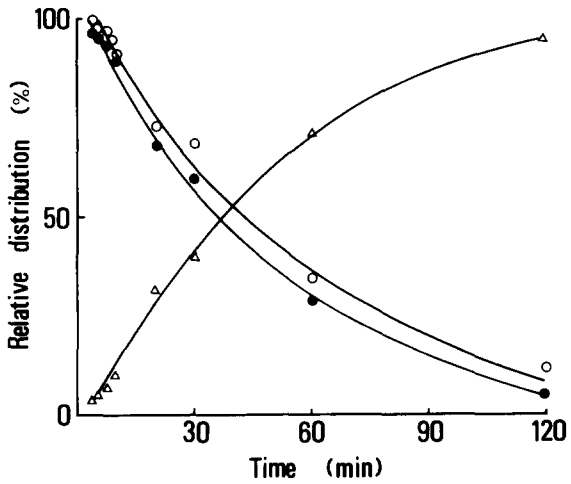

Fig. 4. Time-dependent shift from monomeric α-chain-α_2PI complex (α-α_2PI) to polymerized α-chain-α_2PI complex (nα-α_2PI), and time course of disappearance of monomeric α-chain. Fibrinogen was clotted by thrombin in the presence of FSF, Ca^{2+} and α_2PI. The experimental conditions were the same as in Fig. 1. The relative amounts of α-α_2PI (○) or nα-α_2PI (△) are expressed as percent of the total cross-linked α_2PI. The relative amounts of α-chain monomer (●) are expressed as percent of the whole fibrin clot protein.

Discussion

When blood is clotted, two plasma proteins are covalently cross-linked to the formed fibrin by FSFa which has been activated during the clotting. These plasma proteins are plasma fibronectin [5] and α_2PI [4].

The present studies indicate that these two proteins are independently cross-linked to fibrin without affecting the cross-linking reaction of the other (Fig. 2), and that the cross-linking reactions of these proteins are different from each other in several ways. Fibronectin can be cross-linked to itself by FSFa in the presence of a reducing agent [5], but α_2PI was not cross-linked to itself by FSFa even in the presence of a reducing agent in the present study. Furthermore, no cross-linking between α_2PI and fibronectin was observed with FSFa and a reducing agent. The facts suggest that FSFa shows some differences in substrate specificities for α_2PI and fibronectin.

FSFa catalyzes the acyl transfer reaction where the γ-carboxamide group of the glutamine residue is the acyl donor and the ϵ-amino group of lysine residue serves as the acyl acceptor [6]. This reaction proceeds through the formation of acyl enzyme intermediates and subsequent transfer of acyl groups to the acceptor amine. Probably, fibronectin has both glutamine and lysine residues which are involved in the FSFa-catalyzed acyl transfer reaction, since fibronectin molecules can interact with one another to form cross-linked multimers in the presence of a reducing agent [5]. In contrast, α_2PI molecules did not interact with one another to form cross-linked dimers or multimers even in the presence of a reducing agent in the present study. Since dansylcadaverine, a fluorescent primary amine, can be incorporated into α_2PI by FSFa [4], α_2PI must have one or more glutamine residues which can serve as acyl donors for FSFa. However, α_2PI is not susceptible to self cross-linking by the enzyme because it may not have accessible lysine residues to accept the acyl groups.

The absence of cross-link formation between α_2PI and fibronectin may be explained by a difference in specificities of the acyl enzyme intermediates (α_2PI-FSFa and fibronectin-FSFa) for the amine substrate.

A remarkable difference between the α_2PI and the fibronectin cross-linking reactions to fibrin is that cross-linking of α_2PI rapidly reaches its maximal level

and then plateaus, whereas the cross-linking of fibronectin steadily progresses along with the progression of the cross-linked polymerization of the fibrin α-chain (Fig. 3). Since there was no cross-link formed between fibronectin molecules themselves in the present study in which no reducing agent was used, the progressive increase in cross-linked fibronectin must be due to cross-linking between fibronectin and fibrin. Furthermore, it was previously shown that in the presence of fibrin, fibronectin was cross-linked only to fibrin and the formation of fibronectin multimers did not occur [5].

The extent of cross-linking of fibronectin to fibrin has a direct linear correlation with the extent of cross-linked polymerization of the α-chain, indicating that the cross-linking of fibronectin to fibrin and of fibrin to fibrin proceed at parallel and similar rates (Fig. 3). On the other hand, the cross-linking reaction of α_2PI to fibrin proceeds faster than that of fibrin to fibrin but ceases soon at a relatively low level, showing no fixed correlation between these reactions (Fig. 3).

The maximum plateau level of α_2PI cross-linked to fibrin was approx. 30% of the total α_2PI present, and no further progression of α_2PI cross-linking occurred regardless of the continued progression of the α-chain polymerization (Fig. 2). An addition of FSF only accelerated the reaction and did not significantly change the percentage of α_2PI cross-linked as long as the fibrinogen concentration was more than approx. 2 mg/ml. The percentage of α_2PI cross-linked decreases when fibrinogen concentration was lower than 2 mg/ml, as shown previously [4].

Since the concentrations of fibrinogen, α_2PI and fibronectin used in the present experiments were 2.5 mg/ml (7.4 μM), 60 μg/ml (0.9 μM) and 275 μg/ml (0.63 μM), respectively, it was roughly calculated that only one out of 25 molecules of fibrin was cross-linked to α_2PI, whereas one out of 12 fibrin molecules was cross-linked to fibronectin under physiological conditions.

Most of the α_2PI cross-linked to the fibrin α-chain was in a form of the α-chain monomer-α_2PI complex at the initial stage, but the α-chain monomer-α_2PI complex was gradually transformed to the α-chain polymer-α_2PI complex as the α-chain cross-linking polymerization proceeded (Fig. 4). The decrease of the α-chain monomer-α_2PI complex, which was in an inverse relationship with the increase of the α-chain polymer-α_2PI complex, was parallel to the decrease of the α-chain monomer (Fig. 4). These findings suggest that the α-chain cross-linked to α_2PI and the α-chain without cross-linking to α_2PI are polymerized at the same rate.

α_2PI does not seem to be cross-linked to the γ-chain of fibrin, and seems to be exclusively cross-linked to the α-chain which is most susceptible to plasmin action [18]. This fact together with rapid completion of the cross-linking reaction and the limited and relatively low extent of the cross-linking may imply that the cross-linking of α_2PI to fibrin endows fibrin clots with the only initial resistance to plasmin-catalyzed fibrinolysis.

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References

1 Moroi, M. and Aoki, N. (1976) J. Biol. Chem. 251, 5956–5965
2 Aoki, N. (1979) Prog. Cardiovasc. Dis. 21, 267–286
3 Aoki, N., Saito, H., Kamiya, T., Koie, K., Sakata, Y. and Kobakura, M. (1979) J. Clin. Invest. 63, 877–844
4 Sakata, Y. and Aoki, N. (1980) J. Clin. Invest. 65, 290–297
5 Mosher, D.F. (1975) J. Biol. Chem. 250, 6614–6621
6 Folk, J.E. and Finlayson, J.S. (1977) Adv. Protein Chem. 31, 1–133
7 Aoki, N. and Yamanaka, T. (1978) Clin. Chim. Acta 84, 99–105
8 Vuento, M. and Vaheri, A. (1979) Biochem. J. 183, 331–337
9 Matsuda, M., Yoshida, N., Aoki, N. and Wakabayashi, K. (1978) Ann. N.Y. Acad. Sci. 312, 74–92
10 Blombäck, B. and Blombäck, M. (1956) Ark. Kemi. 10, 415–443
11 Matsuda, M., Iwanaga, S. and Nakamura, S. (1972) Thromb. Res. 1, 619–630
12 Bohn, H. and Haupt, J. (1968) Thromb. Diath. Haemorrh. 19, 309–315
13 Curtis, C.G. and Lorand, L. (1976) Methods Enzymol. 45, 177–191

14 Schwartz, M.L., Pizzo, S.V., Hill, R.L. and McKee, P.A. (1973) J. Biol. Chem. 248, 1395–1407
15 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
16 Lundblad, R.L. (1971) Biochemistry 10, 2501–2505
17 David, G.S. (1972) Biochem. Biophys. Res. Commun. 48, 464–471
18 Pizzo, S.V., Schwartz, M.L., Hill, R.L. and McKee, P.A. (1973) J. Biol. Chem. 248, 4574–4583