Binding of Thrombin to Thrombomodulin Accelerates Inhibition of the Enzyme by Antithrombin III. Evidence for a Heparin-Independent Mechanism†

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ABSTRACT: The endothelial cell surface provides a receptor for thrombin-designated thrombomodulin (TM) which regulates thrombin formation and the activity of the enzyme at the vessel wall surface by serving as a potent cofactor for the activation of protein C by thrombin. Heparin-like structures of the vessel wall have been proposed as another regulatory mechanism catalyzing the inhibition of thrombin by antithrombin III. In the present study, the interaction of antithrombin III with the thrombin–TM complex and its interference with heparin and polycations were investigated by using human components and TM isolated from the microvasculature of rabbit lung. Purified TM bound thrombin and acted as a cofactor for protein C activation. The addition of heparin (0.5 unit/mL) to the reaction mixture interfered neither with the binding of thrombin to TM nor with the activation of protein C. However, the polycations protamine (1 unit/mL) as well as polybrene (0.1 mg/mL) affected the thrombin–TM interaction. This was documented by an increase in the Michaelis constant from 8.3 μM for thrombin alone to 19.5 μM for thrombin–TM with the chromogenic substrate compound S-2238 in the presence of 1 unit/mL protamine. When the inhibition of thrombin by antithrombin III was determined, the second-order rate constant $k_2 = 8.4 \times 10^3$ M^{-1} s^{-1} increased about 8-fold in the presence of TM, implying an accelerative function of TM in this reaction. Although purified TM did not bind to antithrombin III–Sepharose, suggesting the absence of heparin-like structures within the receptor molecule, protamine reversed the accelerative effect of TM in the inhibition reaction. Furthermore, TM was insensitive to heparinase, heparitinase, or periodic acid treatment, indicating that the accelerative effect of TM was not mediated by heparin-like structures of TM. In addition, the presence of TM neither led to the inhibition of thrombin by heparin cofactor II nor did TM accelerate the inhibition of factor Xa by antithrombin III. However, reduction and carboxymethylation of TM, which destroyed its known cofactor activities, also abrogated the accelerative effect of TM in the thrombin–antithrombin III reaction. Thus, the function of TM to alter the specificity of thrombin for macromolecular substrates is not limited to the activation of protein C and to the inhibition of thrombin's procoagulant activity but also includes the increased sensitivity of the enzyme for inactivation by antithrombin III. Although all the activities of the thrombin–TM complex were equally affected by polycations, the interaction between enzyme and receptor was not mediated by heparin-like structures.

The generation of thrombin and its enzymatic activity is balanced by several regulatory mechanisms at the blood endothelium interphase. Procoagulant responses such as release of von Willebrand factor (Levine et al., 1982) and adenine nucleotides (Pearson & Gordon, 1979) as well as anticoagulant responses such as release of prostacyclin (Weksler et al., 1978) and plasminogen activator (Levin et al., 1984) are provoked by the interaction of thrombin with the vessel wall. High- and low-affinity binding sites for the enzyme on endothelial cells have been characterized (Lollar et al., 1980; Owen, 1984) which either involve the active site of thrombin or are active site independent. Cell-bound proteoglycans have been implicated to mediate in part the binding of thrombin (Hatton et al., 1980; Bauer et al., 1983; Shimada & Ozawa, 1985), but 50–60% of thrombin binding to endothelial cells is facilitated by the high-affinity (K_D = 0.5 nM) cell-surface receptor thrombomodulin (TM)[1] (Esmon & Owen, 1981; Maruyama & Majerus, 1985). This receptor is present on the endothelium of veins, arteries, and capillaries (Maruyama et al., 1985; DeBault et al., 1986) but is absent in the microvasculature of the brain (Ishii et al., 1985). TM has been isolated from rabbit (Esmon, N. L., et al., 1982), human (Salem et al., 1984; Kurosawa & Aoki, 1985), and bovine (Jakubowski et al., 1986; Suzuki et al., 1986) origin. The binding of thrombin to TM is associated with the change in specificity of the enzyme for macromolecular substrates and is expressed by the inability to activate platelets (Esmon et al., 1983), to induce fibrin formation, or to activate factor V (Esmon, C. T., et al., 1982). In contrast, the generated thrombin–TM complex very efficiently activates the vitamin K dependent proenzyme protein C (Owen & Esmon, 1982). Activated protein C selectively inactivates the coagulation factors Va and VIIIa (Walker et al., 1979; Fulcher et al., 1984) and thereby expresses potent anticoagulant functions. Thus, the formation of thrombin is regulated by the interaction of the enzyme with its endothelial cell receptor TM.

An additional event which may occur at the endothelial cell site is the accelerated inhibition of cell-bound thrombin by antithrombin III due to heparin-like structures of the vessel wall (Busch & Owen, 1982; Marcum et al., 1984; Marcum & Rosenberg, 1985; Stern et al., 1985). Experiments in a recirculating Langendorff heart preparation (Lollar et al.,

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[1] Abbreviations: AT III, antithrombin III; EDTA, ethylenediaminetetraacetic acid; *i*-Pr_2PF, diisopropyl phosphorofluoridate; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TM, thrombomodulin; Tris, tris(hydroxymethyl)aminomethane.

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1985) have shown, however, that binding of thrombin to the high-affinity sites on the endothelium had no accelerative effect on the reaction of the enzyme with antithrombin III.

This prompted us to investigate the effect of the receptor TM on the inhibition of thrombin by antithrombin III in a purified system. In addition, the influence of heparin and the polycations protamine and polybrene on the functional activity of the thrombin–TM complex was explored.

Experimental Procedures

Materials. DEAE-Sephadex A-50, SP-Sephadex G-50, Sephadex G-25, Sephacryl S-200, and BrCN-activated Sepharose were products from Pharmacia Fine Chemicals, Freiburg, West Germany. DEAE-Trisacryl M was obtained from LKB-Produkter AB, Bromma, Sweden. Reagents for electrophoresis and standard proteins (molecular weights in parentheses), β-galactosidase (120000), phosphorylase *b* (94000), bovine serum albumin (68000), and ovalbumin (43000), were purchased from Bio-Rad Laboratories, Munich, West Germany. Nitrocellulose was from Schleicher & Schuell, Dassel, West Germany. *Flavobacterium* heparinase and heparitinase, Russell's viper venom, *Echis carinatus* venom, bovine and human serum albumin, diisopropyl phosphorofluoridate (*i*-Pr_2PF), phenylmethanesulfonyl fluoride (PMSF), heparin sodium salt from porcine intestinal mucosa, Lubrol PX, protamine sulfate from salmon, and polybrene were obtained from Sigma Chemical Co., Munich, West Germany, and aprotinin was from Bayer, Leverkusen, West Germany. Human fibrinogen and the chromogenic substrate compounds benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide (S-2222), D-Phe-piperolyl-Arg-*p*-nitroanilide (S-2238), and D-Val-Leu-Arg-*p*-nitroanilide (S-2266) were from Kabi AB, Stockholm, Sweden. $Na^{125}I$ (carrier-free) was obtained from New England Nuclear, Dreieich, West Germany. All chemicals used were analytical grade.

Purification of Rabbit Lung Thrombomodulin. The initial steps in the purification of rabbit lung thrombomodulin (TM) were carried out according to Esmon, N. L., et al. (1982). Subsequent to the second gradient elution on *i*-Pr_2PF–thrombin–Sepharose, two additional purification steps were included: The post *i*-Pr_2PF–thrombin–Sepharose pool of TM was dialyzed against 20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, 0.2 mM EDTA, and 0.5% (w/v) Lubrol PX and applied with a flow rate of 30 mL/h on a DEAE-Trisacryl M column (1.5 × 10 cm) equilibrated in the same buffer. TM was eluted with a 2 × 75 mL linear NaCl concentration gradient, 1.2-mL fractions being collected. After dialysis, the TM pool was finally applied on an antithrombin III–Sepharose column (2.5 × 15 cm), equilibrated in 20 mM Tris-HCl, 1 mM $CaCl_2$, and 0.5% (w/v) Lubrol PX. TM was collected in 2-mL fractions in the break-through effluent. Retained material was eluted from the column by increasing the NaCl concentration to 0.6 M. The homogeneity of the final product was demonstrated by SDS–polyacrylamide electrophoresis in a 10% slab gel using the buffer system of Laemmli (1970). In addition, purified TM and the total lung homogenate were transferred from the polyacrylamide gel to nitrocellulose by electroblotting, using the buffer system of Towbin et al. (1979). Following several washes with 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM NaCl, 0.5% (w/v) bovine serum albumin, and 0.1% (w/v) Tween 20, the nitrocellulose was incubated with radiolabeled thrombin (0.3 μCi/mL) for 2 h. After several washes with the same buffer, the matrix was dried, and the distribution of ^{125}I-labeled thrombin was detected by autoradiography.

Monitoring of Thrombomodulin Activity. TM was monitored in column fractions or after elution from polyacrylamide gels with detergent-containing buffer by its ability to function as cofactor for thrombin-catalyzed protein C activation in the presence of calcium ions (Owen & Esmon, 1981) as well as to inhibit thrombin-induced fibrin formation (Esmon, C. T., et al., 1982): (a) A 0.75 μM sample of purified human protein C (see below) was incubated at 37 °C for 15 min with 40 nM thrombin and samples of TM in a total volume of 110 μL containing 20 mM Tris-HCl buffer, pH 7.4, 120 mM NaCl, 3.5 mM $CaCl_2$, 0.5% (w/v) Lubrol PX, and 2.5 mg/mL human serum albumin. Protein C activation was stopped by adjusting the reaction mixture to 50 units/mL hirudin (Pentapharm, Basel, Switzerland). The amount of activated protein C formed was analyzed by diluting the reaction mixture with 20 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, and 0.5 mg/mL bovine serum albumin in a total volume of 700 μL containing 0.275 mM compound S-2266. After incubation at 37 °C for 3 min, the reaction was stopped by the addition of 200 μL of 50% (v/v) acetic acid, and the amount of released *p*-nitroaniline was read at 405 nm in a Uvikon 810 spectrophotometer (Kontron, Munich, West Germany) against a blank, which contained all reagents except TM. (b) Fifty-microliter samples of TM were preincubated with 65 nM thrombin at 37 °C for 2 min in a total volume of 100 μL containing 20 mM Tris-HCl buffer, pH 7.4, 120 mM NaCl, 10 mM $CaCl_2$, and 0.25% (w/v) Lubrol PX and subsequently added to 300 μL of a 1:2 dilution of citrated normal rabbit plasma, prewarmed at 37 °C. Clotting times were recorded in a KC 10 automatic coagulometer (Amelung, Lemgo, West Germany). Concentrations of proteins in the presence of detergent were determined by using the bicinchoninic acid reagent assay (Pierce Chemical Co., Rockford, IL).

Purification of Human Protein C. Protein C was isolated from fresh frozen plasma which had been substituted with the following inhibitors: benzamidine hydrochloride (20 mM), *i*-Pr_2PF (1 mM), PMSF (1 mM), soybean trypsin inhibitor (50 mg/L), and aprotinin (10^6 units/L). After barium citrate adsorption (Stenflo, 1976) and elution, protein C was purified according to Bertina et al. (1982) with the following modifications: After chromatography on DEAE-Sephadex A-50, the first half of the protein C peak which was essentially free of prothrombin was concentrated and applied at a flow rate of 18 mL/h on a Sephacryl S-200 column (2.5 × 120 cm) equilibrated in 20 mM Tris-HCl buffer, pH 7.5, containing 1 M NaCl and 5 mM benzamidine hydrochloride, 2-mL fractions being collected. The effluent was monitored immunochemically for protein C, and protein C activity was analyzed in column fractions by a functional assay described elsewhere (Thiel et al., 1986). Following dialysis against 10 mM triethanolamine hydrochloride buffer, pH 6.4, containing 10 mM benzamidine hydrochloride and 3 mM $CaCl_2$, the protein C pool was applied on a heparin–Sepharose column (Pharmacia) (1.5 × 40 cm) and eluted at a flow rate of 25 mL/h with a 2 × 300 mL linear NaCl concentration gradient in the same buffer, 2-mL fractions being collected. Trace amounts of factor X were removed by passage through an anti-(human factor X)–Sepharose column. The final product was homogeneous as judged by SDS–polyacrylamide gel electrophoresis on a 10% slab gel, exhibiting the two chains of protein C of apparent M_r 40000 and 18000, respectively, under reducing conditions (Kisiel, 1979). Antibodies to human protein C were raised in rabbits.

Other Proteins. Human antithrombin III and human prothrombin were isolated by established procedures in a modified version (Preissner et al., 1985). Prothrombin was

converted to α-thrombin by using the prothrombin activator from *Echis carinatus* venom (Morita & Iwanaga, 1981). Thrombin was isolated by chromatography on SP-Sephadex G-50 essentially as described by Lundblad et al. (1975). Thrombin had a fibrinogen clotting activity between 2200 and 2500 NIH units/mg when determined by the method of Fenton and Fasco (1974). *i*-Pr_2PF–thrombin–Sepharose was prepared according to Esmon, N. L., et al. (1982). Antithrombin III was coupled to BrCN-activated Sepharose at a ratio of 3 mg of protein/mL of swollen gel in 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.0. Radiolabeling of thrombin was carried out by the iodogen procedure (Fraker & Speck, 1978), yielding a specific activity of 1 μCi/μg of protein, and did not affect thrombin activity.

Human factor X and rabbit anti-(human factor X) serum were kindly provided by Dr. N. Heimburger, Behringwerke AG, Marburg, West Germany. Factor X was activated by the use of Russell's viper venom coupled to Sepharose as described by Jesty et al. (1974), and factor Xa was isolated according to Jesty and Esnouf (1973). The IgG fraction of anti-(human factor X) serum was coupled to BrCN-activated Sepharose at a ratio of 8 mg of protein/mL of swollen gel.

Heparin cofactor II which was purified from human plasma according to Tollefsen et al. (1982) was kindly provided by Dr. P. Sié, Centre de Transfusion Sanguine, Toulouse, France.

The isolated proteins were quantified spectrophotometrically by using the following extinction coefficients (ϵ) at 280 nm: protein C, 14.5 (Kisiel, 1979); antithrombin III, 6.5 (Nordenman et al., 1977); thrombin, 18.3 (Fenton et al., 1977); factor X, 11.6 (DiScipio et al., 1977); heparin cofactor II, 11.7 (Tollefsen et al., 1982).

Activation of Protein C by Thrombin–Thrombomodulin. (a) Protein C (0.5 μM) was activated by 14 nM thrombin–TM complex for 15 min at 37 °C in the presence of various concentrations (0–1 unit/mL) of protamine sulfate in a total volume of 320 μL containing 20 mM Tris-HCl buffer, pH 7.4, 120 mM NaCl, 3 mM $CaCl_2$, 0.5% (w/v) Lubrol PX, and 5 mg/mL human serum albumin. In addition, TM was treated with 1 unit/mL heparinase or 1 unit/mL heparitinase in the presence of 10 mM $CaCl_2$ for 2 h at 37 °C, or TM was reduced with 100 mM β-mercaptoethanol for 3 h at 22 °C followed by incubation with 150 mM iodoacetamide for 30 min at 22 °C in the dark. After dialysis, pretreated TM was employed in the same experimental setup.

(b) The activation of protein C was followed in a time course reaction at 37 °C over 90 min, protein C (0.5 μM) was preincubated in the same reaction buffer either in the presence of 0.5 unit/mL heparin, 1 unit/mL protamine sulfate, 0.1 mg/mL polybrene, or 2 μM antithrombin III or without additions, respectively, for 2 min at 37 °C. The reaction was started by the addition of 14 nM thrombin–TM complex. At various time intervals, 40-μL portions were removed from the reaction mixture; protein C activation was stopped by the addition of hirudin (50 units/mL final concentration), and the amount of activated protein C was measured in a chromogenic assay system as described above.

Inhibition of Thrombin-Catalyzed Fibrin Formation by Thrombomodulin. (a) Forty nanomolar thrombin was preincubated with an equimolar concentration of TM at 37 °C for 2 min in the presence of various concentrations of protamine sulfate (0–1 unit/mL) in a total volume of 100 μL containing 20 mM Tris-HCl buffer, pH 7.4, 120 mM NaCl, 10 mM $CaCl_2$, 0.25% (w/v) Lubrol PX, and 5 mg/mL human serum albumin. In addition, TM treated with heparinase, heparitinase as described before, or reduced and carboxymethylated TM was employed under the same assay conditions.

(b) Forty nanomolar thrombin was preincubated in the same buffer with various concentrations (0–60 nM) of TM in the absence and presence of 0.5 unit/mL heparin, 1 unit/mL protamine sulfate, or 0.1 mg/mL polybrene, respectively. Each reaction mixture was transferred into a prewarmed rotating cuvette containing 300 μL of 4.5 μM fibrinogen in 20 mM Tris-HCl buffer, pH 7.4, 120 mM NaCl, and the respective concentrations of heparin, protamine sulfate, polybrene, or buffer control. Clotting times were determined in triplicate in an automatic coagulometer.

Interference of Thrombomodulin with the Inhibition of Thrombin by Antithrombin III. Various concentrations of antithrombin III (0.2–2 μM) were preincubated for 1 min at 37 °C in the absence or presence of 30 nM TM in a total volume of 140 μL containing 20 mM Tris-HCl buffer, pH 7.4, 120 mM NaCl, 3 mM $CaCl_2$, 0.2% (w/v) Lubrol PX, and 5 mg/mL human serum albumin in the absence and presence of 1 unit/mL protamine sulfate. The inhibition reaction was started by the addition of 10 μL of thrombin (30 nM final concentration). At various time intervals, 25-μL portions were removed from the reaction mixture and transferred into a cuvette with 0.2 mM compound S-2238 dissolved in 875 μL of 40 mM Tris-HCl buffer, pH 8.0, containing 120 mM NaCl and 0.1% (w/v) bovine serum albumin and kept at 37 °C. Residual thrombin amidolytic activity was recorded at 405 nm in a Uvikon spectrophotometer. In addition, the following alterations of the initial reaction mixture were made: (a) Prior to use, TM samples were treated with either 1 unit/mL heparinase, 1 unit/mL heparitinase, or 50 mM periodic acid for 2 h at 37 °C; treatments of heparin solutions (0.2 unit/mL) under the same conditions completely destroyed its activity. (b) TM was reduced and carboxymethylated as described above. (c) The inhibitory effect of heparin cofactor II (1.4 μM) in the absence and presence of 30 nM TM was studied. (d) Forty nanomolar factor Xa instead of thrombin was reacted with 1.5 μM antithrombin III in the absence and presence of 30 nM TM, and residual factor Xa amidolytic activity was measured by using 0.2 mM compound S-2222 instead of S-2238.

Enzyme Kinetic Analysis. The effect of TM and protamine sulfate on the interaction of compound S-2238 with thrombin was analyzed in an amidolytic assay system; 1.2 nM thrombin was incubated at 37 °C in the absence and presence of an equimolar concentration of TM with various concentrations (6–100 μM) of compound S-2238 in the absence and presence of 1 unit/mL protamine sulfate in 40 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, 3 mM $CaCl_2$, 0.2% (w/v) Lubrol PX, and 1 mg/mL human serum albumin. The rate of hydrolysis of compound S-2238 was recorded at 405 nm in an spectrophotometer, and values were plotted in a double-reciprocal Lineweaver–Burk plot. An extinction coefficient of $\epsilon = 9.6 \times 10^3$ at 405 nm for *p*-nitroaniline was used.

Results

Purification of Thrombomodulin. The product obtained by affinity chromatography on *i*-Pr_2PF–thrombin–Sepharose was not homogeneous and necessitated further purification steps. After elution of TM with a salt gradient between 23 and 43 mS/cm from the DEAE-Trisacryl column, the protein was finally separated on antithrombin III–Sepharose in order to adsorb heparin-containing material. More than 70% of the protein applied did not retain to the column and contained virtually all TM cofactor activity. This unbound fraction was used in further experiments.

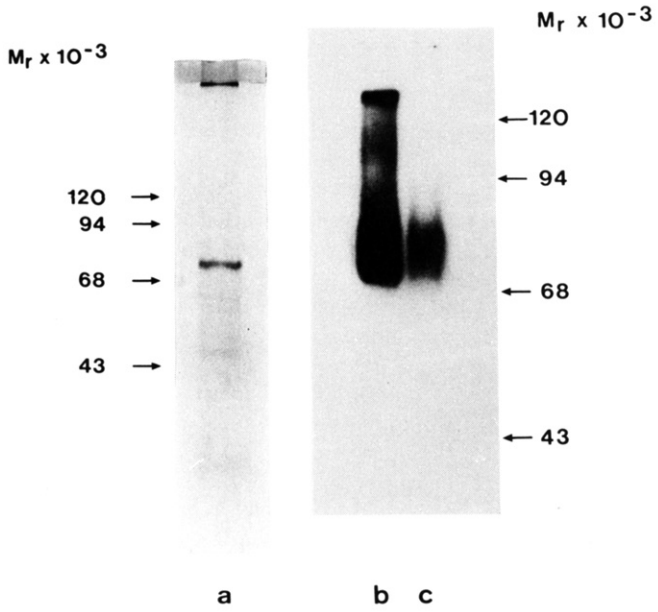

FIGURE 1: Binding of radiolabeled thrombin to thrombomodulin. (a) SDS–polyacrylamide gel electrophoresis of purified thrombomodulin under nonreducing conditions using the buffer system of Laemmli (1970). Approximately 0.5 μg of protein was applied to a 10% gel, which was stained with silver stain. (b) Total lung homogenate and (c) purified thrombomodulin were separated by polyacrylamide gel electrophoresis in the presence of SDS under nonreducing conditions and blotted onto nitrocellulose. An autoradiograph of the blot is shown after incubation of the nitrocellulose with ^{125}I-thrombin. The positions of molecular weight standard proteins are indicated; the anode is at the bottom.

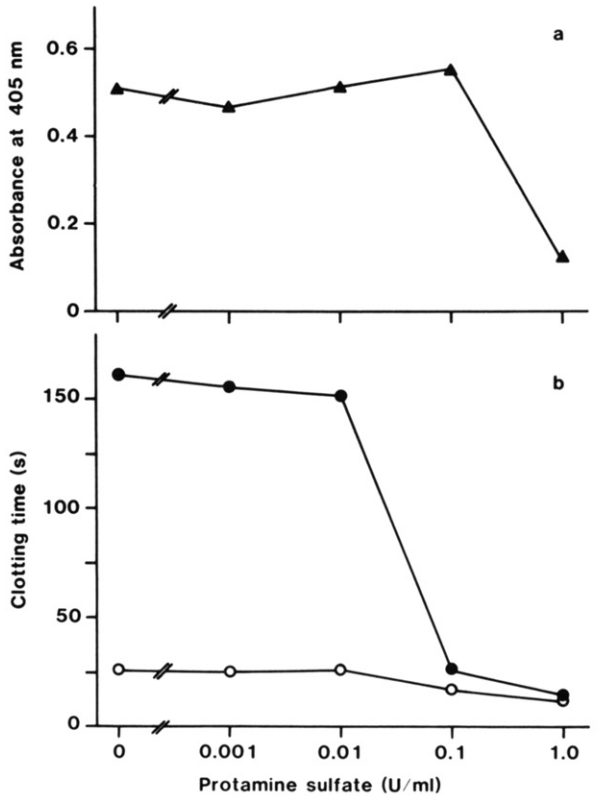

FIGURE 2: Effect of protamine on the activity of the thrombin–thrombomodulin complex. (a) Protein C (0.5 μM) was activated by thrombin (14 nM) and an equimolar concentration of thrombomodulin for 15 min in the presence of various concentrations of protamine. Activated protein C (▲) was determined in a chromogenic assay at 405 nm. (b) The activity of 40 nM thrombin, expressed as fibrinogen clotting time, was analyzed in the presence (●) or absence (○) of an equimolar concentration of thrombomodulin at various protamine concentrations. Each point represents the mean of three determinations.

After electrophoresis on polyacrylamide gels in the presence of SDS under nonreducing conditions, purified TM migrated as a single protein band of apparent M_r 75 000 (Figure 1, lane a). Subsequent electrophoretic transfer of parallel samples onto nitrocellulose allowed the detection of thrombin binding to the endothelial cell receptor. While radiolabeled thrombin bound to several proteins of the starting material in the molecular weight range of 70 000–120 000, a single band of apparent M_r 70 000–80 000 was detectable in the position of purified TM on the autoradiograph (Figure 1, lane c). The functional activity of TM as cofactor for thrombin in the activation of protein C could be recovered after eluting the protein band with M_r 70 000–80 000 from an unfixed and unstained polyacrylamide slab gel run in parallel. No binding of radiolabeled thrombin to samples analyzed under reducing conditions was observed.

Interference of Protamine with Thrombomodulin Activity. The functional activity of TM as cofactor for thrombin in the activation of protein C and as inhibitor of thrombin-catalyzed fibrin formation was analyzed in the absence and presence of various protamine concentrations (Figure 2). Low concentrations of protamine up to 0.1 unit/mL did not affect the activation of protein C by thrombin–TM, but protamine sulfate in a concentration of 1 unit/mL substantially inhibited the cofactor activity (Figure 2a). In a similar fashion, the capacity of TM to inhibit thrombin-induced fibrin formation was affected by protamine. At a concentration of 0.1 unit/mL protamine sulfate and higher, TM activity was abolished, as demonstrated by the decrease in clotting time (Figure 2b). At all concentrations tested, protamine did not interfere with the activity of thrombin in the absence of TM. In further experiments, protamine sulfate was used at a concentration of 1 unit/mL.

Interference of Heparin, Polycations, and Antithrombin III with the Activation of Protein C by Thrombin–Thrombomodulin. The activation of purified human protein C by an equimolar complex of thrombin and TM was followed in a time course reaction. A typical activation profile is shown in Figure 3A. The initial rate of protein C activation was 25 pmol mL^{-1} min^{-1} which was equivalent to the disappearance of protein C with a rate constant k = 0.075 min^{-1} (Figure 3B). The presence of 0.5 unit/mL heparin did not interfere with the protein C activation reaction. However, the addition of 1 unit/mL protamine sulfate reduced the initial rate of protein C activation to 7 pmol mL^{-1} min^{-1} which was equivalent to a reduction of the rate constant to k = 0.020 min^{-1}. In the presence of protamine, the maximal value of activated protein C after 90 min only reached about half of the control value. The presence of 0.1 mg/mL polybrene during the activation reaction resulted in a similar reduction of the production of activated protein C. The presence of 2 μM antithrombin III during the activation reaction totally prevented the production of activated protein C.

Enzyme kinetic analysis for the reaction of thrombin with compound S-2238 was carried out to study the interference of TM and protamine with this reaction. No substantial difference in the Lineweaver–Burk plots was recognized when thrombin (K_m = 8.3 μM, V_{max} = 13.1 μM/min) and thrombin–TM (K_m = 8.9 μM, V_{max} = 15.0 μM/min) were compared (Figure 4); 1 unit/mL protamine sulfate, however, affected the affinity for the substrate compound S-2238 of thrombin in the presence of an equimolar concentration of TM. The presence of the polycation led to an increase of the Michaelis constant for thrombin–TM to K_m = 19.5 μM, with a virtually

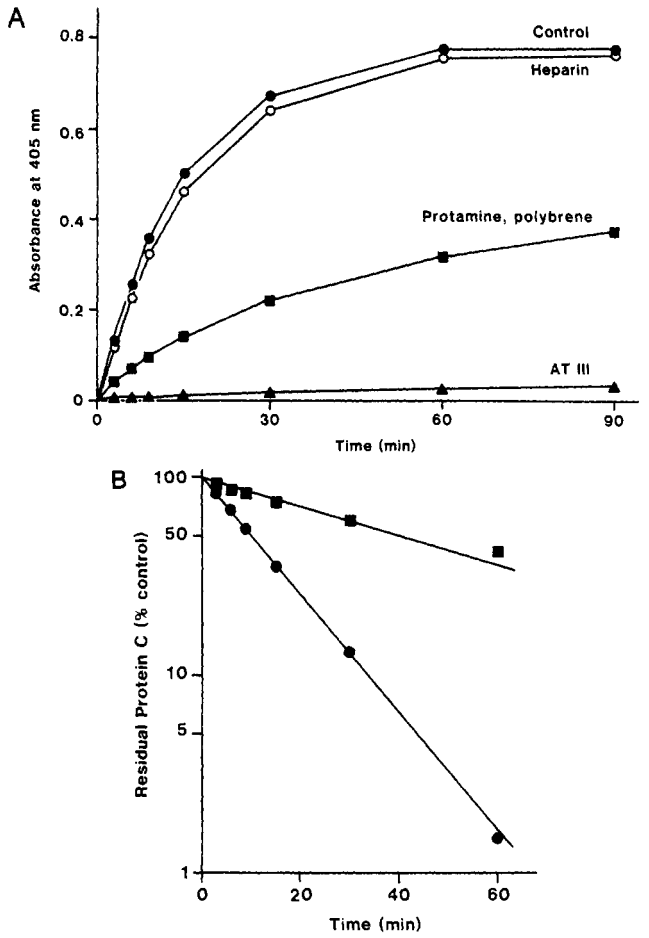

FIGURE 3: Effect of heparin, polycations, and antithrombin III on the activation of protein C by thrombin–thrombomodulin complex. (A) The activation of protein C (0.5 μM) by 14 nM thrombin–thrombomodulin complex was followed in the presence of (○) 0.5 unit/mL heparin, (■) 1 unit/mL protamine sulfate or 0.1 mg/mL polybrene, (▲) 2 μM antithrombin III (AT III), or (●) without any addition. Activated protein C was determined in a chromogenic assay at 405 nm. (B) Plot of percent residual concentration of protein C vs. time; data are taken from Figure 3A: (■) in the presence of 1 unit/mL protamine sulfate; (●) without any addition. From the slopes of the curves, the first-order rate constants for the disappearance of protein C were determined.

unchanged V_{max} = 13.8 μM/min, while protamine did not influence the kinetic constants of thrombin alone.

Effect of Thrombomodulin on Thrombin-Catalyzed Fibrin Formation. The ability of purified TM to affect thrombin-induced fibrin formation was studied in a purified system. Addition of increasing concentrations of TM to a thrombin solution containing a constant concentration of the enzyme resulted in an increase of the thrombin clotting time from 20 s in the absence to more than 150 s in the presence of an concentration of TM equimolar to thrombin (Figure 5). No influence of heparin (0.5 unit/mL) on this concentration-dependent thrombin inhibition was noted. In contrast, the polycations protamine or polybrene abrogated the inhibitory capacity of TM, and no increase in the thrombin-induced clotting time was observed. In the absence of TM, no effect of protamine and polybrene on thrombin activity was recognized. The same qualitative effect of polycations could be documented when normal rabbit plasma instead of a fibrinogen solution was used.

Effect of Thrombomodulin on the Inhibition of Thrombin by Antithrombin III. The hypothesis was tested whether protamine may interfere with the functional activity of TM by neutralizing any heparin-like activity of the receptor. For

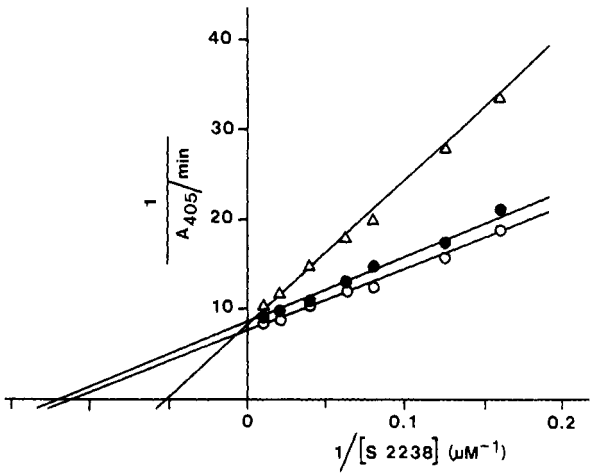

FIGURE 4: Influence of thrombomodulin and protamine on the thrombin-catalyzed hydrolysis of compound S-2238. The initial rates of hydrolysis of compound S-2238 (6–100 μM) by 1.2 nM thrombin alone or in the presence of 1 unit/mL protamine (●) and by 1.2 nM thrombin–thrombomodulin complex in the absence (○) or in the presence (△) of 1 unit/mL protamine sulfate were transformed into a Lineweaver–Burk plot for the determination of K_m and V_{max}.

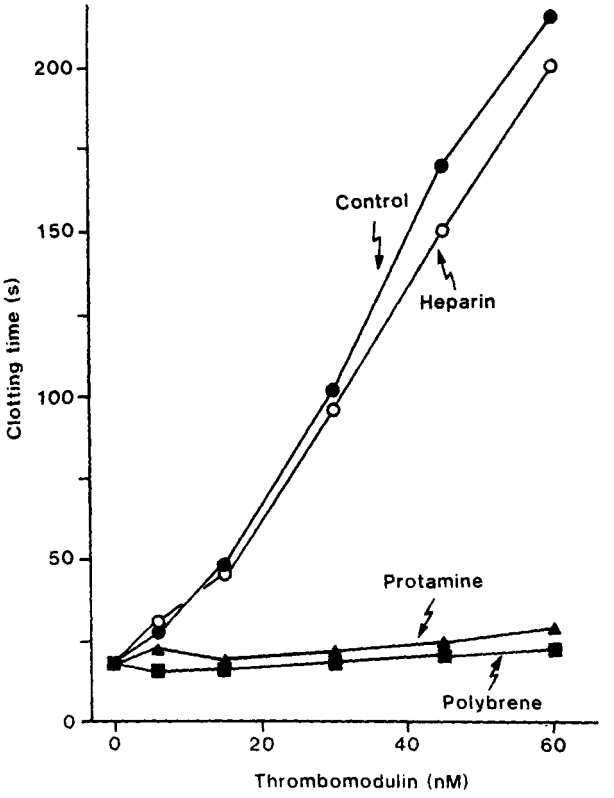

FIGURE 5: Inhibition of thrombin-catalyzed fibrin formation by thrombomodulin. 40 nM thrombin was preincubated with various concentrations of thrombomodulin for 2 min at 37 °C in the presence of (○) 0.5 unit/mL heparin, (▲) 1 unit/mL protamine sulfate, (■) 0.1 mg/mL polybrene, or (●) without any addition. The incubation mixtures were added to solutions containing 4.5 μM fibrinogen, and the clotting times were determined in triplicate.

this purpose, various concentrations of antithrombin III were employed to analyze the kinetics of the inhibition of thrombin by antithrombin III in the presence and absence of TM. Preliminary experiments revealed no difference in the results whether thrombin was added to the TM-containing preincubation mixture or the reaction was started by the addition of the thrombin–TM complex. Pseudo-first-order conditions applied for the inhibition reactions and the apparent rate constants were calculated from the slopes of the linear plots

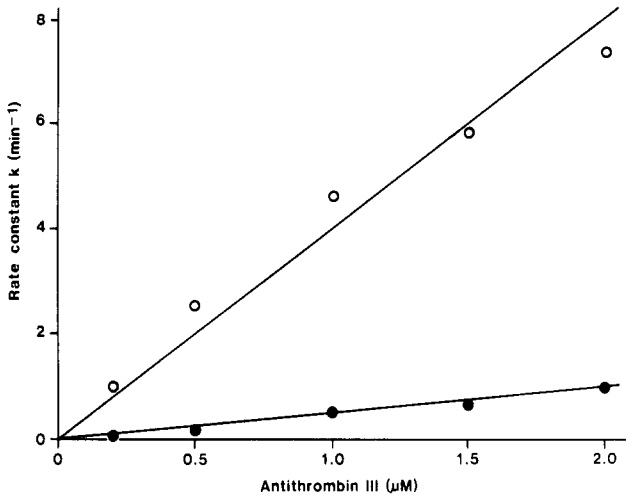

FIGURE 6: Kinetic analysis of the inactivation of thrombin by antithrombin III. The inhibition of amidolytic activity of 30 nM thrombin was measured at different antithrombin III concentrations: (●) in the absence of thrombomodulin; (○) in the presence of 30 nM thrombomodulin. The pseudo-first-order rate constants of the inhibition of thrombin were plotted as a function of antithrombin III concentration. The second-order rate constants were determined from the slopes of the curves.

Table I: Pseudo-First-Order Rate Constants, k (min^{-1}), for Inhibition of Thrombin (IIa) and Factor Xa (Xa) by Antithrombin III (AT III) and Heparin Cofactor II (HC II) in the Absence and Presence of Thrombomodulin (TM)[a]

enzyme	inhibitor	TM	pretreatment of TM	rate constant, k (min^{-1})	n
IIa	AT III	--		0.43 ± 0.07	4
IIa	AT III	++		3.40 ± 0.60	8
IIa	AT III	++	heparinase	3.95 ± 0.53	4
IIa	AT III	++	heparitinase	3.84 ± 0.30	3
IIa	AT III	++	protamine[b]	0.65 ± 0.08	3
IIa	AT III	++	reduction[b]	0.44 ± 0.04	4
IIa	HC II	--		0.01 ± 0.02	3
IIa	HC II	++		0.02 ± 0.05	3
Xa	AT III	--		0.41 ± 0.06	3
Xa	AT III	++		0.48 ± 0.05	3

[a] Mean values ± SD for number of experiments (n) are given. [b] Pretreatment of TM also abrogated its cofactor activity for protein C activation by thrombin and as inhibitor for thrombin-induced fibrin formation.

of the logarithm of residual thrombin amidolytic activity vs. time. While thrombin alone was inhibited by antithrombin III in a slow, progressive fashion, the inhibition rate was accelerated in the presence of TM for each antithrombin III concentration tested (Figure 6). From the slopes of both curves, the second-order rate constants were determined with $k_2 = 8.4 \times 10^3$ M^{-1} s^{-1} for the thrombin–antithrombin III reaction in the absence of TM and $k_2 = 70 \times 10^3$ M^{-1} s^{-1} for the thrombin–antithrombin III reaction in the presence of TM. The addition of 1 unit/mL protamine sulfate counteracted the accelerative effect of TM in this reaction and prevented the fast inactivation of thrombin (Table I). Preincubation neither with heparinase nor with periodic acid affected the rate-enhancing activity of TM, although both treatments abrogated the activity of a control sample containing 0.2 unit/mL heparin. Likewise, preincubation of TM with heparinase did not inactivate its antithrombin III cofactor activity or its cofactor activity for protein C activation and thrombin inhibition. The heparin-dependent inhibitor for thrombin, heparin cofactor II, failed to inactivate the enzyme in the presence of TM, while further addition of 0.02 unit/mL heparin initiated the inactivation of thrombin ($k = 6.5$ min^{-1}). No accelerative effect of TM on the slow, progressive inhibition of factor Xa by antithrombin III was observed as well (Table I). In contrast, reduction and carboxymethylation did affect TM in such a way that modified TM was no longer able to serve as cofactor for protein C activation, as inhibitor of thrombin-induced fibrin formation, or as accelerator of the thrombin–antithrombin III reaction (Table I).

Discussion

Several low- and high-affinity binding sites for thrombin have been recognized on the endothelium, which are involved in the mediation of procoagulant and anticoagulant responses of the vessel wall. However, a direct relationship between a defined cell receptor site and its effect on the function of thrombin could not yet be established. The detection of the endothelial cell membrane protein thrombomodulin (TM) which serves as a high-affinity, active-site-independent receptor for thrombin on endothelial cells (Esmon & Owen, 1981) allowed approach to this question.

A homogeneous TM preparation was obtained by extending the original purification protocol (Esmon, C. T., et al., 1982) by two additional purification steps. Purified rabbit TM did not exhibit affinity for antithrombin III coupled to Sepharose, suggesting the absence of heparin-like structures within the receptor protein. Surprisingly, however, the inhibition of thrombin by antithrombin III was substantially accelerated by TM. Thus, it appeared that the receptor contains an activity catalyzing the thrombin–antithrombin III complex formation. Attempts to dissociate this antithrombin III cofactor activity from TM failed, since all three cofactor activities of TM coeluted upon gel filtration in the presence of 0.1% SDS (not shown). Most interestingly, the functional cofactor activities of TM were blocked by the polycations protamine and polybrene, known to be heparin antagonists.

The possibility that the effect of TM was due either to contaminating heparin or to the presence of heparin activity in TM was excluded by the following experiments: Treatment with neither heparinase, heparitinase, nor periodic acid could affect the accelerative action of TM in the thrombin–antithrombin III reaction. In addition, the heparin-dependent inhibitor for thrombin, heparin cofactor II, did not show any inhibitory effect on the enzyme in the presence of TM. Finally, the slow inhibition of factor Xa by antithrombin III, known to be accelerated by heparin (Yin et al., 1971), was not affected in the presence of TM. Alteration of the molecular integrity of TM by reduction and carboxymethylation, however, not only destroyed the known cofactor activities of TM (Esmon, N. L., et al., 1982) but also abrogated the antithrombin III cofactor activity.

Since it has convincingly been demonstrated that the enzymatic specificity of thrombin is altered due to the interaction with TM (Owen & Esmon, 1981), it is reasonable to assume that the TM-induced acceleration of thrombin inhibition by antithrombin III may also be due to a direct effect of TM on the enzyme. This hypothesis was also supported by the present results that polycations not only reversed the accelerative effect of TM in the inhibition reaction but also blocked the ability of TM to inhibit thrombin-induced fibrin formation and to suppress TM cofactor activity for protein C activation. Thus, binding of thrombin to TM not only is associated with the inhibition of thrombin's procoagulant activity and with an increase in the activation capacity for protein C but also is associated with an increase in sensitivity of thrombin to inactivation by antithrombin III.

Our findings are supported by a recent report (Hofsteenge et al., 1986) in which a 4-fold stimulation of the inactivation

rate of thrombin by antithrombin III in the presence of rabbit TM was found. In particular, the accelerative effect of TM was dependent on the concentration of TM; this is consistent with a mechanism in which free thrombin and thrombin complexed to TM are inactivated at different rates, as proposed in the present study. Conflicting data, however, exist regarding the activity of purified TM from bovine lung employed in similar studies. Suzuki et al. (1986) found neither a difference in the rate of inactivation of thrombin by antithrombin III in the absence or presence of bovine TM nor an interference of antithrombin III with protein C activation by bovine thrombin–TM complex. In contrast, Jakubowski et al. (1986) reported a protective effect of bovine TM against fast inactivation of thrombin by antithrombin III–heparin, while in the absence of heparin the inactivation rate of thrombin remained unaffected by the presence of TM. Likewise, the inhibition rate of thrombin by heparin cofactor II–heparin was found to be reduced in the presence of bovine TM. Similarly, in a system with cultured bovine aortic endothelial cells used instead of purified TM, an appreciable amount of protein C became activated by thrombin on the cell layer despite the presence of an excess of antithrombin III (Delvos et al., 1985; Jakubowski et al., 1986). The discrepancy among these findings may arise from species differences or the fact that large vessel derived TM behaves differently compared with isolated TM from the microvasculature. Unlike bovine TM, rabbit TM may induce a different conformational change in the thrombin molecule, resulting in an alteration in the interaction not only with the substrates fibrinogen and protein C but also with antithrombin III. Since the active site of thrombin is not affected by interaction with either rabbit TM (Esmon, C. T., et al., 1982) or bovine TM (Jakubowski et al., 1986), alterations in the microenvironment of the receptor molecule may account for the observed differences in functional activity. Culture conditions of the endothelial cells or proteolysis of the receptor molecule during isolation may lead to differences in the expression of TM cofactor activity. Finally, the endothelial cell provides additional receptor sites for thrombin which may interfere with the thrombin–TM interaction (Owen, 1984).

Since the thrombin–TM binding is mainly governed by ionic interactions (Esmon, N. L., et al., 1982) and all the activities of the thrombin–TM complex which either are calcium ion dependent or are not were equally affected by protamine or polybrene, these polycations may at least in part compete with the cationic molecule thrombin (Fenton et al., 1977) for binding to TM. The present findings suggest that the hydrophilic binding of thrombin to TM is not mediated by heparin-like structures, since heparin did not displace TM-bound thrombin and, consequently, did not interfere with the functional activities of the enzyme–receptor complex.

In addition, the present study may have clinical implications for the use of heparin antagonists in terminating extracorporal circulation. While the thrombin–TM complex retains its anticoagulant potential during heparin therapy in the patient, treatment with the heparin antagonist protamine at high concentrations does not only result in the neutralization of heparin activity but might additionally weaken the natural anticoagulant capacity of the thrombin–TM mechanism.

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Registry No. Thrombin, 9002-04-4; antithrombin, 9000-94-6; heparin, 9005-49-6; protein C, 60202-16-6; S-2238, 62354-65-8; blood coagulation factor Xa, 9002-05-5; heparin cofactor II, 81604-65-1.

References

Bauer, P. I., Machovich, R., Aranyi, P., Büki, K. G., Csonka, E., & Horvath, I. (1983) *Blood 61*, 368–372.

Bertina, R. M., Broekmans, A. W., van der Linden, I. K., & Mertens, K. (1982) *Thromb. Haemostasis 48*, 1–5.

Busch, C., & Owen, W. G. (1982) *J. Clin. Invest. 69*, 726–729.

DeBault, L. E., Esmon, N. L., Olson, J. R., & Esmon, C. T. (1986) *Lab. Invest. 54*, 172–178.

Delvos, U., Meusel, P., Preissner, K. T., & Müller-Berghaus, G. (1985) *Blood 66*, 355a (Abstr).

DiScipio, R. G., Hermodson, M. A., Yates, S. G., & Davie, E. W. (1977) *Biochemistry 16*, 698–706.

Esmon, C. T., & Owen, W. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2249–2252.

Esmon, C. T., Esmon, N. L., & Harris, K. W. (1982) *J. Biol. Chem. 257*, 7944–7947.

Esmon, N. L., Owen, W. G., & Esmon, C. T. (1982) *J. Biol. Chem. 257*, 859–864.

Esmon, N. L., Carroll, R. C., & Esmon, C. T. (1983) *J. Biol. Chem. 258*, 12238–12242.

Fenton, J. W., & Fasco, M. J. (1974) *Thromb. Res. 4*, 809–817.

Fenton, J. W., Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A., & Finlayson, J. S. (1977) *J. Biol. Chem. 252*, 3587–3598.

Fraker, P. J., & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun. 80*, 849–857.

Fulcher, C. A., Gardiner, J. E., Griffin, J. H., & Zimmerman, T. S. (1984) *Blood 63*, 486–489.

Hatton, M. W. C., Dejana, E., Cazenave, J. P., Regoeczi, E., & Mustard, J. F. (1980) *J. Lab. Clin. Med. 96*, 861–870.

Hofsteenge, J., Taguchi, H., & Stone, S. R. (1986) *Biochem. J. 237*, 243–251.

Ishii, H., Salem, H. H., Bell, C. E., Laposata, E. A., & Majerus, P. W. (1986) *Blood 67*, 362–365.

Jakubowski, H. V., Kline, M. D., & Owen, W. G. (1986) *J. Biol. Chem. 261*, 3876–3882.

Jesty, J., & Esnouf, M. P. (1973) *Biochem. J. 131*, 791–799.

Jesty, J., Spencer, A. K., & Nemerson, Y. (1974) *J. Biol. Chem. 249*, 5614–5622.

Kisiel, W. (1979) *J. Clin. Invest. 64*, 761–769.

Kurosawa, S., & Aoki, N. (1985) *Thromb. Res. 37*, 353–364.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Levin, E. G., Marzec, U., Anderson, J., & Harker, L. A. (1984) *J. Clin. Invest. 74*, 1988–1995.

Levine, J. D., Harlan, J. M., Harker, L. A., Joseph, M. L., & Counts, R. B. (1982) *Blood 60*, 531–534.

Lollar, P., Hoak, J. C., & Owen, W. G. (1980) *J. Biol. Chem. 255*, 10279–10283.

Lollar, P., MacIntosh, S. C., & Owen, W. G. (1984) *J. Biol. Chem. 459*, 4335–4338.

Lundblad, R. L., Uhteg, L. C., Vogel, C. N., Kingdon, H. S., & Mann, K. G. (1975) *Biochem. Biophys. Res. Commun. 66*, 482–489.

Marcum, J. A., & Rosenberg, R. D. (1985) *Biochem. Biophys. Res. Commun. 126*, 365–372.

Marcum, J. A., McKenney, J. B., & Rosenberg, R. D. (1984) *J. Clin. Invest. 74*, 341–350.

Maruyama, I., & Majerus, P. W. (1985) *J. Biol. Chem. 260*, 15432–15438.

Maruyama, I., Bell, C. E., & Majerus, P. W. (1985) *J. Cell Biol. 101*, 363–371.

Morita, T., & Iwanaga, S. (1981) *Methods Enzymol. 80*, 303–311.

Nordenman, B., Nyström, C., & Björk, I. (1977) *Eur. J. Biochem. 78*, 195–203.
Owen, W. G. (1984) in *Biology of Endothelial Cells* (Jaffe, E. A., Ed.) pp 259–267, Martinus Nijhoff Publishers, Boston.
Owen, W. G., & Esmon, C. T. (1981) *J. Biol. Chem. 256*, 5532–5535.
Pearson, J. D., & Gordon, J. L. (1979) *Nature (London) 281*, 384–386.
Preissner, K. T., Wassmuth, R., & Müller-Berghaus, G. (1985) *Biochem. J. 231*, 349–355.
Salem, H. H., Maruyama, I., Ishii, H., & Majerus, P. W. (1984) *J. Biol. Chem. 259*, 12246–12251.
Shimada, K., & Ozawa, T. (1985) *J. Clin. Invest. 75*, 1308–1316.
Stenflo, J. (1976) *J. Biol. Chem. 251*, 355–363.
Stern, D., Nawroth, P., Marcum, J., Handley, D., Kisiel, W., Rosenberg, R. D., & Stern, K. (1985) *J. Clin. Invest. 75*, 272–279.
Suzuki, K., Kusumoto, H., & Hashimoto, S. (1986) *Biochim. Biophys. Acta 882*, 343–352.
Thiel, W., Preissner, K. T., Delvos, U., & Müller-Berghaus, G. (1986) *Blut 52*, 169–177.
Tollefsen, D. M., Majerus, D. W., & Blank, M. K. (1982) *J. Biol. Chem. 257*, 2162–2169.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Walker, F. J., Sexton, P. W., & Esmon, C. T. (1979) *Biochim. Biophys. Acta 571*, 333–342.
Weksler, B. B., Ley, C. W., & Jaffe, E. A. (1978) *J. Clin. Invest. 62*, 923–930.
Yin, E. T., Wessler, S., & Stoll, P. J. (1971) *J. Biol. Chem. 246*, 3703–3711.

Nucleation of Actin Polymerization by Villin and Elongation at Subcritical Monomer Concentration†

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ABSTRACT: We have obtained a quantitative description of villin-nucleated actin polymerization in physiological salt by determining the concentrations of free villin (V), villin–actin monomer (VA), villin–actin dimer (VA_2), and villin–actin oligomer (VA_n). Over a range of actin–villin ratios from 0.1 to 20 we determined the concentration of actin-bound villin by measuring the *low*-intensity pyrenylactin fluorescence of the two terminal actins in each villin–actin polymer. (To this end we first showed that each villin–actin oligomer and polymer contains two low-intensity pyrenylactin molecules.) We determined the concentration of free villin using a calibrated cutting activity assay. The pattern of increase in bound villin together with the pattern of increase in *high*-intensity pyrenylactin fluorescence with increasing G-actin concentration indicated, first, that villin–actin monomers were not formed at detectable levels even at a 12-fold villin excess over actin. Second, there was no stoichiometric villin–actin dimer formation at actin–villin ratios of 2. Instead there was an equilibrium between free villin, VA_2, and VA_n. Defining $K_1 = [VA]/[V][A]$ and $K_2 = [VA_2]/[VA][A]$, a good fit of the data was obtained with $K_1 \ll K_2$ and a value of $K_1K_2 = K_V = 10^{12}$–10^{13} $M^{-2} = [VA_2]/[V][A]^2$, i.e., $1/K_V^{1/2} = (0.3\text{–}1) \times 10^{-6}$ M. We have assumed here that the monomer binding constant of VA_2 to form VA_3 was equal to the monomer binding constant of pointed filament ends, $K_\infty = 1/c_\infty$, obtained as described below. Extending polymerization measurements to actin–villin ratios greater than 100 showed that F-actin increased gradually with increasing G-actin concentrations until a limiting value for the G-actin concentration was obtained, c_∞, approaching $1/K_\infty$ for the pointed filament end. The data could be fitted with the same three constants, K_1, K_2, and K_∞, used for the data in the low range of actin–villin ratios. Retaining the term critical concentration for the limiting G-actin concentration during nucleated polymerization, F-actin is formed at subcritical actin concentrations. In other words, filament assembly no longer has the characteristics of condensation polymerization but instead is described by a series of equilibria that are very similar to those derived by Oosawa for the analogous case of "linear" polymerization.

Actin filaments in nonmuscle cells probably are repeatedly assembled and disassembled to adapt to different functions. Filament formation and breakdown appears to be regulated by actin binding proteins, a large number of which have been isolated [for reviews, see Korn (1982), Craig and Pollard (1982), Weeds (1982), Stossel et al. (1985), and Pollard and Cooper (1986)]. These proteins are capable of bundling filaments, nucleating the formation of new filaments, stabilizing the filaments by capping both ends, or assisting in filament disassembly by stabilization of monomeric actin (G-actin), by filament breakage, and by capping of the preferred end for elongation (Wegner, 1976; Kirschner, 1980).

At this time relatively little is known about the participation of these proteins in intracellular processes. In vitro studies,

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