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FIBRINOGEN AND FIBRIN INTERACTION WITH CONCANAVALIN A DIMER AND ITS INFLUENCE ON COAGULATION

TERESA DÍAZ-MAURIÑO, ROSAURA BLANCO and ARMANDO ALBERT

Instituto de Quimica Fisica 'Rocasolano', Serrano 119, Madrid-6 (Spain)

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Concanavalin A dimer interacts with fibrinogen and soluble fibrin at pH 5.2 Analysis of the binding data shows that there are in both cases four binding sites per molecule and that the dissociation constant does not change by removal of fibrinopeptides A and B. Ultracentrifugal studies show that no aggregates of fibrinogen or fibrin are formed through concanavalin A binding and that up to four molecules of concanavalin A dimer can bind to one molecule of fibrinogen or fibrin. These results imply that the four carbohydrate chains in the molecule are accessible to concanavalin A dimer. There is a diminution in the coagulation of fibrinogen by thrombin at low relative lectin concentrations and an increase at high concentrations. However, the lectin always favours the aggregation of fibrin monomers and does not have any inhibitory effect on the release of fibrinopeptides. We conclude that the electric charge in the neighbourhood of the carbohydrate in both chains, Bβ and γ plays an important role in the attraction between monomeric fibrin and fibrinogen-monomeric fibrin. The different effect of concanavalin A on the coagulation, depending on the relative concentration of the lectin, would be the result of the screening of this electric charge favouring either the interaction of fibrinogen-monomeric fibrin or the polymerization of monomeric fibrin.

Introduction

Fibrinogen is a glycoprotein composed of three pairs of nonidentical polypeptide chains (Aα, Bβ and γ). The Bβ and γ chains are glycosylated [1,2], whereas the Aα chains do not contain carbohydrate residues [3]. The role of carbohydrate in fibrinogen function has been the subject of several studies [4,5] in which contradictory results have been reported. In addition, some dysfibrinogenemias have been associated with an increase in the carbohydrate content [6,7]. It has been shown that the terminal sialic acid of the carbohydrate chains plays a significant role in the clotting process, since asialofibrinogen clots faster than fibrinogen [8,9] probably due to a diminution in the number of negative charges of the asialofibrinogen molecule.

In a previous work [10] on the interaction of fibrinogen and asialofibrinogen with concanavalin A at neutral pH we found that binding of the lectin to both molecules gives rise to an inhibition of the coagulation rate at low concentrations of concanavalin A and an increase at high concentrations. However, asialofibrinogen and its concanavalin A complexes coagulate twice as fast as those of fibrinogen. This suggests that the effect of concanavalin A on the clotting process is not only due to a screening of the negative charge of the sialic acid.

On the other hand, we obtained only two binding sites [10] in the fibrinogen and asialofibrinogen for the lectin, indicating that two of the four possible sites in the molecule were not accessible to concanavalin A tetramer. Furthermore, neuraminidase of *Clostridium perfringens* releases the

sialic acid [9] bound to Bβ chains of fibrinogen much more rapidly than that attached to γ chains. From these results it was concluded that the oligosaccharide in the γ chains of fibrinogen is somehow less accessible to macromolecules than that bound to Bβ chains and that the differences in molecular weight between concanavalin A and neuraminidase (100 000 and less than 70 000 [11,12], respectively) could account for the differences in the binding behaviour of both molecules.

At acidic pH concanavalin A is known to exist as a dimer of M_r 55 000 [13] and fibrin is obtained soluble as independent monomer units [14]. In the present work, the interaction of fibrinogen and fibrin with concanavalin A at pH 5.2 has been studied in order to know if the four carbohydrate chains of fibrinogen are more accessible to the smaller concanavalin A molecule and whether the release of fibrinopeptides has any influence on the accessibility of the lectin to the γ chains. The coagulation by thrombin of the fibrinogen and fibrin-concanavalin A dimer complexes has also been studied and the results compared with those obtained with fibrinogen-concanavalin A tetramer complexes.

Materials and Methods

Human fibrinogen (more than 92% thrombin-clottable) and substrate S-2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride) from Kabi (Stockholm 30, Sweden) were used. Bovine thrombin (300 NIH units/mg protein), concanavalin A, α-methyl-D-glucopyranoside and β-mercaptoethanol were purchased from Sigma Chemical Co (St Louis, MO, USA). Acetonitrile was from Fluka AG, (Buchs). All other chemical used were analytical reagent grade from Merck (Darmstadt).

Fibrin monomers. These were obtained by the following two methods: (a) 1.5 units/ml of thrombin were added to a solution of fibrinogen (1 mg/ml) in 0.018 M phosphate buffer (pH 7.8) containing 0.05 M NaCl and the mixture was allowed to clot at room temperature for 2 h. The clot was collected, washed three times with isotonic saline and dissolved in 5 M urea. The fibrin was exhaustively dialysed against 0.02 M acetate buffer (pH 5.2) containing 0.1 M NaCl for 24 h at 4°C. (b) Fibrinogen solution (1 mg/ml) in 0.02 M acetate buffer (pH 5.2), 0.1 M NaCl was incubated with thrombin (1.5 units/ml) at room temperature for 2 h. At this pH (5.2) the thrombin releases the fibrinopeptides from the fibrinogen but the aggregation of fibrin monomers does not take place as observed by analytical ultracentrifugation. The release of fibrinopeptides was checked using the method of Gralnick et al. [15]. Since both methods gave the same results, the second one was generally used.

Fibrin monomer aggregation. 1-ml solutions of fibrinogen and fibrinogen-concanavalin A complexes (1 mg/ml) at pH 5.2, were brought to pH 6.8 with 0.1 M Tris solution before the addition of thrombin (0.15 units/ml, final concentration). Immediately the increase in absorbance at 350 nm was recorded in a double beam Hitachi 2000 spectrophotometer. In the case of fibrin monomer solutions, the absorbance at 350 nm was recorded after the addition of the 0.1 M Tris solution.

Release of fibrinopeptides. The kinetics of liberation of fibrinopeptides from fibrinogen and fibrinogen-concanavalin A complexes by thrombin was carried out essentially as described by Sellers and Clark [16]. Fibrinopeptides released were quantified on a Water Associates 6000 A liquid chromatography equipped with an automatic sampling system using a reversed-phase Techsil 10 RP-18 column (4 mm × 25 cm; Krattor) and detection wavelength of 210 nm. The mobile phase was 0.025 M ammonium acetate adjusted to pH 6.0 with orthophosphoric acid and mixed with acetonitrile (10 : 1, v/v) [17]. The elution was achieved at room temperature in about 40 min using isocratic conditions with a flow rate of 1.5 ml/min.

Effect of concanavalin A on thrombin activity. Different aliquots of a solution of concanavalin A (10 mg/ml) in 0.15 M NaCl were added to 50 μl of thrombin solution in 0.15 M NaCl (0.47 mg/ml, 150 units/ml). The final concentrations of concanavalin A in the incubation mixture ranged from 1 to 5 mg/mg thrombin. The reactions were carried out at room temperature for various periods of up to 1 h. The concentration of thrombin was estimated spectrophotometrically using a value of $E_{280}^{0.1\%} = 1.83$ [18]. Thrombin activity was assayed

with the substrate S-2238. A 0.1 ml solution of the chromogenic substrate 0.75 mM in distilled water was placed in a quartz semimicro cuvette followed by 0.9 ml of 0.5 M Tris-HCl (pH 8.4). After the addition of the thrombin or thrombin-concanavalin A solution (50 μl) the absorbance at 405 nm was recorded in the spectrophotometer during 5 min against a blank containing buffer and chromogenic substrate.

Other relevant experimental details were essentially as described previously [10], except for the buffer solution, which in the present work was 0.2 M acetate buffer (pH 5.2) containing 0.1 M NaCl.

Results

Binding of ^{125}I-concanavalin A to fibrinogen and fibrin. Analysis of the specific binding data of ^{125}I-concanavalin A to fibrinogen and fibrin at pH 5.2 shows in both cases similar behaviour. Fig. 1 represents the experimental data obtained for fibrinogen and fibrin and the best fit to all the results calculated by the least squares method. The following results were obtained; number of sites was 3.8 and the dissociation constant was $2.9 \cdot 10^{-6}$ M (correlation coefficient = 0.97).

Ultracentrifugal studies. A solution of concanavalin A (2 mg/ml) in 0.02 M acetate buffer (pH 5.2) containing 0.1 M NaCl either in the presence or absence of α-methyl-D-glucopyranoside gave a sedimentation coefficient of $s^0_{20,w} = 3.5$ S.

The sedimentation behaviour of fibrinogen and fibrin-concanavalin A complexes was studied at relative concentrations of the lectin ranging from 60 μg to 1 mg per mg of fibrinogen or fibrin. The apparent sedimentation coefficients varied from 7.8 ± 0.2 S for pure fibrinogen and fibrin to 10.2 ± 0.3 S for the fibrinogen and fibrin-concanavalin A complexes at the highest relative concentration of concanavalin A used (1 mg/mg). In these cases, a slow moving peak with a sedimentation coefficient similar to that of pure concanavalin A could be detected. No higher aggregates or material sedimenting before the 60000 rev/min phase was reached could be detected.

Effect of concanavalin A on the aggregation of moners of fibrin. The effect of the lectin on the aggregation of monomers of fibrin was studied after 30 min of incubation at 20°C [10].

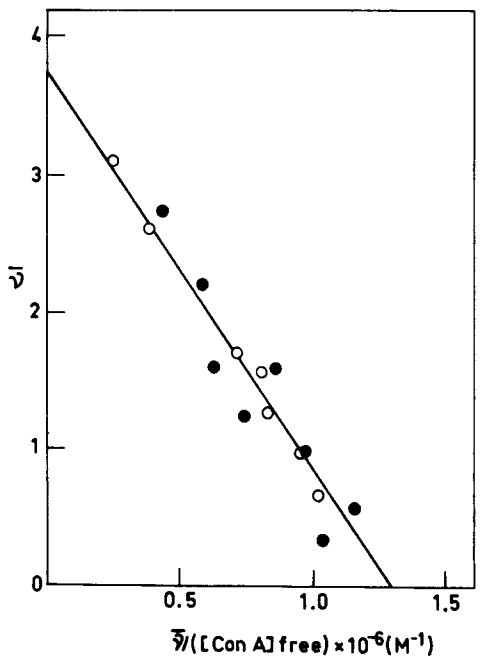

Fig. 1. Scatchard plot for the specific binding of ^{125}I-labelled concanavalin A to fibrinogen (●) and fibrin (○). Concentrations of fibrinogen and fibrin were $3 \cdot 10^{-6}$ M. The molecular weight of concanavalin A dimer used in the calculations of the experimental data was 55000.

Incubation of fibrinogen with low concentration of concanavalin A at pH 5.2 inhibits monomer aggregation. The opposite effect, i.e., an increase in the aggregation rate and a shortage in the usual delay in the aggregation was observed at high concentrations of the lectin (Fig. 2). On the other hand, incubation of soluble monomers of fibrin with concanavalin A produces, even at very small concentrations of the lectin, a progressive increase in their aggregation after the appropriate pH was reached (Fig. 3).

Effect of concanavalin on the release of fibrinopeptides by thrombin. Concanavalin A did not influence the release of fibrinopeptide A by thrombin at pH 5.2, and relative concentrations of concanavalin A/fibrinogen between 20 μg and 260 μg/mg. On the other hand, it was not possible to detect any fibrinopeptide B during the first 50 min when fibrinogen and fibrinogen-concanavalin A complexes were treated with thrombin at pH 5.2.

In our previous work [10] on the interaction of

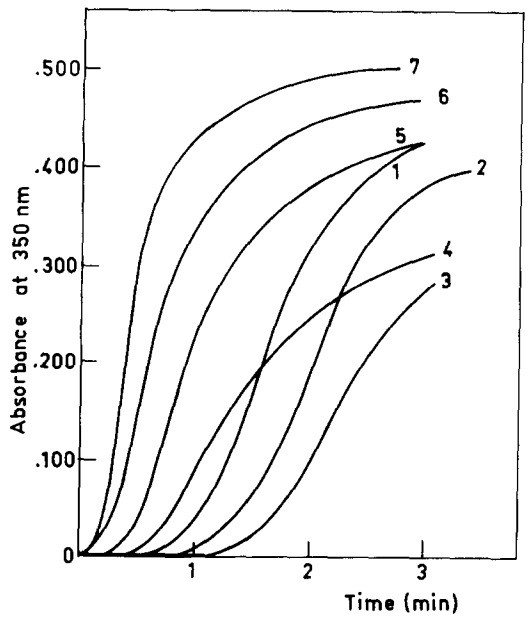

Fig. 2. Variation of the initial aggregation rate of fibrinogen-concanavalin A complexes after thrombin addition as a function of the lectin concentration. Fibrinogen concentration was 1 mg/ml and thrombin 0.15 units/ml. Tracings of the absorbance recording at 350 nm: pure fibrinogen (1) and fibrinogen-concanavalin A complexes; 10 μg/ml (2), 30 μg/ml (3), 90 μg/ml (4), 150 μg/ml (5), 300 μg/ml (6) and 500 μg/ml (7).

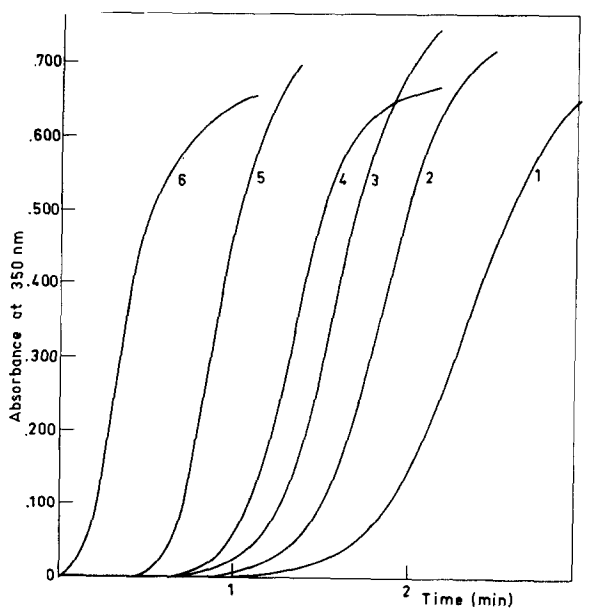

Fig. 3. Variation of initial aggregation rate of fibrin-concanavalin A complexes as a function of the lectin concentration. Fibrin concentration was 1 mg/ml. Tracings of the absorbance recording at 350 nm: pure fibrin (1) and fibrin-concanavalin A complexes: 5 μg/ml (2), 10 μg/ml (3), 16 μg/ml (4), 80 μg/ml (5) and 160 μg/ml (6).

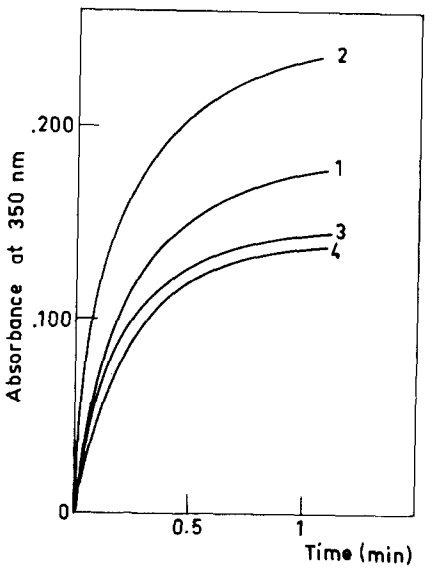

Fig. 4. Effect of concanavalin A on the fibrinogen-fibrin interaction. Fibrinogen solution (0.5 mg/ml in 0.018 M phosphate buffer (pH 7.8) containing 0.05 M NaCl and fibrin solution (0.5 mg/ml) in 0.02 M acetate buffer (pH 5.2) containing 0.1 M NaCl were incubated separately with concanavalin A (100 μg/mg of fibrinogen or fibrin). To 0.2 ml of acetate buffer, 0.3 ml of the fibrinogen solution (with or without concanavalin A) and 0.3 ml of fibrin solution (with or without concanavalin A) were added and the absorbance at 350 nm immediately recorded. Fibrinogen and fibrin (1), fibrinogen and fibrin-concanavalin A (2), fibrinogen-concanavalin A and fibrin (3), fibrinogen-concanavalin A and fibrin-concanavalin A (4).

fibrinogen with concanavalin at pH 7.8, the release of fibrinopeptides by thrombin in presence of concanavalin A was studied by measuring the trichloroacetic acid-soluble arginine, and no appreciable difference was detected with respect to pure fibrinogen. When studied again by HPLC it was found that the release of both fibrinopeptides A and B was slightly increase in presence of concanavalin A; at concentrations of the lectin from 50 to 100 μg per mg of fibrinogen, the amount of fibrinopeptides released was not more than 1.2-times greater than in absence of concanavalin A (data not shown).

On the other hand, preincubation of the lectin with thrombin (up to 5 mg concanavalin A/mg thrombin) for up to 1 h at room temperature did not have any influence on the lytic activity of the thrombin on the synthetic substrate S2238. This result could be influenced by the fact that the thrombin sample has a large proportion of deriva-

tive forms of α-thrombin; however the result is in accordance with the lack of inhibitory effect on the release of fibrinopeptides observed.

Effect of concanavalin A on the fibrinogen-fibrin interaction. The fibrinogen-fibrin interaction is strongly dependent on pH, ionic strength and temperature [19]. Under the present conditions (pH = 7, ionic strength = 0.11 and temperature = 20°C) the influence of concanavalin A on this interaction (Fig. 4) consisted of an increase in the rate of polymerization of fibrin when concanavalin A was incubated previously with the monomers of fibrin and a decrease when the incubation of the lectin was with the fibrinogen. A decrease was also observed when both fibrinogen and fibrin were incubated with concanavalin A.

Discussion

The specific binding of ^{125}I-concanavalin A to fibrinogen and fibrin shows, that under the conditions in which the molecular weight of concanavalin A is only 55000, there are four lectin-binding sites in the molecules of fibrinogen and fibrin. It has been suggested [20] that binding of concanavalin A to cells favours higher stability of the tetramer species even under conditions in which the dimer predominates. If this were the case, it could be possible that a concanavalin A dimer bound to a chain of carbohydrate would bind to another concanavalin A dimer giving place to a concanavalin A tetramer and the apparent four sites would be actually two sites per fibrinogen molecule. However, when concanavalin A at pH 5.2 was studied by analytical ultracentrifugation in the presence and absence of the specific sugar α-methyl-D-glucopyranoside, we found the same sedimentation behaviour, indicating that the binding of the saccharide to concanavalin A does not affect the dimer-tetramer equilibrium in agreement with the results reported by Senear and Teller [21].

Therefore, we conclude that the four chains of carbohydrate present in the fibrinogen molecule are accessible to concanavalin A dimer. This result indicates that steric hinderance regulates the accessibility of the different molecules to the carbohydrate chains of fibrinogen. In this sense concanavalin A tetramer (M_r 110000) only has accessibility to two of the four carbohydrate chains [10] and neuraminidase (M_r < 70000) is able to release the sialic acid from the four carbohydrate chains, but desialylation proceeds much more rapidly in the oligosaccharide chains bound to the Bβ chains [9].

The dissociation constants for the binding of concanavalin A to fibrinogen and fibrin at pH 5.2 are the same, indicating that the release of fibrinopeptides does not have any influence on the accessibility of the lectin to the carbohydrate chains of fibrinogen. This dissociation constant ($2.9 \cdot 10^{-6}$ M) is also equal, within the experimental error, to that obtained for fibrinogen-concanavalin A tetramer interaction [10] ($3.0 \cdot 10^{-6}$ M) suggesting that fibrinogen have the same affinity for concanavalin A dimer than for concanavalin A tetramer.

Binding of concanavalin A dimer to fibrinogen or fibrin leads to the formation of species with molecular weight ranging from 340000 (pure fibrinogen) to 560000 (a complex of one molecule of fibrinogen with upto four concanavalin A dimers), as demonstrated by ultracentrifugation. No aggregates of fibrinogen or fibrin could be detected even at concentrations of the divalent lectin as high as 1 mg per mg fibrinogen (6.25 mol concanavalin A/mol fibrinogen). Therefore, binding of two or more molecules of fibrinogen or fibrin through concanavalin A links does not seem to take place, in contrast to the observations using tetravalent concanavalin A [10].

This behaviour of concanavalin A dimer has already been reported by other authors. Hassing and Goldstein [22] found that below pH 5, concanavalin A dimer is able to bind simple carbohydrate haptens as well as polysaccharides but does not precipitate polysaccharides. Concanavalin A dimer is unable to precipitate glycogen although dimeric and tetrameric species have similar mitogenic activity [23]. Dimers induced by lowered temperature do not agglutinate erythrocytes although electron microscopy showed they could bind soluble glycoproteins [24].

The effect of concanavalin A dimer on the coagulation ratio of fibrinogen is the same as the effect of concanavalin A tetramer, that is, an inhibition at low concentrations of the lectin and an increase at high concentration. This increase is not due to previous formation of aggregates of fibrinogen, since they could not be detected when

fibrinogen is bound to concanavalin A dimer.

On the other hand, concanavalin A does not have any inhibitory effect on the release of fibrinopeptides and always produces an increase in the aggregation of fibrin monomers proportionally to the concentration of the lectin. To explain how the whole reaction is inhibited at low concentrations of concanavalin A whereas the partial reactions are not inhibited, the reaction between monomeric fibrin and fibrinogen that can take place at low thrombin concentrations has to be considered. If fibrinogen molecules bind to monomeric fibrin or to protofibrils the polymerization of fibrin will be inhibited [25].

It has been shown that fibrinogen-concanavalin A complexes inhibit polymerization of fibrin and even of fibrin-concanavalin A complexes. This suggests that binding of concanavalin A to fibrinogen can enhance the interaction fibrinogen-monomeric fibrin giving rise to an inhibition of the following step; the fibrin polymerization.

Taking into account all these considerations it can be concluded that the different effects of concanavalin A on the whole clotting reaction would be the result of favouring either the fibrinogen-fibrin monomer interaction or the fibrin polymerization, giving rise in the first case to an inhibition of the clotting process and in the second one to an acceleration. This can also be applied to concanavalin A tetramer-fibrinogen complexes.

As previously suggested [10], the effect of concanavalin A on the clotting process can not only be due to a simple masking of the electronegative charge of the sialic acid, since asialofibrinogen and its concanavalin A complexes always coagulate faster than those of fibrinogen. Therefore, an explanation of the effect of concanavalin A should include masking of electric charge in the neighbourhood of the carbohydrate. In this sense, it can be mentioned that citraconylation of the ϵ-amino groups that generates additional negative charges into the molecule renders fibrinogen incapable of polymer formation [26].

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