Ca BINDING OF RAT FIBRINOGEN AND FIBRIN(OGEN) DEGRADATION PRODUCTS

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1. Introduction

Insoluble fibrin formation occurs in several steps. In the first step fibrin monomers are formed by partial proteolysis of fibrinogen by thrombin (EC 3.4.21.5). In the next step these fibrin monomers aggregate and Ca accelerates this aggregation [1,2]. In the last step isopeptide bonds (crosslinks) are formed between the aggregated fibrin molecules by the action of a transglutaminase (factor XIIIa, fibrin-stabilizing factor), which requires Ca^{2+} for its activity [3].

Calcium has a protective effect on fibrinogen against heat denaturation [4,5].

Calcium ions also partially protect fibrinogen against plasmic degradation. This is based on our earlier observation [6,7] that in the presence of Ca, only one type of D fragment (D(cate), mol. wt 93 000) was formed which differed in molecular weight but not in amino-terminal amino acids from the D fragment formed in the presence of EGTA (D(EGTA), mol. wt 80 000). It was demonstrated that the difference is due to a difference in the size of the γ-chain remnants of D(cate) and D(EGTA) i.e., 38 000 and 25 000, respectively [6,7]. This led us to the conclusion that Ca protects the C-terminal part of the γ-chain remnants in the D(cate) fragments against further digestion and that the γ-chain remnants are degraded further to mol. wt 25 000 upon sequestration of Ca by EGTA.

When D-dimer formed from fully crosslinked fibrin is incubated with plasmin in the absence of Ca^{2+}, D(EGTA) is formed [6,7]. This indicates that also D-dimer is protected by Ca^{2+} against plasmic degradation.

These observations strongly suggest that fibrinogen D(cate) and D-dimer bind Ca^{2+} within their structures and that the protective effects could be explained by stabilization of a more compact structure.

Three high-affinity binding sites for Ca have been demonstrated in bovine fibrinogen [8].

The purpose of the present investigation was to study the binding of Ca to rat fibrinogen and plasmic fibrin(ogen) degradation products by means of equilibrium dialysis with special reference to the protective effect of Ca^{2+} in the plasmic degradation of fibrinogen.

2. Materials and methods

Rat fibrinogen and fibrin(ogen) degradation products were prepared and purified as in [7–9]. They were freed from Ca by overnight dialysis at 4°C against 0.05 M Tris, pH 7.5, 0.15 M NaCl and 0.003 M EGTA. EGTA was removed by dialysis against this Tris buffer without EGTA. The buffer had been freed from Ca by treatment with chelex (Biorad) as in [8]. Dialysis bags were pretreated as in [8].

Portions, 1 ml, of protein solution (3 mg/ml for fibrinogen and 1 mg/ml for degradation products) were dialysed for 2 days at room temperature against 100 ml Ca^{2+}-free Tris buffer. Experiments were carried out in 10^{-6}–10^{-3} M non-radioactive Ca. To each vessel a constant amount of radioactive Ca (20 μl 72 μg/ml ^{45}Ca solution; Amersham, Bucks., 24.5 Ci/g, batch no. 22 CA) was added. After dialysis 0.100 ml portions of inner and outer solutions were mixed with 10 ml scintillating liquid (Packard Dioxane Scintillator special MI-94) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Aliquots of

the dialysed samples were checked for possible degradation products formed during the experiment. This was done by SDS–polyacrylamide gel electrophoresis as in [9]. No indication for degradation was observed.

3. Results

To evaluate quantitatively the values for the number of Ca binding sites (n) and their association constants, r/C was plotted versus r, according to [10]. According to Scatchard's equation for one class of binding sites: $r/C = nK - Kr$, where r is the av. no. Ca^{2+} bound/mol protein at a free Ca^{2+} concentration C.

If a plot of r/C versus r yields a straight line, then the extrapolation of $r/C \rightarrow 0$ gives the value n, and the slope of the line (K) corresponds with the reciprocal value of the dissociation constant K_d, of this particular class of binding sites. As can be seen from fig.1 (fibrinogen), fig.2 (D(cate)) and fig.3 (D-dimer), no straight

Table 1
Number of calcium binding sites and dissociation constants (K_d) in rat fibrinogen and fibrin(ogen) degradation products

Class / Protein	I		II		III	
	Sites	K_d	Sites	K_d	Sites	K_d
Fibrinogen	3	3.1×10^{-6}	1–2	1.9×10^{-5}	5–8	5.6×10^{-4}
D(cate)	1	3×10^{-6}	1	1.9×10^{-5}	2–3	6×10^{-4}
D-dimer	2	1.5×10^{-6}	1–2	1.5×10^{-5}	not determined	

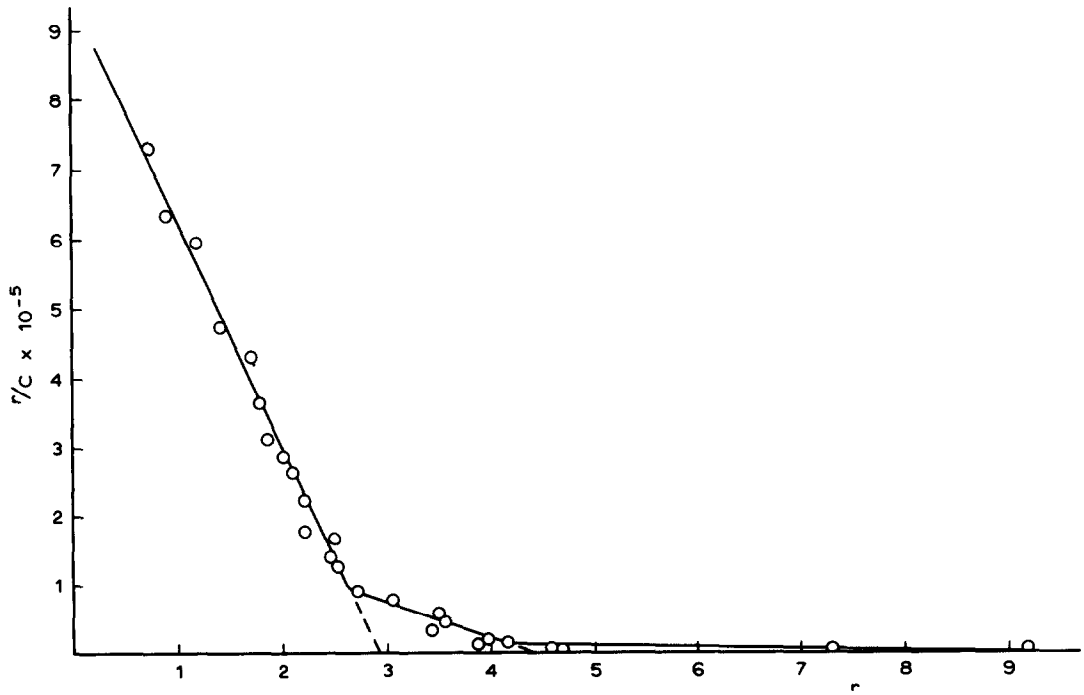

Fig.1. Scatchard plot of no. Ca^{2+} bound/mol rat fibrinogen and free [Ca^{2+}] (details in text).

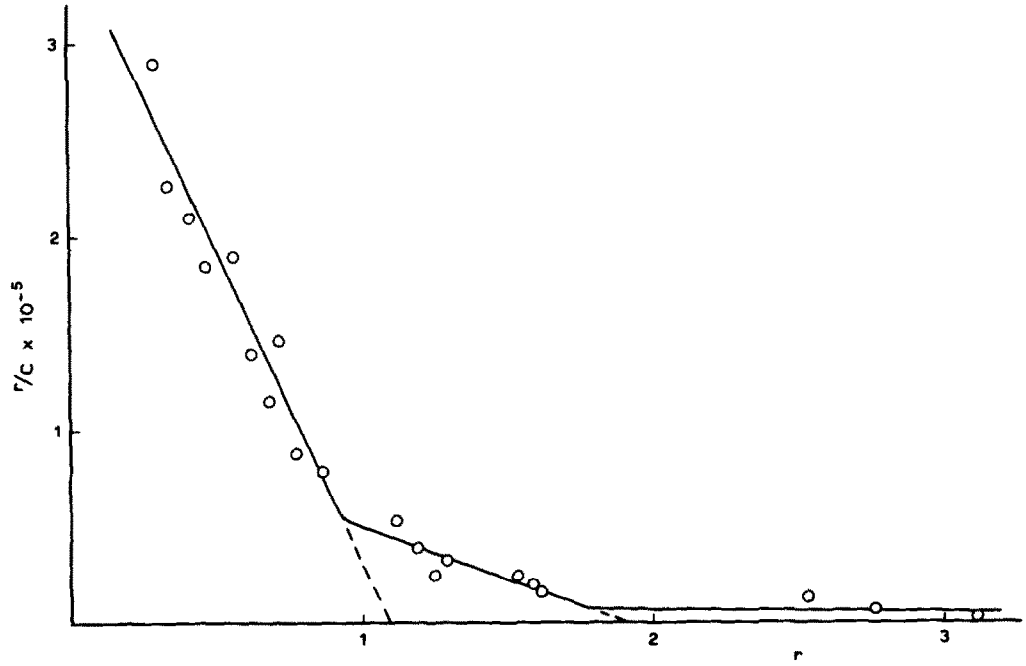

Fig.2. Scatchard plot of no. Ca^{2+} bound/mol rat D(cate) and free $[Ca^{2+}]$ (details in text).

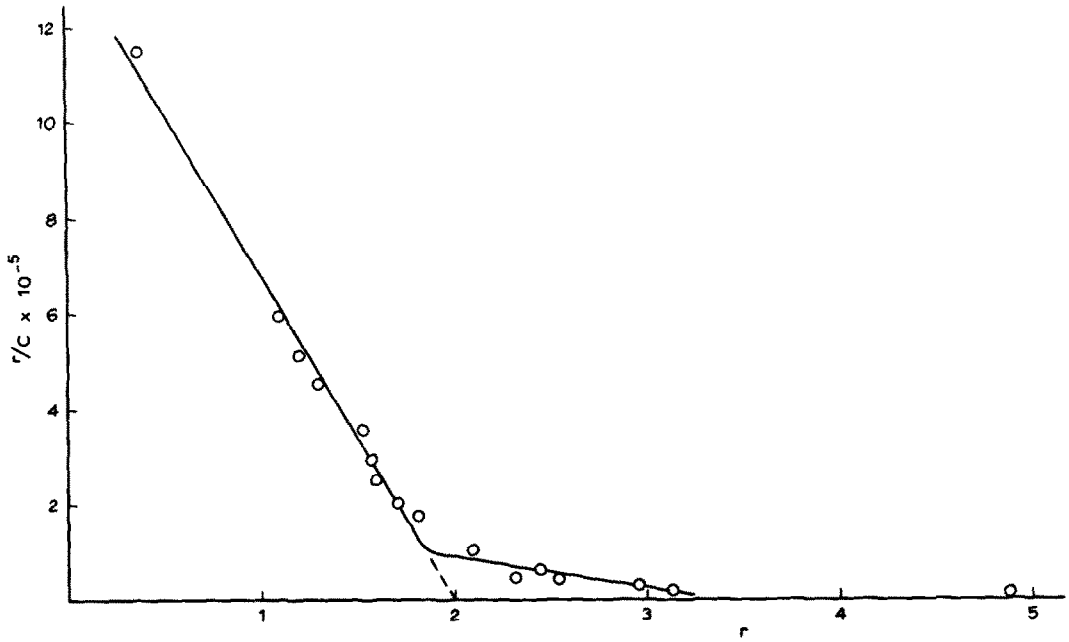

Fig.3. Scatchard plot of no. Ca^{2+} bound/mol rat D-dimer and free $[Ca^{2+}]$ (details in text).

lines are obtained and different classes of binding sites can be discriminated. Table 1 summarizes the results derived from the Scatchard plots for fibrinogen, D(cate) and D-dimer.

No Ca binding sites with K_d values comparable to class I and II were detectable in D(EGTA) and the E fragments derived from fibrinogen and crosslinked fibrin.

4. Discussion

Rat fibrinogen, like bovine fibrinogen [5], has three high-affinity binding sites for Ca. D(cate) has only one high-affinity binding site with nearly the same K. As each fibrinogen molecule comprises 2 mol D(cate) and 1 mol E, one would expect that one of the three Ca^{2+} in fibrinogen is bound by the E-moiety with the same K as found for D(cate). However, E appeared not to contain high-affinity binding sites. When a complete plasmic digest of rat fibrinogen was subjected to equilibrium dialysis, the number of Ca binding sites appeared to be two, when the amount of parent fibrinogen was taken to calculate r.

Obviously then, fibrinogen contains two binding sites structurally related to the D(cate) moieties and a third one, not related to either D(cate) or E, but inactivated because of the plasmic degradation or related to peptide(s) smaller than the cut-off of the dialysis tubing. Our finding that one out of the three high-affinity binding sites has deviating properties is in good agreement with the conclusions in [8]. They found that one out of the three high-affinity binding sites of bovine fibrinogen disappears upon lowering the pH to 6.0 or 5.5. It is peculiar that at pH 7.5 both in rat and bovine fibrinogen the third high-affinity binding site has a K_d which cannot be discriminated from the two identical ones.

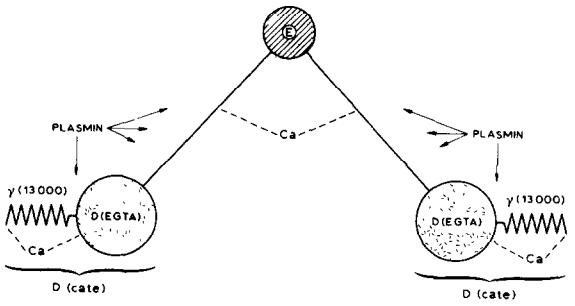

Fig.4. Schematic representation of the three high-affinity binding sites in rat fibrinogen. The solid lines between the schematic E and D(EGTA) regions of the molecule represent parts of all three Aα, Bβ, and γ-chains. The dotted lines indicate a binding of Ca. The arrows represent potential sites of plasmin attack. Their number is not related to the actual number of sites hydrolysed by plasmin. The vertical arrows indicate the minimum plasmic attack in the absence of Ca^{2+}, when D(EGTA) is formed from D(cate).

Interestingly D(EGTA) does not have high-affinity binding sites. This strongly suggests that the 13 000 mol. wt piece of the C-terminal part of the γ-chain in D(cate) is essential for the binding of Ca. This differs from conclusions in [5] that the C-terminal parts of the Aα-chains of fibrinogen, released early in the plasmic degradation are involved. On the basis of our findings fig.4 gives a schematic representation of the high-affinity binding sites in rat fibrinogen.

As was expected from our previous conclusions [8,10], that D-dimer consists of two molecules of D(cate) crosslinked by their γ-chains, D-dimer binds 2 mol Ca/mol protein with high affinity. The affinity is even higher than found for fibrinogen. This could indicate that Ca in fibrin is bound even tighter than in fibrinogen.

In conclusion, the present direct binding studies demonstrate that rat fibrinogen and the plasmic degradation products D(cate) and D-dimer strongly bind Ca^{2+} and that this binding in D(cate) and D-dimer is correlated with a protective effect against further plasmic degradation.

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