BINDING OF Ca^{2+} TO NORMAL AND DICOUMAROL-INDUCED PROTHROMBIN

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SUMMARY: The Ca^{2+} binding properties of normal bovine prothrombin have been studied and compared with those of an abnormal bovine prothrombin induced by dicoumarol. The normal prothrombin binds up to 10-12 Ca^{2+} per mole of protein. The three first Ca^{2+} were bound to sites which exhibited positive cooperativity. A Ca^{2+} dependent conformational change was demonstrated during the binding of the first three Ca^{2+}. In contrast with normal prothrombin, the dicoumarol-induced prothrombin had only one high affinity binding site. No ligand-induced conformational change was detected in this prothrombin.

The physiological activation of prothrombin to thrombin, which is a key step in blood coagulation, is known to require the presence of Ca^{2+} and the presence of phospholipid (1). The activation probably takes place with the protein bound to the surface of phospholipid micelles and Ca^{2+} seems to be necessary for this complex formation (2,3). It has been shown that the electrophoretic mobility of prothrombin in agarose gel varies with the concentration of Ca^{2+} in the buffer indicating that prothrombin binds Ca^{2+} fairly firmly (4).

Recently an abnormal bovine prothrombin induced by dicoumarol has been purified and characterized (5-7). The abnormal prothrombin has no prothrombin activity but shares antigenic determinants with normal prothrombin. Judging from the electrophoretic mobility in buffers containing Ca^{2+}, the abnormal prothrombin does not bind Ca^{2+}. Furthermore quantitative immunoprecipitation curves with Ca^{2+} or EDTA in the buffer have shown that Ca^{2+} induces a conformational change in normal prothrombin, but not in the dicoumarol-induced prothrombin (7).

In the investigation described below the Ca^{2+} binding

properties of the normal and the dicoumarol-induced prothrombin were compared.

MATERIALS AND METHODS

Normal bovine prothrombin was purified according to Ingwall and Scheraga (8) with slight modifications (6). Dicoumarol-induced prothrombin was purified from the plasma from two oxen treated with dicoumarol. The purification procedure described earlier was used (6). The antiserum against normal bovine prothrombin was the same as that used in earlier studies (5-7). $^{45}Ca^{2+}$ was obtained from the Radiochemical Center, Amersham.

In the Ca^{2+} binding studies the flow-dialysis method of Colowick and Womack was used (9). The experiments were performed at ambient temperature (23 ± 1^{o}). The two chambers of the dialysis cell were separated with a Technicon dialysis membrane. At a flow rate of 7.2 ml per min through the lower chamber of the cell a steady state was reached in 90 seconds, after which a 3 ml aliquot was taken and mixed with 10 ml of a gelling scintillator solution (Packard Insta-Gel) for radioactivity counting in a Packard Tricarb liquid-scintillation spectrometer. Before the dialysis experiments the proteins were equilibrated with 0.025 M Tris-HCl, 0.12 M in NaCl, pH 7.4 by filtration through a column of Sephadex G 25. When necessary, the proteins were concentrated in an Amicon ultrafiltration cell using the UM 10 membranes. Before use the dialysis cell and the ultrafiltration cell were washed with water obtained from a Millipore Super Q ultrafiltration plant. The concentrations of the two prothrombins were determined from their absorbancies at 280 nm using an extinction coefficient ($E^{1\%}_{1cm}$) of 14.5 (7). The protein concentration in the dialysis experiments was about 2 mg per ml.

Quantitative immunoprecipitation was performed essentially as described earlier (7). In order to study the effect of Ca^{2+} on the amount of precipitate formed, experiments were first made in 0.025 M Tris-HCl, 0.12 M NaCl pH 7.4 with 5 mM Ca^{2+} or 5 mM EDTA. The antiserum concentration was kept constant and the prothrombin concentration was increased until the difference in amount of precipitate formed with and without Ca^{2+} was largest (7). This concentration of normal prothrombin

(0.15 mg per ml) was chosen for the experiments in which the influence of the Ca^{2+} concentration on the amount of precipitate formed was studied. Before use the antiserum was dialyzed extensively against the buffer to remove Ca^{2+}. The antiserum was decomplemented by incubation with an antigen antibody precipitate prepared from human serum albumin and its corresponding rabbit antiserum (10). No corrections were made for the Ca^{2+} bound to the antiserum, flow dialysis experiments with antiserum only having shown that the amount of Ca^{2+} bound to the antiserum never exceeded 15 % of the total Ca^{2+} concentration.

RESULTS

Typical Scatchard plots of the results of flow-dialysis experiments with the normal and the dicoumarol-induced prothrombin are shown in Fig. 1. At low Ca^{2+} concentrations the normal prothrombin gave a binding curve with a positive slope,

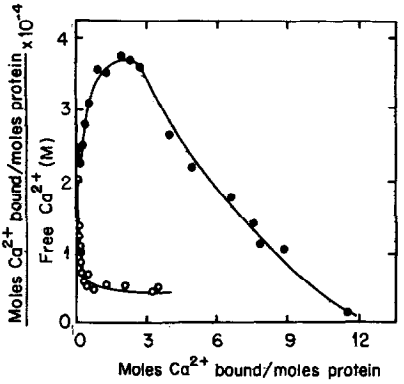

Fig. 1. Scatchard plot of Ca^{2+} binding by the normal and the dicoumarol-induced prothrombin. ●, Normal prothrombin; o, dicoumarol-induced prothrombin.

convex upwards, suggesting a positive cooperativity in the binding of Ca^{2+} with a maximum ratio of bound Ca^{2+}/free Ca^{2+} at 2 to 3 moles Ca^{2+} bound per mole of protein. From the diagram the total number of binding sites was 10 to 12. Assuming ten binding sites plotting of the data in Fig. 1 in a Hill diagram (11) gave two n-values: 1.35 for the binding of the first 3 Ca^{2+} ions and 0.91 for the subsequent binding (Fig. 2). The dicoumarol-induced prothrombin gave quite a different Scatchard plot (Fig. 1), showing the occurrence of both

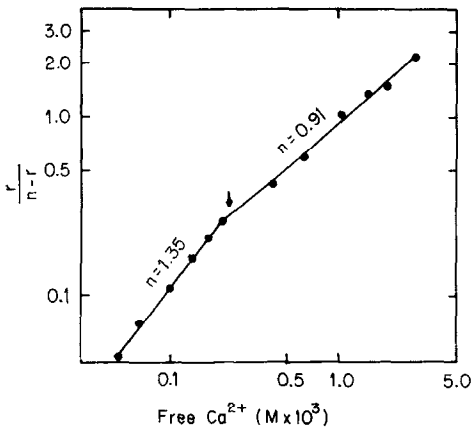

Fig. 2.

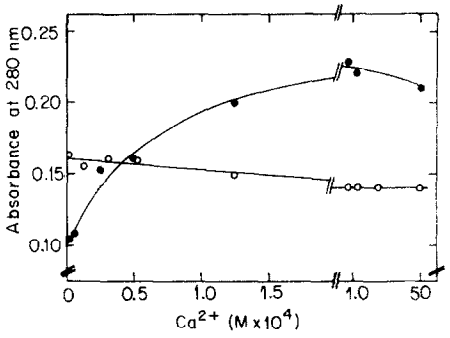

Fig. 3.

Fig. 2. Hill plot of the binding data for the normal prothrombin. r, number of binding sites occupied; n, total number of binding sites. n_H is the Hill coefficient. The arrow denotes the point on the curve corresponding to 3 Ca^{2+} bound.

Fig. 3. Effect of Ca^{2+} concentration on the amount of immunoprecipitate formed of the normal and the dicoumarol-induced prothrombin with anti-bovine prothrombin. For details see "Methods". •, Normal prothrombin; o, dicoumarol-induced prothrombin.

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high-affinity and very low-affinity binding sites. Extrapolation of the first linear segment of the curve to the X-axis gave one binding site with an apparent association constant of approximately 2 x $10^4 M^{-1}$. The second linear segment of the curve suggested several very low affinity sites probably representing nonspecific binding. To ascertain whether the binding curve for normal prothrombin was due to the formation of protein aggregates upon the binding of Ca^{2+} ions, prothrombin samples were subjected to analytical ultracentrifugation at 59780 rpm in wedge cells under the conditions used in the flow dialysis experiments[x] (same buffer, protein concentration and temperature). Quite symmetrical peaks sedimenting with the same velocity were obtained for samples containing Ca^{2+} ions (2 x 10^{-4}M, corresponding to the Ca^{2+} concentration

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[x] These analyses were kindly performed by Ulla-Britt Hansson, Ph.D. at the Chemical Center, University of Lund.

in the peak of the Scatchard plot) and for samples without Ca^{2+} ions.

Quantitative immunoprecipitation experiments were performed in which the concentration of Ca^{2+} was changed, while the antiserum and prothrombin concentrations were kept constant. As shown in Fig. 3, the amount of precipitate formed with the dicoumarol-induced prothrombin was fairly independent of the Ca^{2+} concentration. On the other hand, the amount of precipitate formed with the normal prothrombin increased to a Ca^{2+} concentration of approximately 0.15 to 0.20 mM, which corresponds to 2-3 Ca^{2+} bound per mole of prothrombin. In agreement with the results from the dialysis experiments at higher concentrations the curve plateaus which indicated that the cooperative effect was restricted to the binding of the 3 first Ca^{2+} ions.

DISCUSSION

The Scatchard plot for the binding of Ca^{2+} by the normal prothrombin is remarkable in that is shows a positive slope and is convex upwards at low Ca^{2+} concentrations. Similar binding curves were recently observed for the binding of Ca^{2+} to a protein from sarcotubular membrane under certain conditions (12) and for the binding of Mn^{2+} to transfer RNA (13). The finding suggests a ligand-induced conformational change in the normal prothrombin with positive cooperativity in the Ca^{2+} ion binding. The change in slope of the Hill plot from 1.35 to 0.91 after 3 Ca^{2+} ions have been bound is consistent with positive cooperativity in the binding of the first three Ca^{2+} ions only.

The cooperative Ca^{2+} binding observed in the present study precludes simple estimation of the apparent association constants for the different binding sites. Barton recently studied the Ca^{2+} binding of prothrombin and found a value of 2.9 to 3.1 for log K_a [+]. The total number of binding sites was found to be 10, a figure in good agreement with the present results. In another report the same author (14) proposed that 4 of the binding sites had still higher affinities (log K_a = 4), but he found no signs of cooperativity.

[+] Barton, P.G., Personal communication (1971)

It has been proposed that the role of Ca^{2+} in the prothrombin activation is to form bridges between prothrombin and phospholipids (2,3). If such bonds are responsible for the complex formation, a number of binding sites (carboxylic groups) must be involved, which for sterical reasons must form a cluster on the prothrombin surface. In the absence of phospholipid micelles such a cluster could, by chelation, bind calcium ions firmly to pairs of carboxylic groups. These assumptions are in accord with the finding that the calcium binding groups in human and bovine prothrombin are all present within a minor part of the molecule[&]. In the absence of calcium ions electrostatic repulsion would appear within such a cluster and possibly cause minor conformational changes in the molecule, as found in the quantitative immunoprecipitation experiments. These changes might reduce the affinity of the binding sites for Ca^{2+} ions. However, binding of the first Ca^{2+} ion will reverse the conformational change, with an increase in the affinity of the other binding sites as a result.

The cooperative effect and the conformational changes of the normal prothrombin were seen at very low calcium concentrations never seen in vivo. It therefore seems unlikely that they should serve any purpose in the normal activation of prothrombin.

The normal and the dicoumarol-induced prothrombin are identical with respect to amino acid composition, end group analysis and peptide mapping and both have an approximately identical number of amide groups (7). The difference in Ca^{2+} binding must thus be attributed to minor structural differences not revealed with these methods or to a conformational difference. In contrast with normal prothrombin, the dicoumarol-induced prothrombin bound firmly only one calcium ion, which apparently did not induce any conformational change. This suggests that the differences demonstrated between the two prothrombins are confined to that part of the molecule harbouring the Ca^{2+} binding sites, which has recently been shown to be the amino terminal part of the molecule[&]. Like normal prothrombin,

[&] Stenflo, J., To be published.

the dicoumarol-induced prothrombin can be activated to thrombin by trypsin (15) and staphylocoagulase (16). This implies that the dicoumarol-induced prothrombin is not activated in normal blood coagulation (6,17) because of a deficient Ca^{2+} ion binding and inability to form complexes with phospholipid.

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