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Mode of Action of Vitamin K. Calcium Binding Properties of Bovine Prothrombin†

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ABSTRACT: Due to recent observations which suggest that the calcium binding property of prothrombin results from the action of vitamin K, the binding of calcium to prothrombin has been examined more carefully. The binding curve is sigmoidal, and at 1 mM calcium, 4 mol of calcium is bound per mol of prothrombin. There was a strong dependence of calcium binding on pH with maximum binding occurring between pH's 7.5 and 9.5. The loss of calcium binding on either side of this range is very marked, suggesting an ionization event at these pH's. A biologically inactive prothrombin produced by dicumarol-treated cows has a lower calcium binding affinity, and at 1 mM less than 1 mol of calcium is bound per mol. In contrast to normal prothrombin, this abnormal prothrombin does not adsorb onto barium citrate, and this property appears to be related to the same sites that cause calcium binding. Modification of prothrombin with 8 M urea or 8 M urea plus reduction of disulfide bonds revealed that tertiary structure and disulfide bonds are necessary for maximum calcium binding. However, if the proteins were allowed to refold and reoxidize, calcium binding was fully restored. Similar treatment of the abnormal prothrombin did not result in any detectable calcium binding. These observations indicate that the abnormal prothrombin differs from the normal protein in some way other than its disulfide-bond arrangement or tertiary structure.

The hepatic biosynthesis of prothrombin and three other blood-clotting enzymes (factors VII, IX and X) is dependent upon the vitamin K status of the animal. Evidence has been presented to suggest that the vitamin acts to convert a preformed polypeptide chain to active prothrombin (Suttie, 1970; Shah and Suttie, 1971). In support of this general mechanism, Stenflo (1970) has reported the existence, and partial purification of an inactive prothrombin which is found in the circulatory system of cows treated with the vitamin K antagonist, dicumarol. There have been a number of other reports of new proteins or prothrombin of low specific activity in the plasma of vitamin K deficient or dicumarol-treated animals (Dulock and Kolmen, 1968; Hemker and Muller, 1968; Ganrot and Niléhn, 1968; Josso *et al.*, 1968; Malhotra and Carter, 1971; Morrissey *et al.*, 1972; Johnson *et al.*, 1972; Pereira and Couri, 1971, 1972). What relationship all of these have to the protein described by Stenflo is not yet clear.

We have reported the purification of the protein described by Stenflo, and have demonstrated that its amino acid and carbohydrate content is similar to that of normal prothrombin (Nelsestuen and Suttie, 1972b). Of the many properties tested the only differences observed between this abnormal prothrombin[1] and prothrombin are the biological activity, the failure of abnormal prothrombin to adsorb quantitatively onto barium citrate, and, as indicated by Stenflo (1970) and Josso *et al.* (1970), the alteration of the electrophoretical mobility of prothrombin in the presence of calcium ions.

The observations on the properties of normal and abnormal prothrombin indicate that the major difference between the two proteins is in the calcium binding site. The available data also suggest that formation of this calcium binding site, which is required to bind phospholipids to prothrombin (Bull *et al.*, 1972), may result from vitamin K action. We report here studies on the calcium binding by the prothrombin proteins.

Materials and Methods

Prothrombin. Bovine prothrombin was purified by a modification (Nelsestuen and Suttie, 1972a) of the method of Ingwall and Scheraga (1969) and by acrylamide gel electrophoretic analysis contained no single contaminating protein which accounted for as much as 1% of the total protein. The abnormal prothrombin was purified as previously described (Nelsestuen and Suttie, 1972b) from the plasma of dicumarol-treated cows. The purity of this protein approached that of normal prothrombin as judged by a quantitative antibody assay, by acrylamide gel electrophoretic analysis, and by

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[1] The inactive protein produced by dicumarol-treated cows will be referred to simply as abnormal prothrombin while the normal enzyme will be referred to as prothrombin or normal prothrombin.

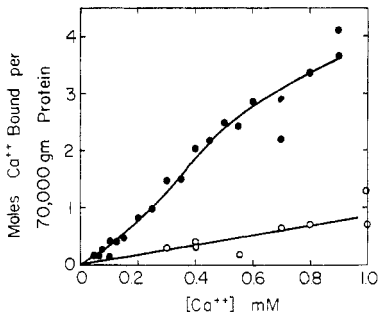

FIGURE 1: Calcium binding by normal and abnormal prothrombin. The general procedure used is described in the Methods section. All points were obtained in Tris buffer (pH 7.95) with 140 μg of protein in a total volume of 200 μl. Calcium bound by normal prothrombin (●); calcium bound by abnormal prothrombin (○).

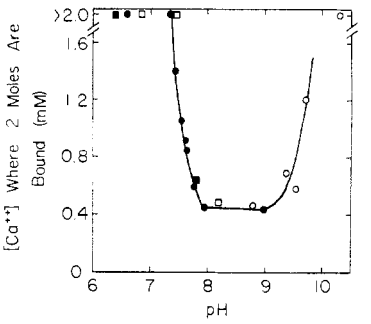

FIGURE 2: The pH dependence of calcium binding by prothrombin. The calcium concentration where 2 mol of calcium was bound per mol of prothrombin was determined from calcium binding curves run at various pH's. At least six calcium concentrations were used to define the curve at each pH. The general procedure used is described in the Methods section. All experimental points were obtained with 140 μg of protein in a total volume of 200 μl. Tris-buffered solutions (●); glycine-buffered solutions (○); cacodylate-buffered solutions (□); imidazole-buffered solutions (■).

the amount of thrombin activity generated by *Echis carinatus* venom. However, when assayed by the two-stage method of Shapiro and Waugh (1966), prothrombin had a final specific activity of 2200 units/mg while the abnormal prothrombin contained only 78 units/mg thus demonstrating the lack of biological activity of this protein. Protein concentrations were based on an $E_{280}^{1\%} = 16.5$ (Ingwall and Scheraga, 1969).

Calcium Binding Measurements. Binding of ^{45}Ca was determined by the general procedure for measuring radioactive ligand binding to proteins by ultrafiltration as described by Paulus (1969). Prothrombin and abnormal prothrombin in 5 mM sodium acetate (pH 6.0) were mixed with the appropriate amounts of concentrated buffer, radioactive $CaCl_2$ solutions, and NaCl to give solutions which contained the indicated Ca^{2+} concentration, 0.15 M NaCl, and 0.1 M of the various buffers used. All solutions contained ^{45}Ca at a specific activity of 174 cpm/nmol. The solutions were forced through 7 mm diameter UM-10 Diaflo membranes in a Metaloglass, Inc., apparatus by the use of nitrogen at a pressure of 40 psi. After filtration was complete, the bottom side of membranes were washed with ethylene glycol and the membranes removed. The washed membranes were suspended in 0.5 ml of NCS solubilizer (Nuclear-Chicago Corp.) and 15 ml of counting solution (5 g of 2,5-diphenyloxazole and 0.12 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. of toluene) was added. Radioactivity was determined in the ^{14}C window of a Packard Model 3375 Tri-Carb scintillation spectrometer. Blanks run without protein demonstrated that the background count was proportional to the original calcium concentration and was independent of the buffer used.

Adsorption of Proteins onto Barium Citrate. Proteins were diluted with 0.02 M sodium citrate to a 280-nm absorbance of 0.25. One-twelfth volume of 1 M barium chloride was added with mixing and the precipitate was centrifuged down after 10 min at 0°. The absorbance of the supernatant was determined at 280 nm, corrected for dilution by the barium chloride solution, and the per cent of the protein adsorbed was calculated. Spectral analyses of the protein solutions after barium citrate adsorption indicated that the 280-nm absorbance was due to protein and not to light scattered from residual barium citrate particles.

Modifications of Prothrombin. Solutions of prothrombin or the abnormal prothrombin (1.4 mg/ml) in 0.1 M Tris buffer (pH 8.0) were made 8 M in urea by the addition of solid urea with or without the addition of 10 mM dithiothreitol, and allowed to stand at 4° for 20 hr. The solutions were dialyzed for 24 hr against several changes of 0.15 M NaCl–5 mM sodium acetate (pH 6.0) and analyzed. The sulfhydryl groups were quantitated by treatment with 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959) and 8 M urea in 0.1 M phosphate (pH 7.0). An extinction coefficient of 13,600 at 412 nm was used for quantitation.

Results

Calcium Binding. The ability of both the normal and abnormal prothrombin to bind calcium is shown in Figure 1. Apparent calcium binding attributable to the Donnan effect would be negligible at the high ionic strengths used. Normal prothrombin bound calcium much more effectively, and at a calcium concentration of 1 mM about 4 mol of calcium was bound per mol of protein (mol wt 70,000) (Ingwall and Scheraga, 1969). At calcium concentrations and pH's which resulted in more than 4 mol of calcium bound a great deal of variability was observed, and the highest calcium binding ever observed in many runs over a wide range of pH was 6 mol/70,000 g of protein. The sigmoidal characteristic of the binding curve, which suggests some cooperative effect, did not allow the extrapolation of reciprocal plots to determine the maximum calcium bound by normal prothrombin.

The extent to which each of these proteins was adsorbed onto barium citrate was also examined. Although normal prothrombin was always quantitatively adsorbed, only 24–40% of the abnormal prothrombin was adsorbed in a number of trials. Under the same conditions, diluted plasma proteins were found to adsorb to an extent of 20%. This low adsorption of non-prothrombin proteins would probably be removed by washing the barium citrate during the normal isolation procedure for prothrombin (Moore *et al.*, 1965; Nelsestuen and Suttie, 1972a).

pH Dependence of Calcium Binding. Due to the sigmoidal nature of the calcium binding (Figure 1) and the lack of a clearly defined maximum value, it was not possible to directly plot calcium binding *vs.* pH. Rather, partial binding curves were determined at various pH's and the calcium concentration at which 2 mol of calcium was bound per mol of prothrombin was calculated from the curves and plotted against pH (Figure 2). The sigmoidal properties of the binding were observed at all pH's where calcium was detectably bound. The value of 2 mol of calcium bound was chosen as it was in

the most accurately measurable portion of the binding curve and it should bear some resemblance to a dissociation constant. Calcium concentrations over 2 mM were not studied since the abnormal protein binds nearly 2 mol of calcium at this concentration.

As can be seen from Figure 2, the maximum calcium binding occurred over a pH range of about 7.5–9.5. The sharp, reversible loss of binding outside of this range suggests that some group associated with the calcium binding is ionizing at these pH's. The use of several different buffers demonstrates that the pH dependence is not related to the buffer used.

Barium citrate adsorption was not so strikingly dependent on pH but was at least partially pH dependent, as shown by the respective percent of prothrombin adsorbed with the following buffers: imidazole (pH 6.7), 59% adsorbed; imidazole (pH 7.8), 95% adsorbed; glycine (pH 9.7), 94% adsorbed; and glycine (pH 10.3), 65% adsorbed.

Calcium Binding to Modified Prothrombin. It has been suggested that the mechanism of action of vitamin K may involve a rearrangement of tertiary structure or a rearrangement of disulfide bonds in prothrombin. These possibilities were tested by determining if they could account for the differences in calcium binding observed between normal and abnormal prothrombin.

The results of calcium binding by prothrombin which was modified by denaturation in urea and dithiothreitol are shown in Figure 3. As can be seen, the partially oxidized protein-bound calcium slightly less tightly than native prothrombin. The addition of 10 mM mercaptoethanol to these proteins during ultrafiltration had no effect on calcium binding by the denatured prothrombin but slightly inhibited calcium binding by normal prothrombin. After complete reoxidation of the sulfhydryls had occurred, the calcium binding was almost completely restored with 2 mol bound at 0.46 mM calcium. Therefore, it is apparent that while intact tertiary and disulfide structures are necessary for maximum calcium binding to normal prothrombin, the spontaneously formed structures are adequate. The spontaneously formed structures for the abnormal prothrombin are however still inadequate to cause calcium binding. Consequently, alterations in these structural properties can not explain the calcium binding differences betweed the two proteins. In addition, this treatment did not alter the barium citrate adsorption properties of these proteins. No biological activity was recovered in either of these modified proteins as determined in the two-stage assay system.

One effect which denaturation had on the calcium binding by normal prothrombin (Figure 3) occurred above the point where 3–4 mol of calcium is bound. The loss of ordered calcium binding evident for normal prothrombin under these conditions is apparent in Figure 3 and was observed to occur when tested at pH 7.9, 8.5, and 9.0. The denatured protein showed no such loss and the determinations were all within the experimental error of the line drawn in Figure 3 (at 3.5 mM calcium, the background subtraction is 50% of the total counts observed). The important parameter in the loss of ordered binding is the number of ligands bound and not calcium concentration. The experiments in Figure 2 often involved high calcium concentrations without loss of reproducibility, but the total amount bound never exceeded 4 mol. At high calcium concentrations (greater than 1.0 mM) normal prothrombin sometimes precipitated on the walls of the ultrafiltration apparatus. This would result in a loss of observed binding since the insoluble protein was not on the filter, and

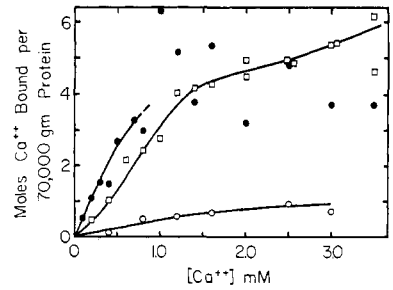

FIGURE 3: Calcium binding by modified prothrombins. Both normal and abnormal prothrombin were treated with urea and dithiothreitol and dialyzed as described in the Methods section. Calcium binding to the proteins was determined by ultrafiltration in pH 8.5 Tris buffer as described in the Methods section. At the beginning of the ultrafiltration the modified normal prothrombin contained 15 free sulfhydryls/70,000 and the abnormal prothrombin was completely reoxidized. All points were obtained with 140 μg of protein in a total volume of 200 μl. Untreated normal prothrombin (●); abnormal prothrombin treated as indicated (○); and normal prothrombin treated as indicated (□).

radioactive calcium associated with it would not be detected in the assay. This explanation alone would not appear to satisfactorily explain the observations, but it is apparent that high calcium binding causes a change in the prothrombin molecule which does occur in the denatured protein. Exactly what protein structural changes may be involved are not known, but it has been demonstrated that excess calcium inhibits the prothrombin activation reaction (Yue *et al.*, 1972). Perhaps this phenomenon is related to the observations we have made and that under conditions of excess calcium, prothrombin is no longer a good substrate for the activating enzymes.

Discussion

The results of these studies extend the observations of Stenflo (1970) and Josso *et al.* (1970) that the normal and abnormal prothrombins differ in their interactions with calcium ions. The results demonstrate the following. (1) Normal prothrombin binds calcium much more avidly than abnormal prothrombin. (2) This calcium binding is dependent on pH. (3) The native tertiary and disulfide structures are necessary for maximum calcium binding but the spontaneously formed structures are adequate for substantial calcium binding. (4) The adsorption of prothrombin by barium citrate appears to be closely related to the ability of the protein to bind calcium.

These data suggest that the various differences observed between the normal and abnormal prothrombin are due to the same property of the protein and that this is a result of the action of vitamin K. That is, calcium binding is essential for activation of prothrombin to thrombin, and the calcium binding sites are also responsible for the quantitative adsorption of prothrombin onto barium citrate. If this is true, it would follow from the above observations that vitamin K does not function in tertiary structure rearrangement or disulfide bond rearrangement during the synthesis of the protein, and that there must be a chemical difference between these two proteins. The vitamin K sensitive step could be an unknown amino acid alteration, the attachment of a prosthetic group, or possibly a proteolytic cleavage. The latter possibility would necessarily involve a small peptide since the molecular weights are indistinguishable (Nelsestuen

and Suttie, 1972b; Stenflo, 1970). The change mediated by the vitamin would necessarily involve the chemical features responsible for calcium binding by prothrombin and may account for some of the observed properties of calcium binding such as the pH dependence.

The recent report by Yue *et al.* (1972) on the calcium binding by bovine factor X, another vitamin K dependent protein, indicates that there are 3 mol of calcium bound with a dissociation constant of 2.2×10^{-4} l./mol and that an additional 25 mol of calcium can be bound with a dissociation constant of 2.5×10^{-3} l./mol. Although the binding curves we obtained do not allow calculations of this type for direct comparison, an extrapolation of the slope of the binding curve for the denatured prothrombin at high calcium concentrations (Figure 3) to zero calcium concentration indicates that between 3 and 4 mol is bound tightly. Additional calcium is bound loosely or about to the extent that calcium binds to the abnormal protein. Since the synthesis of both factor X and prothrombin is vitamin K dependent, the more tightly bound calcium in each case may be due to the same groups on each enzyme and the studies on these two enzymes may be directly comparable.

The rapid loss of calcium binding at pH 7.5 and 9.7 suggests that there may be ionizable groups associated with calcium binding which have pK values in this region. There are several possible amino acid functional groups ionizing at the higher value but only a few that ionize at neutral pH. A recent report by Klarman *et al.* (1972) on calcium binding by the protein hemocyanin, implicates histidine in a binding role which would be consistent with an ionization near neutral pH. Phosphate could also be an effective calcium binder, and has an ionization near neutrality. An analysis of the phosphate content of prothrombin (Nelsestuen and Suttie, 1972b) has, however, indicated that there is not sufficient phosphate present to account for the calcium binding we have observed.

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