The binding of Gla-containing proteins to phospholipids

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It is demonstrated here that osteocalcin, the Gla-containing protein from bone, is unable to interfere with the binding of the blood coagulation factors to phospholipid vesicles. Therefore, it seems that besides the Gla residues other structural features of the coagulation factors are required for their effective binding to phospholipid surfaces.

Vitamin K *γ-Carboxyglutamic acid* *Prothrombin* *Carboxylase* *Osteocalcin* *Blood coagulation*

1. INTRODUCTION

Since the discovery of Gla it has been frequently suggested that the Gla residues in the 4 'vitamin K-dependent' coagulation factors are directly involved in the binding of these factors to negatively charged phospholipid surfaces. The presumed binding of the Gla residues was thought to occur via Ca^{2+} [1–4]. Since the number of high-affinity calcium-binding sites more or less equals the amount of Gla residues in the coagulation factors [1–5], it was assumed that one Ca^{2+} is centered between the two carboxyl groups of Gla and two negatively charged phospholipids, as represented in fig.1. Although the formation of this hypothetical complex is not theoretically impossible, the question remains as to how one Ca^{2+} can sufficiently neutralize the 4 negative charges in this complex. One would rather think that after Ca^{2+} has been bound to a Gla residue, the resulting $Gla^{2-} \cdot Ca^{2+}$ complex has no net charge and hence is not involved in the binding of the protein to negatively charged phospholipid surfaces.

Secondly, a consequence of the model proposed in fig.1 is that Gla-containing proteins which are not involved in blood coagulation should also be able to bind to phospholipids and compete with the coagulation factors for binding sites on the phospholipid surface. We have examined this possibility by determining whether the activation of blood coagulation factors at limiting phospholipid concentrations is inhibited by purified osteocalcin and present here the results.

2. MATERIALS AND METHODS

Bovine osteocalcin was prepared as described for decarboxylated osteocalcin [6] except that the

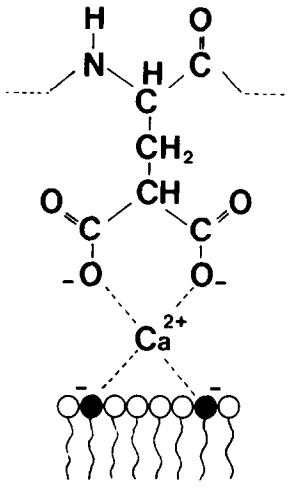

Fig.1. Hypothetical model for the binding of a Gla residue to negatively charged phospholipids.

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decarboxylation step was omitted. The presence of osteocalcin in the various preparations was tested with the aid of a radioimmunoassay (Immunonuclear).

Bovine coagulation factors II (prothrombin) and X were prepared as in [7] and factor X was activated with Russell's viper venom [8]. The activation of prothrombin in a purified system was measured with a chromogenic assay [7] at 37°C in reaction mixtures containing 0.175 M NaCl, 0.05 M Tris–HCl (pH 7.9), 0.25 μM prothrombin, 1 nM factor Xa, 10 mM $CaCl_2$, 50 μM phospholipid (sonicated vesicles containing 20% 18:1 phosphatidylserine and 80% 18:1 phosphatidylcholine, 0.47 mM chromogenic substrate S2238 (AB Kabi Diagnostica) and osteocalcin as indicated. The reaction mixtures were preincubated for 5 min at 37°C in the absence of factor Xa.

The coagulation times in plasma were determined with a one-stage coagulation assay using normal citrated bovine plasma and various dilutions of bovine thromboplastin [9].

3. RESULTS

3.1. *The effect of osteocalcin in a purified prothrombinase assay system*

Using purified bovine coagulation factors and a limiting concentration of phospholipid vesicles, the activation of prothrombin by activated factor X was measured in the absence and presence of various concentrations of osteocalcin. The rate of thrombin formation was measured in duplicate experiments and after 3 different incubation periods (table 1). At the highest concentration the excess of osteocalcin was 800-fold with respect to prothrombin and 200000-fold with respect to activated factor X. Nevertheless these amounts of osteocalcin did not influence the rate of prothrombin activation.

Table 1

Activation of prothrombin in the absence and presence of osteocalcin

Time elapsed since start of the reaction (min)	Prothrombin activation rate (mol IIa formed/min per mol Xa)		
	A	B	C
2	1.21	1.26	1.26
4	1.21	1.24	1.30
6	1.17	1.22	1.25

The osteocalcin concentrations were 0 μM (A), 10 μM (B) and 200 μM (C). The data are the means of two duplicate experiments. Experimental details are given in section 2

3.2. *The effect of osteocalcin on the coagulation time of plasma*

Using bovine plasma and decreasing concentrations of thromboplastin we also measured the effect of osteocalcin on the rate of clot formation in whole plasma. By using various dilutions of thromboplastin, in this experiment a system was also created in which the concentration of phospholipids was rate-determining. It was checked that the addition of purified phospholipids (vesicles containing 4 μM phosphatidylserine and 16 μM phosphatidylcholine) to such a system caused a substantial decrease in clotting time. As shown in fig.2, up to a concentration of 0.5 mM osteocalcin did not influence the coagulation time of plasma. Since the plasma concentrations of prothrombin, factor X and factor VII are 2, 0.2 and less than 0.02 μM, respectively [3,8,10], this experiment demonstrates again that even if osteocalcin does possess a certain affinity towards phospholipids, this affinity is more than 3–5

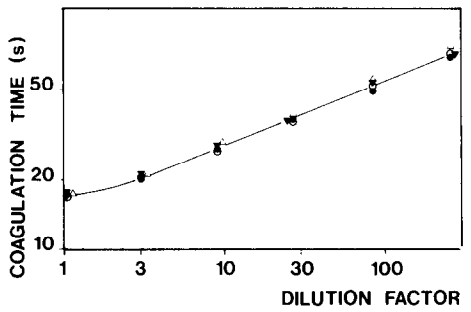

Fig.2. The influence of osteocalcin on the coagulation time of plasma. The osteocalcin concentrations were 0 mM (●—●), 0.1 mM (○—○), 0.25 mM (▼—▼) and 0.5 mM (△—△). The one-stage coagulation times were measured at increasing dilutions of thromboplastin in buffer A (0.1 M NaCl, 0.05 M Tris–HCl, pH 7.0). The reaction mixtures contained 0.1 ml plasma, 0.1 ml diluted thromboplastin, 0.1 ml osteocalcin in buffer A or buffer A alone and 0.1 ml 30 mM $CaCl_2$.

orders of magnitude lower than that of the Gla-containing coagulation factors.

4. DISCUSSION

Osteocalcin is a protein of M_r ~5000 and contains 3 Gla residues on the same face of one helix, spaced at intervals of about 5.4 Å [11]. The M_r values of the 4 vitamin K-dependent coagulation factors range between 50000 and 70000 and these proteins contain 10–12 Gla residues per molecule [3,10]. From these data it may be calculated that, when expressed as a percentage of the total amount of amino acid residues, the Gla content of osteocalcin is 3–4-times higher than that of the coagulation factors. When the presence of Gla residues is the only requirement for a protein to be able to bind to negatively charged phospholipids, one might therefore expect that the affinity of osteocalcin for phospholipid vesicles should not differ by many orders of magnitude from that of the coagulation factors. Nevertheless we have not been able to detect any competition between the phospholipid binding of coagulation factors and that of osteocalcin, although the latter was present in great excess. Therefore, we believe that an alternative explanation for the role of Gla residues in the protein–phospholipid interaction should be considered.

From the amino acid sequence in bovine prothrombin [12] it may be readily seen that among the first 48 amino acid residues 10 Gla residues are found, together with 6 amino acids containing a positively charged side chain (fig.3). At a neutral pH, the net charge of this part of the molecule is strongly negative (−15), whereas that of the rest of the fragment-1 region is slightly positive (+3). Obviously, saturation of the Gla residues with Ca^{2+} will have a profound impact on the conformation and properties of the NH_2-terminal part of the prothrombin molecule, because the net charge changes from −15 to +5. One of the results of this binding will be that the net charge of fragment-1 as a whole is now +8, with most of the positively charged side chains positioned at the NH_2-terminus. Without doubt this structure will have a high affinity towards negatively charged phospholipids. The model also enables us to explain why descarboxyprothrombin is inactive in the blood coagulation process: because of the relatively weak interaction

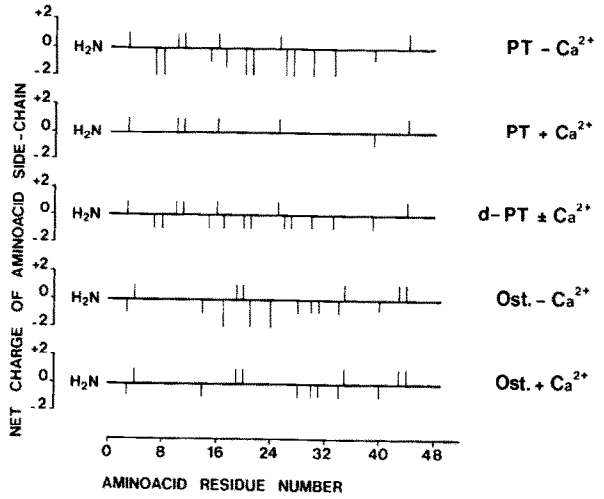

Fig.3. Charge distribution in the NH_2-terminal part of various proteins. Top to bottom: prothrombin in the absence of Ca^{2+}, prothrombin in the presence of Ca^{2+}, descarboxyprothrombin in the absence and presence of Ca^{2+}, osteocalcin in the absence of Ca^{2+} and osteocalcin in the presence of Ca^{2+}. The amino acid sequence of prothrombin is from [12] and that of osteocalcin from [11].

between Glu residues (instead of Gla) and Ca^{2+}, these residues are not neutralized at physiological Ca^{2+} concentrations. As a result the net charge of the first 48 amino acid residues will be −5 and no binding to phospholipids will occur.

Similarly, it may be seen that the net charge of osteocalcin is −7 and after neutralization of its 3 Gla residues a slightly negatively charged molecule still remains. So it is not expected that conditions can be found under which osteocalcin will bind to negatively charged phospholipids. It seems possible, therefore, that Gla residues in a certain protein only serve to bind Ca^{2+}, whereas the eventual affinity of the protein towards negatively charged phospholipids is related to the charge distribution in the rest of the molecule. Although the present results are far from complete and may hardly be used in favor of one of the models for protein–phospholipid interaction, we believe that the model explained in fig.3 should be taken into consideration for future investigations.

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