Vol. 142, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1987 Pages 113-119

THE EFFECT OF GLA-CONTAINING PROTEINS ON THE PRECIPITATION OF INSOLUBLE SALTS

Peet G.F. van de Loo, Berry A.M. Soute, L. Johan M. van Haarlem, and Cees Vermeer▼

Department of Biochemistry, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands

Received November 24, 1986

The precipitation of insoluble salts containing divalent metal ions is inhibited by Gla-containing proteins of various origin. In this paper we demonstrate that:
1. Gla-residues are required for the inhibitory activity;
2. the inhibition is effected by a protein which in vivo is bound to calcified tissue (osteocalcin) as well as by proteins occurring in blood plasma (factor X) and urine (the urinary Gla-protein);
3. The inhibitor concentration required for 50% precipitation-inhibition varied slightly from one salt to the other, but no marked differences were observed between the effects of the various Gla-containing proteins used;
4. Precipitation-inhibition occurred in all phosphates (Be, Ca, Mn and Zn) and in all calcium salts (phosphate, oxalate and carbonate) tested.

Vitamin K is involved in the vitamin K-dependent post-translational modification of proteins. The reaction at issue is the carboxylation of protein-bound glutamic acid residues into γ-carboxyglutamic acid (Gla) residues. Vitamin K in its hydroquinone form acts as a coenzyme for the carboxylating enzyme system, which is found in the microsomal fraction of various mammalian tissues [1-3]. The function of Gla-residues in proteins is the relatively strong binding of Ca^{2+}.

Numerous Gla-containing proteins have been discovered during the last few years, but those which have been characterized most extensively may be subdivided into two groups. The first group comprises the Gla-containing proteins which are produced by the liver (the blood coagulation factors II, VII, IX and X, the coagulation inhibiting proteins C and S), and which mainly occur in blood plasma [4]. The second group is formed by Gla-containing proteins mainly occurring in calcified tissues such as bone, dentin, renal stones and hardened atherosclerotic plaques [5-8]. It seems probable that the Gla-containing protein found in renal stones is similar to the Glycoprotein Crystallization Inhibitor (GCI) described by Nakagawa et al. [9], which was obtained from human urine.

▼To whom all correspondence should be addressed.
Abbreviation used: Gla = γ-carboxyglutamic acid.

0006-291X/87 $1.50

It has been reported that osteocalcin, the Gla-containing protein from bone, stongly inhibits the precipitation of calcium phosphate from supersaturated solutions of calcium and phosphate [5]. Furthermore GCI was demonstrated to inhibit the calcium oxalate crystal growth [9]. We have investigated a) if different Gla-containing proteins (blood coagulation factor X, osteocalcin and the urinary Gla-protein) inhibit the precipitation of calcium phosphate to a similar extent; b) if these proteins also inhibit the precipitation of insoluble phosphates from other divalent metal ions and c) if they inhibit the precipitation of other insoluble calcium salts. The results of these investigations are presented in this paper.

MATERIALS AND METHODS

Chemicals. $^{7}BeCl_2$ (350 Ci/mmol), $^{45}CaCl_2$ (1.8 Ci/mmol), $^{54}MnCl_2$ (5.4 mCi/mmol) and $^{65}ZnCl_2$ (65 Ci/mmol) were purchased from Amersham (UK) and Atomlight from New England Nuclear (GFR). Bovine serum albumin was obtained from Sigma (USA). All chemicals were of the highest quality commercially available.

Proteins. Blood coagulation factor X from bovine plasma was purified according to Fujikawa et al. [10] and osteocalcin from bovine bone as described earlier [11]. The urinary Gla-protein was purified from human urine using the method described by Nakagawa et al. [9], except that the DEAE column chromatography was replaced by a batchwise adsorption to Ba-citrate, which was then washed with buffered saline and dissolved in a small volume of 0.5 M EDTA, pH 8.0, and dialyzed against 0.1 M NaCl in 50 mM Tris/HCl, pH 7.0 with at least three changes of the buffer.

Precipitation-inhibition assays. Non-labeled $CaCl_2$ was supplemented with 20 000 dpm of the tracer ($^{45}CaCl_2$) and the Gla-protein to be tested. The reaction was started with a sodium phosphate buffer, pH 6.0. The final concentrations in the reaction mixtures (1 ml) were 5 mM $CaCl_2$, 50 mM phosphate and 0.15 M NaCl. The mixtures were incubated in Eppendorff tubes for 2 h at 37 °C and the precipitate which had formed during this period (if any) was spun down at 2000 x g. From the supernatant 0.5 ml was taken and counted. The same scheme was used for precipitation-inhibition studies with other phosphates, except that the concentration of the divalent kation varied slightly: $BeCl_2$, 0.6 mM; $MnCl_2$, 2.5 mM and $ZnCl_2$, 0.6 mM. ^{7}Be was counted in a Packard 5360 Autogamma counter, the other isotopes in a Beckman LS1801 liquid scintillation counter using Atomlight as a scintillation liquid. The precipitation studies with calcium oxalate were performed in reaction mixtures containing 0.5 mM $CaCl_2$, 150 mM NaCl, 3.75 mM sodium oxalate, 0.1 M bis-Tris/HCl, pH 6.0 and proteins as indicated. For the precipitation studies with calcium carbonate we used a freshly prepared solution containing 1 M $NaHCO_3$, the pH of which had been adjusted to 6.5 by slowly adding 0.2 M HCl. This solution was added to the rest of the reaction mixture in a 1:1 ratio (v/v) to give a final concentration of 5 mM $CaCl_2$, 0.15 M NaCl, 0.5 M $NaHCO_3$ and proteins as indicated. The extent to which precipitation had occurred was calculated as follows. The radiolabel left in the supernatants of the precipitates formed in the absence of any protein added was regarded as a blanc value and was subtracted from all other data. Subsequently the radiolabel in the supernatant of precipitates formed in the presence of the various proteins was expressed as a percentage of the total amount of label added to the mixture. The IC-50 is defined as the protein concentration required for a 50% inhibition of precipitation and it was estimated from the in vitro protein concentration vs. precipitation-inhibition curves.

Other assays. Protein concentrations were measured according to Sedmak and Grossberg [12] and Gla-residues were detected after alkaline hydrolysis as

described by Kuwada and Katayama [13]. Thermal decarboxylation of Gla-containing proteins was accomplished by the method of Poser and Price [14].

RESULTS

A. Inhibition of calcium phosphate precipitation by three different Gla-containing proteins.

The Gla-containing proteins used in these experiments were chosen rather arbitrarily: bovine coagulation factor X, bovine osteocalcin and the human urinary Gla-protein. The Gla-content of the purified preparations was 11.8, 2.9 and 2.4 residues per molecule, respectively. The effect of these three proteins on the precipitation of calcium phosphate from a supersaturated solution of calcium and phosphate is shown in fig. 1. The optimal conditions to visualize the effect were: 5 mM $CaCl_2$, 0.15 M NaCl and 50 mM sodium phosphate, pH 6.0 and they were similar for all three proteins tested. It is clear that all three proteins strongly inhibited the rate of calcium phosphate precipitation and that, when expressed on a molar base, the effects of the three proteins were closely similar. An incubation time of 2 h was chosen because during this period the precipitation of calcium phosphate in the absence of protein had amply reached plateau values. On the other hand it should be noted that also in the presence of Gla-containing proteins some precipitate was formed after prolonged incubations (24h). No inhibition of precipitation was observed in the presence of bovine serum albumin (fig. 1). Also after thermal decarboxylation of the Gla-residues in the three different proteins, no inhibitory activity could be detected, even at protein concentrations up to 30 μM (data not shown).

B. Precipitation-inhibition of insoluble phosphates.

The optimal concentrations for visualizing the effect of the Gla-containing proteins on the calcium phosphate precipitation are not the same as

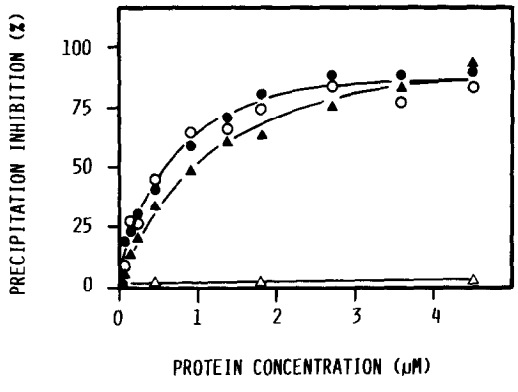

Fig. 1. Precipitation of calcium phosphate: inhibition by various proteins. Explanation of symbols: ●—●, osteocalcin; O—O, urinary Gla-protein; ▲—▲, coagulation factor X; △—△, bovine serum albumin.

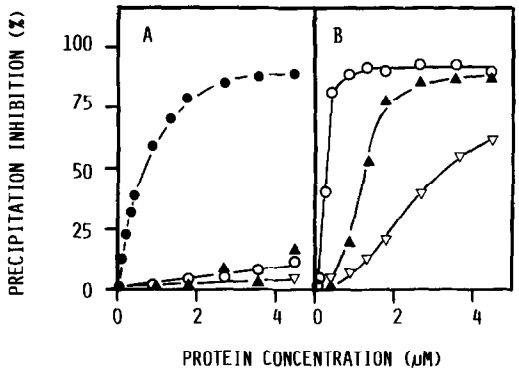

Fig. 2. Precipitation of calcium salts under various conditions. All experiments were performed in 0.15 M NaCl and 0.05 M sodium phosphate, pH 6.0. In A the concentration of each of the divalent kations was 5 mM. In B the concentrations were: 0.6 mM $_2$ $BeCl_2$, 2.5 $_2$ mM $MnCl_2$ and 0.6 mM $ZnCl_2$. Explanation of symbols: ●–●, Ca^{2+}; ○–○, Zn^{2+}; ▽–▽, Mn^{2+}; ▲–▲, Be^{2+}.

those required for other salts. This is demonstrated in fig. 2A, where we have plotted the effect of osteocalcin on the precipitation of various insoluble phosphates under the conditions known to be optimal for calcium phosphate (5 mM of the divalent metal ion in 0.15 M NaCl and 50 mM sodium phosphate, pH 6.0). By slightly adapting the kation concentration, however, the precipitation-inhibtion could be demonstrated for all divalent metal ions tested (fig. 2B). Although the optimal conditions for performing these precipitation studies were different for the various salts, they were independent of the type of Gla-protein used.

In this way we have measured the IC-50 for all three Gla-containing proteins and the results are summarized in table I. Without doubt the proteins tested inhibit the precipitation of all four phosphates to a comparable degree.

C. Precipitation-inhibition of insoluble calcium salts

Finally we have also measured if the nature of the anion in a number of insoluble calcium salts might play a role in the extent to which the three

Table I. Precipitation of insoluble phosphates: effect of various Gla-containing proteins

Divalent metal ion	IC-50 (μM) of:		
	factor X	osteocalcin	UGP
Be^{2+}	0.7	1.3	1.2
Ca^{2+}	0.9	0.6	0.6
Zn^{2+}	0.7	0.3	0.3
Mn^{2+}	4.0	3.4	2.5

Reaction mixtures contained: 0.15 M NaCl, 50 mM sodium phosphate, pH 6.0 and either $BeSO_4$ (0.6 mM), $CaCl_2$ (5 mM), $ZnCl_2$ (0.6 mM) or $MnCl_2$ (2.5 mM). Radiolabeled compounds were added in trace amounts and all incubations were performed for 2h at 37 °C. UGP stands for Urinary Gla-Protein.

Table II. Precipitation of insoluble calcium salts: effect of various Gla-containing proteins

Anion	IC-50 (μM) of: factor X	osteocalcin	UGP
phosphate	0.9	0.6	0.6
carbonate	0.2	0.1	0.1
oxalate	1.5	2.4	2.8

The reaction conditions were as described in Materials and Methods and in the legend to table I. UGP stands for Urinary Gla-Protein.

Gla-containing proteins inhibit the salt precipitation. The three salts tested were calcium phosphate, calcium carbonate and calcium oxalate. The results of these experiments are shown in table II and it is clear that again all three proteins inhibit the precipitation. It should be noted, that phosphate and oxalate were tested at pH 6.0 but that for carbonate a slightly higher pH (6.5) was used. It is unlikely that the fact that the calcium carbonate precipitation shows the highest sensitivity towards Gla-containing proteins may be explained by the different reaction conditions, since control experiments with calcium phosphate and calcium oxalate (data not shown) demonstrated that at higher pH values increasing amount of the Gla-containing proteins are required to inhibit the precipitation of these salts.

DISCUSSION

Osteocalcin is a protein consisting of 49 aminoacid residues, three of which are Gla-residues. It has been demonstrated some years ago [5] that even at very low protein concentrations osteocalcin is able to inhibit the precipitation of calcium phosphate from supersaturated solutions of calcium and phosphate. The urinary protein GCI has been characterized less thoroughly, but this protein was demonstrated to inhibit the calcium oxalate crystal growth [9]. Here we have shown that it affects the precipitation of amorphous calcium phosphate as well. We were interested in the factors which play a role in the inhibition of calcium phosphate precipitation and we have investigated to which extent the following variables contribute to the inhibitory effect: 1) the nature of the Gla-containing protein; 2) the presence of Gla-residues in these proteins; 3) the type of the divalent metal ion and 4) the type of the anion.

It turned out that precipitation-inhibition occurred with all salts investigated, but that the conditions required to visualize the effect may vary slightly. Possibly these differences are related to the differences which exist between the various solubility products of the salts. Although the three Gla-containing proteins investigated were widely different with respect to their aminoacid sequence and composition, their molecular mass and

Gla-content, the precipitation-inhibition by these proteins was of the same order of magnitude when expressed on a molar base. This is in agreement with the observation of Romberg et al. [15] who showed that osteocalcin and prothrombin inhibit the hydroxylapatite crystal growth to a similar extent. The importance of the Gla-residues is clear from the fact that the inhibitory potency of the proteins was lost after they had been submitted to thermal decarboxylation. Since factor X contains 12 Gla-residues and osteocalcin and GCI only 3 it would be interesting to know whether partially carboxylated factor X (containing 3-11 Gla-residues) might have retained its full inhibitory activity.

From the results presented here we conclude that, although from a physiological point of view the interaction of Gla-residues with Ca^{2+} is more important than that with other metal ions, this interaction is far less specific than generally is assumed. Moreover, if it is true that osteocalcin is involved in the regulation of the calcium phosphate precipitation in vertebrate bone, similar proteins might be used by invertebrates for the regulation of $CaCO_3$ precipitation. Unfortunately, the soluble matrix protein which has been suggested to regulate shell growth in oysters has not yet been tested for its Gla-content [16], but on the other hand it is at least striking that a Gla-containing protein has been found in hermatypic corals [17].

Since all three Gla-containing proteins inhibit the salt precipitation, even if these salts are present in a thousand fold excess, it seems that the proteins may interact with the nucleation site or the surface of a forming crystal in such a way that they prevent further accretion of the mineral. The fact that this protein-salt association takes place indicates that the proteins must have a much higher affinity for the precipitating salts than for the free metal ions, because otherwise one would expect that the Gla-residues in the proteins would be saturated with the divalent kations in solution.

ACKNOWLEDGMENTS

The authors wish to thank Dr. K. Hamulyāk for his critical remarks and Mrs. M. Molenaar-van de Voort for typing the manuscript. Our research is supported by grant 28-1150.7 from the Praeventiefonds, grant 84.463 from the Dutch Kidney Foundation and grant 13-50-52 from the Division for Health Research TNO.

REFERENCES

1. Suttie, J.W. (1985) Ann. Rev. Biochem. 54, 459-477
2. Olson, R.E. (1984) Ann. Rev. Nutr. 4, 281-337
3. Vermeer, C. and de Boer-van den Berg, M.A.G. (1985) Haematologia 18, 71-97
4. Prydz, H. (1977) Sem. Thrombos. Hemostas. 4, 1-14
5. Price, P.A., Otsuka, A.S., Poser, J.W., Kristaponis, J. and Raman, N. (1976) Proc. Natl. Acad. Sci. USA 73, 1447-1451

6. Linde, A., Bhown, M., Cothran, W.C., Höglund, A. and Butler, W.T. (1982) Biochim. Biophys. Acta 704, 235-239
7. Lian, J.B., Prien, E.L., Glimcher, M.J. and Gallop, P.M. (1977) J. Clin. Invest. 59, 1151-1157
8. Levy, R.J., Howard, S.L. and Oshry, L.J. (1986) Atherosclerosis 59, 155-160
9. Nakagawa, Y., Abram, V., Kézdy, F.J., Kaiser, E.T. and Coe, F.L. (1983) J. Biol. Chem. 258, 12594-12600
10. Fujikawa, K., Legaz, M.E. and Davie, E.W. (1972) Biochemistry 11, 4882-4891
11. Vermeer, C. (1984) FEBS Lett. 173, 169-172
12. Sedmak, J.J. and Grossberg, S.E. (1977) Anal. Biochem. 131, 173-179
13. Kuwada, M. and Katayama, K. (1983) Anal. Biochem. 131, 173-179
14. Poser, J.W. and Price, P.A. (1979) J. Biol. Chem. 254, 431-436
15. Romberg, R.W., Werness, P.G., Riggs, B.L. and Mann, K.G. (1986) Biochemistry 25, 1176-1180
16. Wheeler, A.P., George, J.W. and Evans, C.A. (1981) Science 212, 1397-1398
17. Hamilton, S.E., King, G., Tesch, D., Riddles, P.W., Keough, D.T., Jell, J. and Zerner, B. (1982) Biochem. Biophys. Res. Commun. 108, 610-613