Characterization of a Gla-containing Protein from Calcified Human Atherosclerotic Plaques

Birgit L.M.G. Gijsbers, L. Johan M. van Haarlem, Berry A.M. Soute, Rob H.M. Ebberink, and Cees Vermeer

In this article we describe the isolation of a 4-carboxyglutamic acid (Gla)-containing protein from calcified human atherosclerotic plaques. The protein was extracted from pulverized calcified plaques by demineralization with ethylenediaminetetraacetic acid and was subsequently purified by anion-exchange fast protein and high-performance liquid chromatography by using ion-exchange and gel-filtration columns. The protein was designated as plaque Gla protein (PGP) and has an apparent mass of 23 kD as estimated from sodium dodecyl sulfate-polyacrylamide gel analysis. By determining the sequence of its first six amino acid residues, the protein was unequivocally demonstrated to be not related to any other known protein. Moreover, no immunological relationship (as tested by Western blot analysis) was found between PGP and other known Gla-containing proteins.

(Arteriosclerosis 10:991–995, November/December 1990)

Proteins containing the unusual amino acid 4-carboxyglutamic acid (Gla) have been detected in blood plasma and in a variety of calcified tissues, such as bone, dentin, renal stones, and hardened atherosclerotic plaques. The only known function of the Gla residues in these proteins is the binding of calcium ions. The Gla-containing proteins of hepatic origin were characterized several years ago, and their role in blood coagulation is fairly well understood. The function of the nonhepatic Gla-containing proteins, on the other hand, has remained obscure. Most of the information available has been obtained from osteocalcin, the Gla-containing protein produced by the osteoblasts in bone tissue.

In 1979, Levy et al. showed that a Gla-containing protein could also be isolated from hardened atherosclerotic plaques, and because of its calcium-binding properties, this protein was called atherocalcin. The mass of this protein was found to be 80 kD and was dissimilar from any of the blood coagulation factors. Recently, however, Levy et al. reported that atherocalcin is merely a complex consisting of albumin and osteocalcin. Also, trace amounts of nonbound osteocalcin (5.8 kD) and other nonidentified Gla-containing proteins (6 to 8 kD) seem to occur in mineralized heart valves and in calcified atherosclerotic lesions. The origins of the latter proteins are currently unknown, but it has been suggested that they are proteolytically degraded coagulation factors.

We have developed an extraction procedure by which essentially all proteins are released from the mineralized atherosclerotic plaques, and we investigated whether other Gla-containing proteins may be found in the extract. The results of these experiments are presented in this article.

Methods

Materials

Bovine serum albumin, aprotinin, and guanidine hydrochloride (guanidine HCl) were obtained from Sigma (Saint Louis, MO). CNBr-activated Sepharose 4B, QAE-Sephadex, DEAE-Sephacel, prepacked polyacrylamide PhastGel gradient gels, and the Low Molecular Weight protein calibration kit were products of Pharmacia (Uppsala, Sweden). Benzamidine hydrochloride was purchased from Janssen Chimica (Beerse, Belgium), and Keyhole Limpet Haemocyanin (KLH) was from Pacific Bio-Marine Supply (Venice, CA). Purified human prothrombin and factor X were obtained from Johan W.P.H. Soons (University of Limburg); human factor IX and the proteins C, S, and Z, as well as antibodies against the various coagulation factors, were kindly supplied by Rogier M. Bertina (University Hospital, Leiden, The Netherlands). Human osteocalcin was purified as described by Poser et al., and the antibodies were raised in rabbits. All chemicals were of analytical grade or better. The Diaflo PM-50 ultrafiltration system was purchased from Amicon (Oosterhout, The Netherlands), and Spectrapor dialysis tubing was from Spectrum Industries (Los Angeles, CA).

Plaque Gla Protein

Human atherosclerotic aortas were obtained at autopsy between 5 and 24 hours after death and were promptly frozen. The protocol of investigation had been approved by the University Hospital Ethics Committee.
Table 1. Purification of Plaque Gla Protein from Calcified Human Plaques

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Gla (nmol/mg protein)</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Proteins released</td>
<td>330</td>
<td>ND</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>after EDTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. QAE-Sephadex eluate</td>
<td>51.7</td>
<td>4</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>3. Mono-Q FPLC</td>
<td>2.93</td>
<td>42.8</td>
<td>10.7</td>
<td>60.6</td>
</tr>
<tr>
<td>4. Superose FPLC</td>
<td>0.63</td>
<td>139.5</td>
<td>34.9</td>
<td>42.5</td>
</tr>
<tr>
<td>5. TSK HPLC</td>
<td>0.22</td>
<td>224.7</td>
<td>56.2</td>
<td>23.9</td>
</tr>
</tbody>
</table>

The starting material used for this purification scheme was obtained from 74 aortas. ND, not detectable because of the large excess of other proteins. Therefore, the purification and yield are expressed relative to step 2. Gla=4-carboxyglutamic acid, FPLC=fast protein liquid chromatography, HPLC=high-performance liquid chromatography.

The donors had not been treated with oral anticoagulants. For a routine preparation of plaque Gla protein, 60 to 80 aortas were thawed, and areas of discrete calcification were carefully dissected from the surrounding tissue and pooled. After extensive washing of the calcified plaques with buffer A (0.15 M NaCl, 50 mM Tris/HC1, pH 8.0) and a second examination to remove the last traces of tissue, a liquid nitrogen milled powder was prepared, resulting in approximately 50 g of powder. Proteins were extracted from the calcified material by stirring the powder in 1 l of buffer B (1.0 M ethylenediaminetetraacetate, 0.2 M KCl, 4.0 M guanidine HC1 (pH 8.0), 0.01 M benzamidine HC1, and 50 mg/l aprotinin) for 2 days at 4°C. Insoluble material was collected by centrifugation at 10 000 g for 15 minutes at 4°C and was subjected to two more extraction cycles of 2 days each. The pooled extracts were diluted 20-fold with distilled water, were adsorbed batchwise to QAE-Sephadex (10 ml of preswollen slurry in buffer A per l of diluted extract), and the adsorbed proteins were eluted with 1.0 M NaCl in buffer A. The calcium plaque extract thus obtained was fractionated further by fast protein liquid chromatography (FPLC) on a Mono-Q anion-exchange column. The proteins were applied in buffer C (20 mM Bis-Tris/HC1, pH 7.0) and were eluted with a linear gradient from 0 to 1 M NaCl in buffer C. The Gla-containing fractions were pooled and fractionated further on a Superose 12 size-exclusion column (FPLC) and on a TSK G-2000 SW column (high-performance liquid chromatography, HPLC). All protein-bound Gla residues were recovered in a protein peak eluting at a position corresponding to a mass of 23 kD. This protein was designated as plaque Gla protein (PGP).

Anti-plaque Gla Protein Antibodies

The highly immunogenic KLH was conjugated to purified PGP (3.5 mg KLH per mg PGP) according to the method of Tanaka et al. Portions containing 75 μg PGP...
Table 2. Amino Acid Composition of Human Plaque Gla Protein

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amount* (pmol/sample)</th>
<th>Molar fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-Carboxyglutamic acid†</td>
<td>108</td>
<td>5.2</td>
</tr>
<tr>
<td>Aspartic acid+asparagine</td>
<td>44</td>
<td>6.5</td>
</tr>
<tr>
<td>Serine</td>
<td>70</td>
<td>10.3</td>
</tr>
<tr>
<td>Glutamic acid+glutamine+gla</td>
<td>115</td>
<td>17.0</td>
</tr>
<tr>
<td>Proline</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Glycine</td>
<td>91</td>
<td>13.4</td>
</tr>
<tr>
<td>Alanine</td>
<td>39</td>
<td>5.8</td>
</tr>
<tr>
<td>Valine</td>
<td>49</td>
<td>7.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>5</td>
<td>0.7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>19</td>
<td>2.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>41</td>
<td>6.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>27</td>
<td>4.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>19</td>
<td>2.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>9</td>
<td>1.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>14</td>
<td>2.1</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6</td>
<td>0.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>21</td>
<td>3.1</td>
</tr>
<tr>
<td>Cysteine</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Total residues</td>
<td>–</td>
<td>100</td>
</tr>
</tbody>
</table>

*All values are the averages of duplicate determinations. †Determined after acid hydrolysis. Calculated after alkaline hydrolysis and Gla detection. ND = not determined.

Figure 2. Sodium dodecyl sulfate–polyacrylamide gel analysis of human plaque Gla protein. Lane A. Marker proteins reduced. Lane B. 5 μg reduced purified plaque Gla protein. Lane C. 5 μg nonreduced purified plaque Gla protein.

were emulsified with Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and used for the immunization of rabbits through multiple intracutaneous injections. Booster injections were given in Freund's incomplete adjuvant at 3-week intervals. Immunoglobulin G (IgG) was purified from the immune sera by (NH₄)₂SO₄ precipitation and DEAE-Sephacel column chromatography, and the presence of IgG in the various fractions was measured with an assay based on horseradish peroxidase (HRP)-labeled swine anti-rabbit total IgG (Dako, Copenhagen, Denmark). The proteins were subsequently concentrated in an Amicon cell by using a PM-50 membrane filter. Immunoelectrophoretic analysis of the sample revealed one precipitin line, indicating that the preparation consisted of homogenous IgG.

Various Assays

Polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate (SDS) was performed with prepacked Phast-Gel gradient gels (10% to 15% and 8% to 25% cross-linking) in combination with PhastGel SDS and native buffer strips. The gels were either stained with Coomassie brilliant blue or subjected to Western blotting with HRP-labeled swine anti-rabbit immunoglobulin as a second antibody.

Protein concentrations were determined as described by Sedmak and Grossberg. Gla and Glu residues in the various proteins were determined after alkaline hydrolysis according to the method of Kuwada and Katayama. This method is based on the separation of derivatized amino acids by HPLC. The various peaks were recorded, and the surface areas were integrated automatically by using a Kratos Spectroflow 980 fluorescence detector. These surface areas were regarded as a measure for the Gla concentration in the sample. A reference curve was constructed with known amounts of purified Gla and was used for the quantitation of the Gla concentration in the various samples.

The amino acid composition of PGP was established after acid hydrolysis by use of an LKB 4400 amino acid analyzer. Amino acid sequences were determined with an Applied Biosystems 477A Protein Sequencer, which was on-line equipped with a Model 120 A PTH Analyzer. Protein sequences were compared in the Fast P data bank (Austin Code Works, Austin, TX).

Osteocalcin was assayed with a commercial radioimmunoassay kit (INCSTAR, Stillwater, MN).

Results

PGP was isolated from the calcified plaques of human aortas, and the purification procedure is summarized in Table 1. The last three steps were anion-exchange and size-exclusion chromatography; the elution patterns are shown in Figure 1. Rechromatography of the final preparation on a Superose 12 or TSK column showed one single peak eluting at a position of 23 kD. Purified PGP was analyzed by electrophoresis on polyacrylamide gels in SDS (Figure 2). Under both reducing and nonreducing conditions, one band was found with an Mᵣ of 23 kD. From these results, we concluded that our PGP prepara-
Figure 3. Western blot analysis of human plaque Gla protein. The various proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to nitrocellulose paper as described in the Methods section. The blots were treated with either anti-plaque Gla protein immunoglobulin G (PGP IgG) (A), anti-prothrombin IgG (B), or anti-osteocalcin IgG (C). Lanes 1, 7, and 8. 10 ng prothrombin. Lanes 2 and 3. 1 and 25 ng PGP. Lanes 4, 6, and 9. 5 ng PGP. Lanes 5, 10, and 11. 5 μg osteocalcin.

Discussion

In this article, we have described the isolation of a hitherto-unidentified Gla-containing protein from calcified atherosclerotic plaques. The protein was called PGP (plaque Gla protein), and biochemical characterization showed it to be a single-chain molecule with a mass of 23 kD. The amino acid composition determination showed that PGP contains 5.2 Gla residues per molecule, appreciable amounts of serine and threonine, and probably not more than one residue of methionine. These data exclude the possibility that PGP is somehow related to osteocalcin (which does not contain serine, threonine, or methionine). The sequence of the first six NH$_2$-terminal amino acid residues in PGP clearly shows that it is dissimilar from any of the Gla proteins characterized until now. In addition, the sequence was compared with those in a commercial data bank, and it was found that PGP is not related to any other protein of known primary structure.

Immunochromatography characterization of PGP was performed by Western blot analysis, and no cross-reaction between PGP and antibodies against other Gla proteins was detected. With the aid of a commercial radioimmunoassay RIA (lower detection limit: 1 ng/ml), it was demonstrated that the contamination of PGP (1000 ng tested) by osteocalcin antigen was less than 0.1%. The origin of PGP is not known. Obviously, the protein might be adsorbed from the blood stream to the calcifying vessel wall, but in that case, it remains to be explained why other Gla-containing proteins, such as prothrombin, are not detectable in the calcified plaque extract. Alternatively, PGP might also be synthesized by the vessel wall itself. In that respect, it is suggestive that recently the arterial intima was found to contain relatively high amounts of vitamin K-dependent carboxylase, the enzyme required for Gla formation. Also, in cultured...
endothelial cells of arterial and venous origin, the carboxylating enzyme system was detected. 19
Nothing is known about the in vivo function of PGP. In vitro the protein is extremely potent in inhibiting the precipitation of various calcium salts, 12 but whether a similar function is its main physiological role remains a matter of speculation. Our present hypothesis is that PGP is synthesized in the vessel wall and that the continuous flow of PGP from this tissue to the bloodstream is one means of preventing the deposition of calcium salts. If for some reason calcification does occur, PGP is obviously expected to bind to the crystal surface, since all Gla-containing proteins are known to have a high affinity for insoluble barium and calcium salts. This hypothesis implies that PGP will probably also occur in plasma. Techniques to detect PGP in plasma and to isolate it from thereon are presently being developed in our laboratory.

Acknowledgments
The authors thank H. Coenraad Hemker and Karly Hamulyak for their stimulating discussions. We are grateful to Marjo H.J. Knapen and Monique M.C.L. Groenen for excellent technical assistance and to Mariet Molenaar-van de Voort for typing this manuscript. Hans van Eijck is acknowledged for analyzing the manuscript. The authors also thank the Departments of Pathology from the St. Maartensgasthuis in Venlo and the St. Jozef Hospital in Kerkrade for supplying us with human aortas.

References

10. Levy RJ, Gundberg CM, Schelman R. The identification of the vitamin K-dependent bone protein, osteocalcin as one of the γ-carboxyglutamatic acid-containing proteins present in calcified atherosclerotic plaque and mineralized heart valves. Atherosclerosis 1983;46:49–56

Index Terms: 4-carboxyglutamic acid • vitamin K • atherosclerosis • calcification
Characterization of a Gla-containing protein from calcified human atherosclerotic plaques.

B L Gijsbers, L J van Haarlem, B A Soute, R H Ebberink and C Vermeer

_Arterioscler Thromb Vasc Biol._ 1990;10:991-995
doi: 10.1161/01.ATV.10.6.991

_Arteriosclerosis, Thrombosis, and Vascular Biology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/10/6/991

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Arteriosclerosis, Thrombosis, and Vascular Biology_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Arteriosclerosis, Thrombosis, and Vascular Biology_ is online at:
http://atvb.ahajournals.org//subscriptions/