Decarboxylated bone Gla-protein as a substrate for hepatic vitamin K-dependent carboxylase

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Bovine bone Gla-protein (B.G.P.) was prepared and decarboxylated into descarboxy-B.G.P. (d-B.G.P.). The latter was purified and identified as decarboxylated osteocalcin. Both crude and purified d-B.G.P. are good substrates for vitamin K-dependent carboxylase. Because the K_m of this enzyme for d-B.G.P. is low, the latter is a better substrate than the frequently used pentapeptide FLEEL or exogenous protein substrates such as descarboxyprothrombin.

Carboxylase *Vitamin K* *Warfarin* *γ-Carboxyglutamic acid* *Osteocalcin*

1. INTRODUCTION

Vitamin K-dependent carboxylase has been detected in the liver [1,2] and in many other mammalian tissues [3,4]. It is involved in the post-translational carboxylation of glutamic acid (Glu) residues into γ-carboxyglutamic acid (Gla) residues [5]. Except for the vitamin K-dependent clotting factors, the function of these Gla-containing proteins is not completely understood, but it is generally believed that in all cases the Gla residues are required for the binding of Ca^{2+}.

Although much work has been done already to elucidate the mechanism of the vitamin K-dependent reaction, the latter is still a matter of debate [6,7]. In most experiments concerning this subject the synthetic pentapeptide Phe–Leu–Glu–Glu–Leu (FLEEL) is used as an exogenous substrate for carboxylase. Because of its low M_r this peptide can be added to carboxylase in high concentrations (10–20 mM). Disadvantages are, however, that it has a high K_m (4–6 mM) for carboxylase [8,9] and that it is extremely expensive. Until now only one exogenous substrate has been described with a low K_m for carboxylase: Fragment Su, a peptide which is obtained after proteolytical degradation of descarboxyprothrombin with subtilisin and which corresponds to the amino acids 13–29 in descarboxyprothrombin [9]. This substrate, however, is not commercially available and its preparation is both expensive and laborious.

We describe here the preparation of another exogenous substrate for carboxylase. It combines the advantage of a low K_m for carboxylase with the fact that it is readily prepared in large quantities at a low cost.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Vitamin K_1 was obtained from Hoffmann-La Roche and vitamin K hydroquinone was prepared as in [10]. Dithiothreitol (DTT), benzamidine, aprotinin and 3-([3-cholamidopropyl]dimethylammoniol)-1-propanesulfonate (CHAPS) were from Sigma and the pentapeptide FLEEL from Vega Fox. $NaH^{14}CO_3$ (40–60 Ci/mol) was purchased from Amersham and Sephadex G-25 from Pharmacia. All other chemicals were obtained from Merck.

2.2. *Preparation of decarboxylated bone Gla-protein*

Fresh bovine femurs and tibias were obtained from the Maastricht Slaughter House, cut into

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slices and demarrowed. The bones were carefully cleaned, heated at 120°C overnight and ground to powder mechanically. Powder (100 g) was extracted by stirring overnight with 200 ml buffer A (1 M EDTA, 0.2 M KCl, 0.1 M benzamidine, pH 8.0). Insoluble material was removed by filtration and the solution was desalted on a Sephadex G-25 column (60 × 5 cm) in water. The protein peak was collected and the pH was adjusted to 2.5 by adding 1 M HCl. The preparation was subsequently lyophilized and decarboxylated by heating at 110°C for 5 h under vacuum [11]. The resulting material was dissolved in buffer B (0.1 M KCl, 0.02 M Tris–HCl, pH 7.4) to a final concentration of 5 mg/ml. Since after the decarboxylation step the proteins remain acidic, it is important that the pH of the solution is checked at this stage. The preparation thus obtained is referred to as crude decarboxylated bone Gla-protein (d-B.G.P.).

2.3. *Various assays*

Vitamin K-dependent carboxylase was prepared from normal cow tissues [3] and carboxylase activity was measured by the incorporation of $^{14}CO_2$ into exogenous substrates in the presence of vitamin K hydroquinone [10]. High-performance liquid chromatography (HPLC) was carried out using a Beckmann-112 system and a Mono Q column (Pharmacia) for anion exchange chromatography and a Spherogel-TSK 2000 SW column (Beckmann, 300 × 7.5 mm) as a molecular sieve. Protein concentrations were determined as in [12] and the N-terminal amino acid was identified as in [13]. Gla and Glu residues were determined by alkaline hydrolysis of the protein and subsequent HPLC on a Nucleosil 5SB column (Chrompack) as in [14].

3. RESULTS

During the preparation of bone Gla-protein (B.G.P.) it turned out that the bone powder could be extracted a number of times and that after each extraction about 70 mg protein was present in the extract. Five subsequent extracts obtained from one portion of 100 g bone powder were separately processed for the production of d-B.G.P. The Gla : Glu ratio in these extracts was 0.03 before and 0 after decarboxylation. The decarboxylated preparations were used as substrates for carboxylase and in all cases the incorporation of $^{14}CO_2$ amounted to about 120000 dpm/mg substrate after 3 h incubation at 25°C under standard conditions. Since no differences were found between the various preparations they were pooled and used for the purification of d-B.G.P.

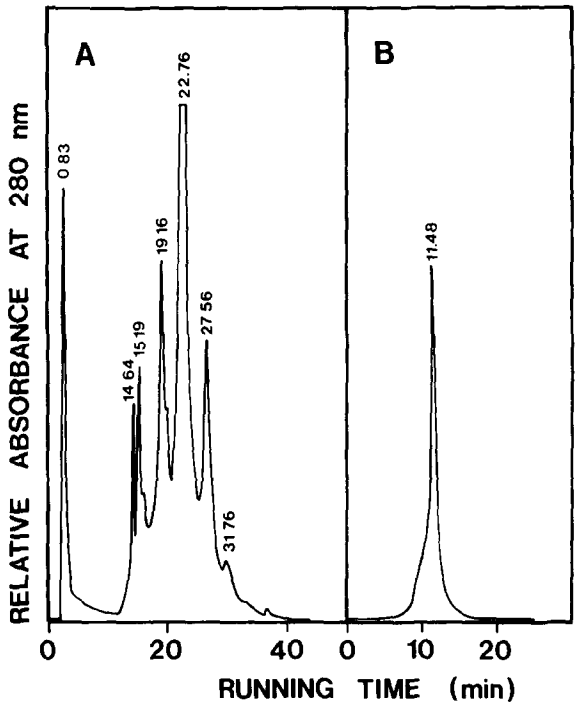

Fig.1. Fractionation of d-B.G.P. (A) One mg of crude d-B.G.P. was adsorbed to a Mono Q ion exchange column and eluted with a linear salt gradient from 0.05 to 0.5 M NaCl in 20 mM Tris–HCl, pH 7.4. The eluate was recorded at 280 nm and the start of the tracing represents the start of the gradient. The various peaks were collected and tested with the carboxylase assay. All d-B.G.P. activity was recovered in the peak eluting after 22.76 min. (B) The eluted d-B.G.P. was analyzed on a TSK-2000 molecular sieve and all activity was recovered in the peak eluting after 11.48 min. Trasylol eluted after 11.56 min.

The crude d-B.G.P. was purified by HPLC on an ion exchange column (fig.1A). When analyzed on a size exclusion column the preparation thus obtained eluted in a single peak (fig.1B) shortly before trasylol (aprotinin), and its apparent M_r was about 6800; the NH_2-terminal amino acid was identified as tyrosine. Since similar characteristics have been reported for bovine osteocalcin [15,16] it is highly probable that d-B.G.P. is similar to

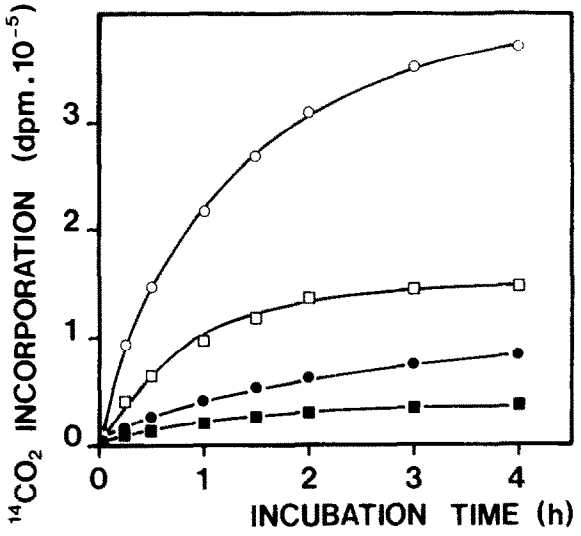

Fig.2. Effect of $(NH_4)_2SO_4$ on the carboxylation of d-B.G.P. and FLEEL. The carboxylation of both substrates was monitored after several intervals of time in the presence and absence of 1 M $(NH_4)_2SO_4$: (●—●) FLEEL (4 mM); (○—○) FLEEL (4 mM) + $(NH_4)_2SO_4$; (■—■) d-B.G.P. (0.03 mM); (□—□) d-B.G.P. (0.03 mM) + $(NH_4)_2SO_4$.

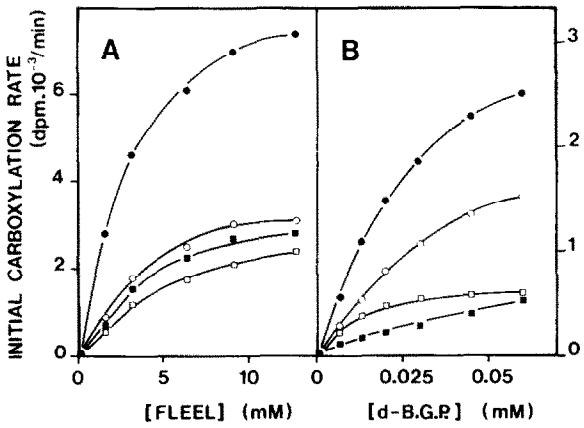

Fig.3. The carboxylation of FLEEL and d-B.G.P. in various carboxylating systems. The initial reaction rate was measured for the carboxylation of FLEEL (10 mM, A) and d-B.G.P. (0.05 mM, B). The incubations were performed in standard reaction mixtures containing 1 M $(NH_4)_2SO_4$. The sources of carboxylase were bovine liver (●—●), kidney (○—○), testis (■—■) and aorta (□—□). Note the different scales for A and B.

decarboxylated osteocalcin. The optimal reaction conditions for the carboxylation of d-B.G.P. were compared with those for the carboxylation of FLEEL and it turned out that they were similar for both substrates. Therefore the following conditions were used in all experiments described below: 0.25 ml reaction mixtures containing 4 mg microsomal proteins, 0.4 M KCl, 0.05 M Tris–HCl (pH 7.4), 0.4% CHAPS, 12% ethylene glycol, 16 mM DTT, 0.1 mM vitamin K hydroquinone and 0.01 mCi $NaH^{14}CO_3$ were incubated at 25°C. Under these conditions the carboxylation of both substrates was markedly enhanced by the addition of $(NH_4)_2SO_4$. Unless stated otherwise we therefore added $(NH_4)_2SO_4$ to our reaction mixtures to a final concentration of 1 M.

When we compared the kinetic constants of the two substrates we found the K_m of FLEEL to be 4.8 mM and that of d-B.G.P. 0.025 mM. The respective maximal reaction rates (V_{max}) were 14375 and 4825 dpm/min. It was checked that the presence of $(NH_4)_2SO_4$ did not affect the K_m of the two substrates but only increased the V_{max}. The fact that the V_{max} of FLEEL is higher than that of d-B.P.G. probably results from its low M_r, which will be accompanied by a high diffusion rate.

Time courses of the carboxylation of the two substrates (both in the presence and absence of $(NH_4)_2SO_4$) are shown in fig.2 and it is clear that $(NH_4)_2SO_4$ not only increases the initial carboxylation rate 4–5-fold, but also at the end point of the reaction (after 4 h) the amounts of incorporated $^{14}CO_2$ are 5-times higher in the presence of $(NH_4)_2SO_4$ than in its absence. Hence $(NH_4)_2SO_4$ induces an increase of the total amount of carboxylation events that can be accomplished under our experimental conditions. From these types of experiments we have calculated the efficiency of the carboxylation reaction and it turned out that at limiting substrate concentrations 0.008 mol CO_2 is bound per mol FLEEL. For d-B.G.P. this value is 0.16.

We have also checked whether d-B.G.P. is carboxylated in non-hepatic systems. In fig.3 the carboxylation of FLEEL and that of d-B.G.P. are compared in carboxylating systems from liver, kidney, testis and aorta and it is clear that both substrates are carboxylated in all systems. It should be noted that most of the carboxylation reactions described above (except the determina-

tion of the kinetic constants) were performed with both purified and crude d-B.G.P. In none of these experiments have we detected any difference between crude and purified d-B.G.P. This demonstrates that the contaminating proteins in crude d-B.G.P. do not influence the carboxylation reaction, so that in most cases no further purification of the substrate is required.

4. DISCUSSION

The first exogenous substrate for vitamin K-dependent carboxylase that has been described in the literature is the synthetic pentapeptide FLEEL [17]. Since it has been derived from an amino acid sequence in prothrombin it may be regarded as a homologous substrate for hepatic carboxylase (prothrombin is synthesized in the liver) but it is also readily carboxylated in non-hepatic systems [3]. Although FLEEL has proven to be very helpful in investigations concerning various carboxylating enzyme systems, its main disadvantages are that it is expensive and that it has a high K_m for carboxylase. To overcome at least this last drawback, derivatives of FLEEL as well as other Glu-containing peptides have been synthesized [18]. Unfortunately none of these substrates proved to be a better substrate than FLEEL is.

Until now the only known exogenous substrate with a low K_m for carboxylase was Fragment Su, a Glu-containing peptide which is obtained from descarboxyprothrombin by proteolytic cleavage with subtilisin [9]. Unfortunately, its preparation is both laborious and expensive and it is not commercially available.

We have described here the preparation of a protein from bovine bone which, in its decarboxylated form, may serve as a substrate for both hepatic as well as various non-hepatic carboxylases. The protein could be identified as osteocalcin and its K_m for hepatic carboxylase was more than 200-fold lower than that of FLEEL. Osteocalcin, however, is synthesized in bone cells [19] and therefore it is the first exogenous substrate described to be well carboxylated in a heterologous system. The preparation procedure for the crude bone extract is quick, easy and cheap and since crude d-B.G.P., when used as a substrate for carboxylase, has the same characteristics as those of the purified material, in most cases it will not be necessary to purify the material further.

We have compared the optimal reaction conditions for the carboxylation of FLEEL and d-B.G.P. and no differences were found. Also the addition of $(NH_4)_2SO_4$ to the reaction mixtures stimulated the carboxylation of both substrates to the same extent. Therefore we recommend the use of d-B.G.P. as a substrate for vitamin K-dependent carboxylase.

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