Osteocalcin binds tightly to the γ-glutamylcarboxylase at a site distinct from that of the other known vitamin K-dependent proteins

Roger J. T. J. HOUBEN*, Dayun JIN†, Darrel W. STAFFORD†, Paul PROOST‡, Rob H. M. EBBERINK§, Cees VERMEER* and Berry A. M. SOUTE*[1]

*Department of Biochemistry and Cardiovascular Research Institute, Maastricht University, P.O. Box 616, 6200 MD, Maastricht, The Netherlands, †Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280, U.S.A., ‡Rega Institute, Department of Molecular Immunology, Catholic University of Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium, and §Perkin-Elmer Netherlands Ltd., P.O. Box 305, 2910 AH Nieuwerkerk aan de IJssel, The Netherlands

Vitamin K-dependent proteins contain a propeptide that is required for recognition by the enzyme γ-glutamylcarboxylase. Substrates used *in vitro* for carboxylation studies lacking a prosequence are characterized by K_m values in the millimolar range, whereas the K_m for peptides containing a prosequence is three or four orders of magnitude smaller. Here we report that descarboxy-osteocalcin is an exception in this respect. With descarboxy-osteocalcin in purified propeptide-free recombinant carboxylase, the K_m was 1.8 μM. Furthermore, osteocalcin was an inhibitor of descarboxy-osteocalcin carboxylation with a K_i of 76 μM. In contrast with the other vitamin K-dependent proteins, free propeptides do not inhibit descarboxy-osteocalcin carboxylation. Moreover, propeptide-containing substrates were inhibited neither by osteocalcin nor by its propeptide. From our studies we conclude that descarboxy-osteocalcin must have an internal recognition sequence that binds to γ-glutamylcarboxylase at a site different from the propeptide-recognition site.

Key words: bone Gla-protein, γ-carboxyglutamate, phylloquinone, post-translational processing.

INTRODUCTION

Osteocalcin (OC), also called bone γ-carboxyglutamate (Gla)-protein (BGP) [1], is the most abundant non-collagenous protein in bone [2]. The mature protein consists of 49 amino acid residues and has a molecular mass of 5700 Da [3,4]. During its biosynthesis, OC undergoes a series of post-translational modifications including the conversion of three glutamate residues into Gla residues. In this process vitamin K serves as a coenzyme for γ-glutamylcarboxylase, an integral membrane protein of 94 kDa residing in the rough endoplasmic reticulum of various mammalian cells [5,6]. Natural substrates for γ-glutamylcarboxylase include precursors of proteins involved in blood coagulation (prothrombin, factor VII, factor IX, factor X, protein C, protein S and protein Z) [7], those involved in tissue mineralization [OC and matrix Gla-protein (MGP)] [8,9], and the growth-arrest-specific gene 6 protein, Gas6 [10].

Other than MGP, which has an internal recognition site for carboxylase, all Gla-proteins characterized so far are synthesized as preproproteins [11]. The presequence is required for translocation across the endoplasmic reticulum membrane, whereas the prosequence functions as a high-affinity recognition site for carboxylase [12,13]. After carboxylation, the protein is transported through the cell to the *trans*-Golgi network. There the propeptide is cleaved from the protein by an as yet unknown proconvertase, after which the fully modified, mature Gla-protein is secreted into the circulation [14]. Therefore circulating mature Gla-proteins lack the propeptide domain. By using carboxylating systems *in vitro* it was shown that small synthetic peptides lacking the propeptide [e.g. the pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL)] are poor substrates with K_m values in the millimolar range [15,16]. Under similar conditions, K_m values for peptides that did contain the prosequence of either prothrombin or factor IX were three or four orders of magnitude lower [17]. In addition, the binding of prosequence-containing substrates led to a 90–95% decrease in K_m for the cofactor vitamin K hydroquinone, suggesting an allosteric effect of the prodomain on binding to carboxylase [18]. Remarkably, the addition of free propeptide did not affect the K_m for vitamin K hydroquinone, and resulted in only a minor decrease (less than 90%) in the K_m for small peptide substrates [19].

During recent years it has been shown that heat-decarboxylated bovine OC can serve as a substrate for bovine liver carboxylase [11,20]. The protein is exceptional because, despite its lack of a prodomain, reported K_m values are comparable with those of prodomain-containing substrates [21,22]. Because heat-decarboxylation can cause unwanted and undefined damage to the protein molecule, we have prepared synthetic human OC, in both its carboxylated and non-carboxylated (descarboxy) forms; the preparations are designated here as OC and d-OC respectively. In the present study we compared the substrate properties of d-OC with those of other well-known substrates for carboxylase and we tested the extent to which OC is capable of inhibiting the carboxylation of various substrates. Finally we investigated whether the low K_m values for d-OC are the result of an unusually high affinity of its Gla motif for the carboxylase active

Abbreviations used: bEELOMe, tripeptide t-butoxycarbonyl-Glu-Glu-Leu-OMe; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonic acid; d-OC, descarboxy-osteocalcin; d-$OC^{13–25}$ and d-$OC^{22–31}$, synthetic descarboxy-osteocalcin residues 13–25 and 22–31 respectively; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; Fmoc, fluoren-9-ylmethoxycarbonyl; Gla, γ-carboxyglutamate; HPC4, Glu-Asp-Gln-Val-Asp-Pro-Arg-Leu-Ile-Asp-Gly-Lys; KH_2, vitamin K hydroquinone; MGP, matrix Gla-protein; OC, osteocalcin; $OC^{1–16}$, $OC^{13–25}$, $OC^{22–31}$ and $OC^{29–43}$, synthetic osteocalcin residues 1–16, 13–25, 22–31 and 29–43 respectively; Pro^{FIX} and Pro^{OC}, synthetic propeptides (−18 to −1) of human factor IX and human osteocalcin respectively; $Pro(Glu)_{10}$, synthetic human prothrombin propeptide covalently attached to 10 Glu residues; ProIX-59 Q/S, 59 amino acid peptide containing the human factor IX propeptide and the first 41 residues of factor IX γ-carboxyglutamate domain (sequence −18 to 41) with an Arg → Gln mutation at the −4 position and an Arg → Ser mutation at position −1; ProPT-28 F/A, synthetic peptide consisting of residues −18 to +10 of human descarboxy-prothrombin with a Phe → Ala mutation at position −16.

[1] To whom correspondence should be addressed (e-mail b.soute@bioch.unimaas.nl).

site or, alternatively, of an internal docking sequence located elsewhere in the molecule.

MATERIALS AND METHODS

Reagents

All chemicals were of analytical grade or higher. Vitamin K_1 (Konakion®, mixed micelles) was purchased from Hoffmann-La Roche (Basel, Switzerland), $NaH^{14}CO_3$ with a specific radioactivity of 50 mCi/mmol was from New England Nuclear (Boston, MA, U.S.A.) and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonic acid (CHAPS) was from Sigma (St. Louis, MO, U.S.A.). The insect cell expression vector (pVL1392) was purchased from PharMingen (San Diego, CA, U.S.A.) and the baculovirus viral DNA (BacVector 3000) was purchased from Novagen (Madison, WI, U.S.A.). The Sf9 (*Spodoptera frugiperda*) cells were obtained from the Lineburger Cancer Center at the University of North Carolina–Chapel Hill, and the High Five (*Trichoplusia ni*) cells were a gift from Dr. Thomas Kost (Glaxo-Wellcome, Research Triangle Park, NC, U.S.A.). Affinity resin with a Glu-Asp-Gln-Val-Asp-Pro-Arg-Leu-Ile-Asp-Gly-Lys (HPC4) antibody tag was provided by Dr. Charles T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A.). The Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG) antibody epitope and the FLAG peptide standards were purchased from Sigma.

Peptides

Synthetic OC residues 1–16, 13–25, 22–31 and 29–43 ($OC^{1–16}$, $OC^{13–25}$, $OC^{22–31}$, $OC^{29–43}$ respectively), synthetic descarboxy-osteocalcin (d-OC) residues 13–25 and 22–31 (d-$OC^{13–25}$ and d-$OC^{22–31}$ respectively) and synthetic propeptides (-18 to -1) of human factor IX and human OC (Pro^{FIX} and Pro^{OC} respectively) were synthesized by Pepscan Systems (Lelystad, The Netherlands). Both Phe-Leu-Glu-Glu-Leu (FLEEL) and t-butoxy-carbonyl-Glu-Glu-Leu-OMe (bEELOMe) were purchased from Bachem (Bubendorf, Switzerland). A recombinant 59-residue peptide containing the human factor IX propeptide and the first 41 residues of factor IX γ-carboxyglutamate domain (sequence -18 to 41) with an Arg → Gln mutation at the -4 position and an Arg → Ser mutation at position -1 (ProIX-59 Q/S) was produced and purified as described previously [17]. Synthetic human prothrombin propeptide covalently attached to 10 Glu residues [$Pro(Glu)_{10}$] and a synthetic peptide consisting of residues -18 to $+10$ of human descarboxy-prothrombin with a Phe → Ala mutation at position -16 (ProPT-28 F/A) were synthesized by Perkin Elmer (Nieuwerkerk aan de IJssel, The Netherlands). The amino acid sequences of the various peptides are listed in Table 1.

Synthesis of OC and d-OC

Mature 49-residue OC and d-OC (Table 1) were synthesized by solid-phase peptide synthesis by using amino acids with fluoren-9-ylmethoxycarbonyl (Fmoc)-protected α-amino groups on a 431A peptide synthesizer (Perkin Elmer, Foster City, CA, U.S.A.) with standard FastMoc programmes. The side-chain-protecting groups used were: trityl for Asn, Cys, Gln and His; t-butyl for Tyr; 2,2,5,7,8-pentamethyl-chroman-6-sulphonyl for Arg; and t-butyl ester for Asp, Gla and Glu. All reagents and amino acids, except for the protected amino acid Gla (Bachem), were peptide-synthesis grade from Perkin Elmer. After each coupling step, the free N-termini of non-reacted peptides were capped by acetic anhydride to avoid the elongation of incompletely synthesized peptides. To cleave the peptide from the resin and to remove the side-chain-protecting groups, the resin-bound protein was stirred for 90 min at room temperature in a cleavage mixture containing 0.75 g of crystalline phenol, 250 μl of ethanedithiol, 500 μl of thioanisole, 500 μl of doubly distilled water and 10 ml of trifluoroacetic acid. The peptide was separated from the resin by filtration through a medium-porosity glass filter and precipitated in cold methyl t-butyl ether. The peptide was washed with methyl t-butyl ether, dissolved in water and dried. The amino acid

Table 1 Sequences of various peptide substrates and inhibitors

Position	-16	-10	-1	1	5	10	15	20	25	30	35	40	45	49
Propeptide Factor IX(Pro^{FIX})	AVFLDHENANKILNRPKR													
Propeptide Osteocalcin (Pro^{OC})	AAFVSKQEGSEVVKRPRR													
Descarboxy Osteocalcin				YLYQWLGAPVPYPDPLEPRREVCELNPDCDELADHIGFQEAYRRFYGPV										
Osteocalcin				YLYQWLGAPVPYPDPLγPRRγVCγLNPDCDELADHIGFQEAYRRFYGPV										
Osteocalcin $^{1-16}$				YLYQWLGAPVPYPDPL										
Descarboxy Osteocalcin $^{13-25}$							PDPLEPRREVCEL							
Osteocalcin $^{13-25}$							PDPLγPRRγVCγL							
Descarboxy Osteocalcin $^{22-31}$								VCELNPDCDE						
Osteocalcin $^{22-31}$								VCγLNPDCDE						
Osteocalcin $^{29-43}$										CDELADHIGFQEAYR				
ProIX-59 Q/S	AVFLDHENANKILNQPKS			YNSGKLEEFVQGNLERECIEEKCSFEEAREVFNTERTNEF										
$Pro(Glu)_{10}$	HVFLAPQQARSLLQRVRR			EEEEEEEEEE										
ProPT-28 F/A	HVFLAPQQARSLLQRVRR			ANTFLEEVRK										

Abbreviations used: ProIX-59 Q/S, the propeptide and Gla-domain of human descarboxyfactor IX, in which R^{-4} has been replaced by Q, and R^{-1} has been replaced by S; $Pro(Glu)_{10}$, the prosequence of human prothrombin covalently bound to ten Glu residues; proPT28 F/A, the prosequence of human descarboxyprothrombin with the first 10 residues of the Gla-domain, in which F^{-16} has been replaced by A; γ stands for gammacarboxyglutamate.

sequence of the synthetic proteins was confirmed by Edman degradation on a 477A/120A protein sequencer (Perkin Elmer).

MS of OC and d-OC

To verify the purity and the full carboxylation at positions 17, 21 and 24 of the synthetic OC and the purity of d-OC, electrospray ionization MS was performed on a Micromass Platform II (Micromass UK Ltd., Altrincham, Cheshire, U.K.) single-quadrupole benchtop mass spectrometer operating in positive ionization mode. For the MS full-scan spectra, data were acquired in continuum mode over the range m/z 600–3000 in 2 s at unit mass resolution. A cone voltage of 35 V was set, which produces predominantly MH^+ ions with little evidence of fragmentation for this type of peptide. Instrumental control, data acquisition and data processing were performed with the MASSLYNX software package (version 2.3). The observed average m/z of OC of 5930.85 units was compared with the calculated MH^+ average mass of 5931.55 units. Also the observed average m/z of d-OC of 5800.05 units was compared with its calculated MH^+ average mass of 5799.45 units. The calculated masses of both OC and d-OC (with a free N-terminus and a carboxy group at the C-terminus) did not differ significantly from the measured masses and we therefore conclude (1) that the OC was pure and fully carboxylated and (2) that d-OC was pure and not fragmented.

Expression of recombinant human carboxylase in *Trichoplusia ni* High Five cells

The cDNA encoding the human vitamin K-dependent carboxylase was sub-cloned into the *Eco*RI site of the pVL1392 vector. The sequence coding for the FLAG antibody epitope was introduced at the N-terminal end of the carboxylase and an HPC4 antibody tag (Glu-Asp-Gln-Val-Asp-Pro-Arg-Leu-Ile-Asp-Gly-Lys) [23] was added at the C-terminal end. The engineered vector was co-transfected with baculovirus BacVector 3000 triple-cut virus DNA into Sf9 (*Spodoptera frugiperda*) cells. Recombinant virus was isolated by plaque purification, amplified and titred by plaque assay in accordance with the manufacturer's instructions (PharMingen). The expression of carboxylase was done by infection of approx. 2×10^6/ml High Five cells with the recombinant virus at a multiplicity of infection of approx. 1. Cells were collected after 48 h by centrifugation, then stored at −80 °C.

Preparation of microsomes from High Five cells

A total of 5×10^8 cells expressing the recombinant human carboxylase were washed with buffer A [25 mM Tris/HCl (pH 7.4)/150 mM NaCl] and a protease inhibitor cocktail [24] consisting of EDTA (2 mM), dithiothreitol (2 mM), Phe-Pro-Arg-chloromethane (0.125 μg/ml), Phe-Phe-Arg-chloromethane (0.125 μg/ml), leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), PMSF (34 μg/ml) and aprotinin (2 μg/ml) together with 10% (v/v) glycerol and resuspended in 50 ml of buffer A. The sample was homogenized by five strokes with a Dounce homogenizer and then sonicated with twenty 5 s pulses and an interval time of 25 s with an Ultrasonic Heat Systems sonicator. Cellular debris was removed by centrifugation at 4000 ***g*** for 15 min. The supernatant was then centrifuged at 105000 ***g*** for 1 h. The microsomal pellet was resuspended in buffer A with a final protein concentration of 20 mg/ml, 1 × protease inhibitor cocktail and 10% (v/v) glycerol, aliquotted and stored at −80 °C until further use.

Purification of recombinant human carboxylase with HPC4 antibody resin

Microsomes were diluted to a final protein concentration of 12 mg/ml and solubilized by the addition of an equal volume of solubilization buffer [50 mM Tris/HCl (pH 7.4)/0.15 M NaCl/1% (v/v) CHAPS/0.2% phosphatidylcholine/10% (v/v) glycerol/1 × protease inhibitor cocktail] at 4 °C for 1 h. The solubilized microsomes were centrifuged at 105000 ***g*** for 1 h and the pellet was discarded. A total of 10 ml of HPC4 resin was equilibrated with wash buffer [20 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.5% CHAPS/0.2% phosphatidylcholine/1 × protease inhibitor cocktail] and added to the solubilization supernatant along with a final concentration of 5 mM $CaCl_2$, then incubated overnight with gentle stirring. The resin was centrifuged and poured into a column, washed with 50 ml of wash buffer plus 5 mM $CaCl_2$ and eluted with wash buffer plus 10 mM EDTA. Carboxylase samples were collected, aliquotted and stored at −80 °C. Enzyme concentration was estimated from dot-blots of carboxylase by using the FLAG antibody and known concentrations of FLAG antibody standard.

Carboxylase assay

Standard reaction mixtures (125 μl) contained: 6 nmol of HPC4-purified γ-glutamylcarboxylase, 25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.2% (v/v) CHAPS, 0.2% phosphatidylcholine, 5 μCi of $NaH^{14}CO_3$, 222 μM vitamin K hydroquinone (KH_2) and 5 mM dithiothreitol. Substrates were added as indicated. The mixture was incubated at 20 °C for 30 min in sealed tubes. Kinetic analysis was performed on samples drawn at 0, 15 and 30 min. All reactions were stopped by the addition of 0.8 ml of 5% (w/v) trichloroacetic acid to 100 μl of reaction mixture; unbound $^{14}CO_2$ was removed by boiling for 3 min. After cooling of the mixture, 5 ml of Formula 989 (Packard Bioscience, Groningen, The Netherlands) was added. Finally samples were counted with a Wallac (Turku, Finland) 1414 WinSpectral liquid-scintillation counter. Results are expressed as pmol of $^{14}CO_2$ incorporated per minute.

RESULTS AND DISCUSSION

Kinetic constants for d-OC carboxylation

Whereas bovine liver microsomal carboxylase can be contaminated with propeptide-containing endogenous substrates, the affinity-purified enzyme is saturated with free propeptide as a consequence of its elution from the affinity ligand. Neither of these systems is suitable for determining the kinetic constants for OC carboxylation or the effects thereon of various propeptides. Therefore we used purified recombinant human carboxylase expressed in insect cells known to be free of endogenous precursor proteins. Carboxylase was eluted from the affinity column in the absence of free propeptide, thereby generating carboxylase completely devoid of interfering propeptide-containing proteins. Using this system we have demonstrated for the first time that, in the absence of propeptide, d-OC is a good substrate for carboxylase. Kinetic constants of various OC-derived substrates were compared with other well-known substrates for carboxylase (Table 2). Substrates containing a propeptide sequence were characterized by K_m values between 0.4 and 5 μM, whereas the K_ms for propeptide-deficient peptides were in the millimolar range. d-OC, which lacks an N-terminal propeptide, is an exception in this respect with a K_m of 1.7 μM. Remarkably, the corresponding peptide, d-$OC^{13–25}$, was a poor substrate for

Table 2 Kinetic constants for different peptide substrates and the cofactor KH_2 during carboxylation

Reactions were performed as detailed in the Materials and methods section. Kinetic parameters were determined by fitting the data to the Michaelis–Menten equation. Values are means ± S.D. for at least three independent determinations.

Substrate	K_m (μM)	V_{max} (pmol $^{14}CO_2$/h)	K_m for KH_2 (μM)
ProIX-59 Q/S	0.51 ± 0.09	90 ± 13	5 ± 0.5
ProPT-28 F/A	5.35 ± 0.77	183 ± 18	3 ± 0.4
$Pro(Glu)_{10}$	0.56 ± 0.18	119 ± 15	9 ± 1.1
d-OC	1.71 ± 0.35	287 ± 17	13 ± 0.9
d-$OC^{13–25}$	9100 ± 2050	470 ± 57	61 ± 3.4
bEELOMe	2000 ± 100	182 ± 11	39 ± 2.3
FLEEL	2400 ± 200	401 ± 32	34 ± 1.7

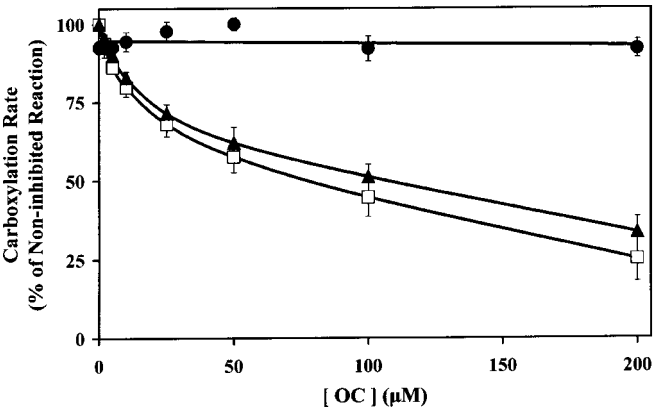

Figure 1 Effect of OC on the carboxylation of different substrates

All incubations were at 20 °C for 30 min. All values are means for at least three independent measurements, and are expressed as a percentage (± S.D.) of the non-inhibited reaction rate. Symbols: □, carboxylation of 2 μM d-OC; ●, carboxylation of 1 μM of ProIX-59 Q/S; △, carboxylation of 5 mM bEELOMe at increasing concentrations of OC. If error bars are not visible they were smaller than the symbol used.

Table 3 Effect of propeptides on apparent K_m for various substrates in the vitamin K-dependent carboxylation reaction

Reactions were performed as detailed in the Materials and methods section. Kinetic parameters were determined by fitting the data to the Michaelis–Menten equation. Values are means ± S.D. for at least three independent determinations.

	K_m (μM)		
Substrate	No addition	With 20 μM Pro^{FIX}	With 20 μM Pro^{OC}
d-OC	1.7 ± 0.35	1.9 ± 0.24	1.8 ± 0.37
d-$OC^{13–25}$	9100 ± 2050	4000 ± 900	10000 ± 1900
ProIX-59 Q/S	0.5 ± 0.10	2.6 ± 0.35	0.4 ± 0.26
bEELOMe	2100 ± 100	600 ± 400	2200 ± 800

carboxylase with a K_m three orders of magnitude higher than that for d-OC, similar to short peptide substrates such as FLEEL. These experiments demonstrate that d-OC contains an internal recognition site for carboxylase, permitting its efficient carboxylation. Because this recognition site was not present in d-$OC^{13–25}$ (containing the entire OC Gla motif) it seems that a sequence outside its Gla motif contributes to the recognition of mature OC by carboxylase. We have also tested d-$OC^{22–31}$ for its ability to serve as a substrate for carboxylase. However, this peptide was not measurably carboxylated, nor was carboxylation observed when $OC^{1–16}$ was added (results not shown). It has been shown previously that for the carboxylation of short peptide substrates the K_m for KH_2 is relatively high but that it is substantially decreased on binding of propeptide-containing substrates [18]. The data in Table 2 show that in this respect d-OC only partly mimics the propeptide-containing substrates, with a K_m for KH_2 that is approx. 3-fold that for the carboxylation of ProIX-59 Q/S, but almost 80 % lower than that for the carboxylation of d-$OC^{13–25}$. These results demonstrate that the presence of the prosequence is not an absolute requirement for increasing the affinity between carboxylase and its cofactor KH_2. The possibility cannot yet be excluded that the function of the prosequence in OC precursors is to induce a more pronounced interaction between carboxylase and KH_2, thereby lowering the K_m for KH_2 to the same values as those observed for the other propeptide-containing substrates. It has been shown previously that the prosequence in propeptide-containing substrates possesses the remarkable property of decreasing the K_m for KH_2 by 10–20-fold [18]; this might contribute to a more efficient utilization of KH_2 in the carboxylation of OC. An explanation for this phenomenon might be found in postulating an allosteric conformational change of carboxylase on binding of the prosequence. Definite proof of this hypothesis might be obtained from a prosequence-containing OC molecule. However, high-efficiency long-chain synthesis by Fmoc chemistry is limited to approx. 50 residues in our hands. Only a few successful long-chain syntheses have been reported so far with the linear Fmoc protocol [25–28], indicating the problems accompanying the synthesis of these long peptide chains (more than 50 residues).

Inhibitory effect of OC on d-OC and bEELOMe carboxylation

Because of the high degree of sequence similarity of d-OC and OC, it seemed feasible that both molecules might have an affinity for carboxylase. In that case OC would be expected to act as an inhibitor of d-OC carboxylation. We therefore investigated the potential inhibitory effect of OC on the carboxylation of various substrates. As is shown in Figure 1, the carboxylation of the propeptide-containing ProIX-59 Q/S (1 μM) was not inhibited, even at a 200-fold molar excess of OC. The carboxylation of d-OC was inhibited with a K_i of 76 μM (approx. 45-fold the K_m for d-OC), and bEELOMe with a K_i of 104 μM (approx. 0.05-fold the K_m for bEELOMe). These results show that OC is not an inhibitor for propeptide-containing substrates, whereas it inhibits propeptide-deficient substrates with high and low sequence similarities to similar extents, suggesting that elements of recognition are shared by both substrate and product. In an attempt to locate more precisely the sequence responsible for the high affinity of d-OC for carboxylase, we tested the potential inhibitory effects of a number of peptides derived from OC: $OC^{1–16}$, $OC^{13–25}$, $OC^{22–31}$ and $OC^{29–43}$. However, none of these peptides, whether alone or in combination, inhibited the carboxylation of d-OC.

Effect of free propeptides on carboxylation of d-OC and ProIX-59 Q/S

As has previously been shown [29], the presence of free propeptide stimulates substrate carboxylation *in vitro* by lowering the K_m for

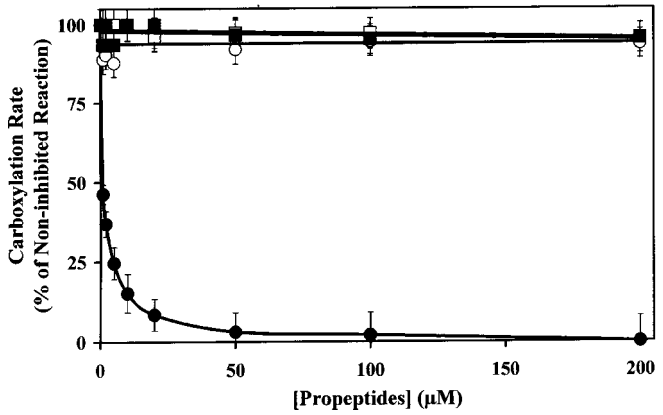

Figure 2 Effect of propeptides on substrate carboxylation

The carboxylation of 1 μM ProIX-59 Q/S was measured at various concentrations of either Pro^{FIX} (●) or Pro^{OC} (○); the carboxylation of 2 μM of d-OC was measured at various concentrations of either Pro^{FIX} (■) or Pro^{OC} (□). Incubations were performed at 20 °C for 30 min. All values are means for at least three independent measurements, and are expressed as a percentage (±S.D.) of the non-inhibited reaction rate. If error bars are not visible they were smaller than the symbol used.

substrates derived from blood-clotting factors. In the present study we investigated whether propeptides have a similar effect on substrates derived from OC. In addition, we examined to what extent free propeptides might act as competitive inhibitors for propeptide-containing substrates during substrate recognition by carboxylase. The propeptides used for these studies were Pro^{FIX} and Pro^{OC}; we measured the effects of these propeptides on the carboxylation of four different substrates. The data shown in Table 3 were obtained in the presence of either 20 μM Pro^{FIX} or 20 μM Pro^{OC} and demonstrate that Pro^{FIX} acted as an inhibitor for the propeptide-containing ProIX-59 Q/S, and that it mildly stimulated the propeptide-deficient bEELOMe. Its effects on OC-derived substrates were negligible. In contrast, Pro^{OC} did not affect the carboxylation of either substrate. At increasing concentrations of Pro^{FIX}, a dose-dependent inhibition with a K_i of 0.8 μM of ProIX-59 Q/S was observed (Figure 2), whereas even at 200 μM of Pro^{FIX} the carboxylation of d-OC remained unaffected. In contrast, Pro^{OC} did not inhibit either substrate even at very high concentrations. The fact that Pro^{OC} did not inhibit ProIX-59 Q/S carboxylation is consistent with its reported low affinity for carboxylase (T. Stanley, D. Jin, P.-J. Lin and D. W. Stafford, unpublished work). From these results we conclude (1) that the affinity of carboxylase for Pro^{OC} must be substantially lower than that for Pro^{FIX}, and (2) that carboxylase must have at least two different docking sites: a high-affinity site for d-OC and a site for propeptide-containing substrates. In our opinion it is unlikely that an attached propeptide lowers the K_m for OC even further. Results reported by Benton et al. [20] showed only a 3-fold difference between propeptide-lacking and propeptide-containing d-OC (0.72 μM compared with 0.24 μM respectively). Comparison of these results with ours is hampered by the fact that Benton et al. studied the kinetic parameters in purified bovine carboxylase that contained 100 μM free human factor X propeptide. OC itself might contain an internal recognition site for carboxylase. This is also seen in MGP, another Gla-protein synthesized in bone. Because OC and MGP have significant sequence similarities, it has been suggested that both proteins have developed from a common ancestor protein [30,31]. However, no sequence homology between the internal propeptide of MGP and mature OC is seen; we therefore suggest the presence of a different binding site on carboxylase.

In conclusion, we have been able to demonstrate that d-OC binds with high affinity to carboxylase, and that the OC prosequence does not have a noticeable role in the enzyme–substrate interaction. Moreover, the results strongly suggest the presence of two substrate-docking sites on carboxylase: one recognizing the prosequence in clotting-factor-type substrates, and another with high affinity for OC precursors. However, it is still not known which structural determinants of OC contribute to its recognition by carboxylase.

We thank Dr. Dirk T. S. Rijkers (Department of Medicinal Chemistry, Institute for Pharmaceutical Sciences, Utrecht University, the Netherlands) for providing the electrospray MS results for synthetic OC. P.P. is a senior research assistant of the Fund for Scientific Research (FWO-Vlaanderen).

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Received 5 February 1999/12 April 1999; accepted 5 May 1999