Osteocalcin: posttranslational gamma carboxylation of a unique vitamin K-dependent protein

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Posttranslational gamma carboxylation of a unique
vitamin K-dependent protein

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**Osteocalcin:
Posttranslational gamma carboxylation of a unique
vitamin K-dependent protein**

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. Dr. A.C. Nieuwenhuijzen Kruseman
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 16 december 1999 om 16.00 uur

door

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*What is man, when you come to think upon him, but
a minutely set, ingenious machine for turning, with
infinite artfulness, the red wine of Shiraz into urine ?*

Isak Dinesen (Karen Blixen)

Seven Gothic Tales (1934) "The Dreamers"

Voor Jacqui,

Voor mijn ouders en Erwin.

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Abbreviations

bEELoMe	The tripeptide boc-glutamate-glutamate-leucine-methyl
BGP	Bone Gla protein
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
d-...	Refers to a descarboxy protein
DHFR	Dihydroxy folate reductase
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
FII	Prothrombin
FIX	Coagulation factor IX
FIXproGLA41	59 Amino acid peptide containing the human factor IX propeptide and first 41 residues of factor IX gla domain (Sequence -18 to 41)
FIXproGLA46	64 Amino acid peptide containing the human factor IX propeptide and the entire gla domain and hydrophobic stack sequence (Sequence -18 to 46)
FIXproNonGLA	52 Amino acid peptide containing the factor IX propeptide followed by a 34 amino acid non-gla domain sequence
FLEEL	The pentapeptide phenylalanine-leucine-glutamate-glutamate-leucine
FLEEV	The pentapeptide phenylalanine-leucine-glutamate-glutamate-valine
FVII	Coagulation factor VII
FX	Coagulation factor X
γ-CRS	Gamma glutamyl carboxylase recognition sequence
Gla	Gamma-carboxyglutamic acid
Glu	Glutamic acid
HPLC	High performance liquid chromatography
K ₁	Phylloquinone

K ₂	Groupname for Menaquinones
K ₃	Menadione
kDa	KiloDalton
KH ₂	Vitamin K hydroquinone
KO	Vitamin K 2,3 epoxide
MBP	Maltose binding protein
MGP	Matrix Gla protein
MK-n	Menaquinones
MOPS	3-(N-morpholino)propane-sulfonic acid
MW	Molecular weight
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) reduced form
(NH ₄) ₂ SO ₄	Ammonium sulphate
OC	Osteocalcin
PAA	Polyacryl amide
PAGE	Polyacryl amide gel electrophoresis
PC	Phosphatidylcholine
Pro	Propeptide
Pro(Glu) ₁₀	The prosequence of human prothrombin covalently bound to ten Glu residues
ProFIX	Full length uncarboxylated human factor IX with the propeptide still attached
Pro ^{FIX}	Propeptide of human coagulation factor IX precursor molecule
ProFIX(GLA)	Full length carboxylated human factor IX with the propeptide still attached
ProFIX19	AVFLDHENANKILNRPKRY
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 ^{OC}	Propeptide of human osteocalcin precursor molecule
ProPT-28 F/A	The prosequence of human descarboxyprothombin with the first

Abbreviations

	10 residues of the Gla-domain, in which F ⁻¹⁶ has been replaced by A
RER	Rough endoplasmic reticulum
S-MeTPT	Methylated tripeptide
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
ucOC	Undercarboxylated osteocalcin not bound to hydroxyapatite

Chapter 1

GENERAL INTRODUCTION

Vitamin K and its antagonists in a historical perspective

The first reports about the possibility of a new and so far unknown vitamin involved in blood coagulation were made by the Danish biochemist Henrik Dam who studied the effect of a cholesterol depleted diet on chickens ¹. Cholesterol was removed from the chicken feed by ether-extraction after which the food was supplemented with the other known essential fat-soluble components and vitamins. After several weeks, the chickens showed spontaneous bleedings, delayed blood-clotting, and finally died within a month. Administration of purified cholesterol and / or extra vitamins to the diet did not result in abrogation of the bleeding tendency. Eventually, in 1935, Dam was able to prove that a deficiency of a yet unknown fat soluble vitamin was the sole cause of this hemorrhagic disease. He called this fat-soluble, anti-hemorrhagic vitamin "Vitamin K", after the first letter of the Danish word Koagulation².

The observations made by Henrik Dam had a remarkable similarity with those made in cattle in the early 1920's ³. It turned out that cattle fed on improperly cured sweet clover hay suffered from spontaneous bleedings comparable to those observed in vitamin K deficient chickens. The component in the hay responsible for these phenomenons was identified as 3,3'-methylene-bis-(4 - hydroxycoumarin) and called dicoumarol (figure 1) ^{4,5}. This component is also present in fresh sweet clover where it is chemically bound, and thus inactive. However, after dry-storage of the hay it may be liberated and become active. Additional research showed that dicoumarol and some of its derivatives act as an antagonist of vitamin K action. Presently they are used as pharmacological agents for anticoagulation and as rodenticides. In humans coumarin derivatives serve for the prevention of thrombotic events through their inhibition of the hepatic synthesis of the coagulation factors prothrombin, VII, IX and X. Since during recent years a number of vitamin K-dependent proteins have been discovered with regulatory functions in other physiological processes than blood coagulation ⁶⁻¹¹, unexpected side-effects of anticoagulant therapy cannot be excluded. An example of such side-effects is the fetal warfarin embryopathy in which severe bone / cartilage malformations occur in the developing fetus resulting in abnormal cartilage calcification, nasal hypoplasia and abnormalities of the spine ¹².

Figure 1: Structure of several vitamin K antagonists

Various forms of vitamin K

Vitamin K is a generic term for a group of distinguishable compounds that possess "vitamin K activity", and are called phyloquinone (K_1), menaquinones (K_2) and menadione (K_3). Phyloquinone and menaquinones are naturally occurring vitamers, whereas menadione is a synthetic analogue of K_1 .

All K vitamers have a 2-methyl-1,4-naphtoquinone ring structure in common but they differ in the length and degree of saturation of an aliphatic side chain, located at the 3-position. Phyloquinone is produced by green algae and in green leafy plants, where it functions as an electron carrier in chloroplasts¹³. Its phytyl side chain consists of 4 isoprenoid residues of which the first one is unsaturated.

Menaquinones are products of bacterial metabolism; they are generated in mammalian intestines and also occur in fermented food products such as sauerkraut,

cheese, curd-cheese and natto (fermented soy beans). The various menaquinones differ in their phytyl side chain length which may vary from 4 up to 13 isoprenoid units. According to the generally accepted nomenclature menaquinones are designated as MK-n, where n stands for the number of isoprenoid units in the side chain. At increasing side chain length the hydrophobicity of the menaquinones will increase proportionally.

Menadione, or 2-methyl-1,4-naphthoquinone, is a synthetic product and does not occur in nature. Menadione is water soluble, and has to be metabolized *in vivo* before it obtaining vitamin K activity. As a therapeutic agent, however, menadione is not applied any more in humans because of its possible adverse side effects such as haemolysis^{14,15}. Several authors have reported *in vitro* suppression of tumor cell growth by menadione¹⁶⁻²⁰. Menadione is currently used on a large scale as a supplement in

Vitamin K1

Menaquinon -n

Menadion

Figure 2: Chemical structures of vitamin K₁ (phylloquinone), menaquinone (vitamin K₂) and menadione (vitamin K₃)

animal food (figure 2).

Functions of vitamin K

Vitamin K is involved as a co-factor in the post-translational processing of proteins. It was already postulated in 1963 by Hemker et al ²¹ that the blood plasma of patients receiving anticoagulant therapy contained an abnormal form of prothrombin (factor II) which they called preprothrombin, the so-called precursor molecule of the active prothrombin. This hypothesis was supported by both Ganrot ²² and Josso ²³ who demonstrated a protein in plasma of anticoagulated patients which behaved immunochemically like prothrombin but immunoelectrophoretically showed differences when compared to prothrombin. Moreover, it remained inactive in an *in vitro* coagulation assay because it was unable to bind Ca^{2+} ions. However, it took until 1974 before several groups reported independently ²⁴⁻²⁶ that these so called PIVKA's (Protein Induced by Vitamin K Antagonists) were lacking an uncommon amino acid: γ -carboxyglutamic acid (Gla). The absence of this highly negatively charged amino acid was the reason that PIVKA-prothrombin (PIVKA-II) was incapable of binding Ca^{2+} ions.

Figure 3: Structures of glutamic acid (Glu) and γ -carboxy glutamic acid (Gla)

Subsequent investigations showed that the Gla-residues bind Ca^{2+} , which is necessary for the biological activity of various coagulation factors, and that vitamin K promotes the formation of specific glutamic acid residues (Glu) into Gla during the posttranslational modification stage of protein biosynthesis (figure 3) ^{24,25}.

For a long time it has been thought that prothrombin and the factors VII, IX, and X were the only proteins that required vitamin K for their synthesis. However, during the last 25 years various vitamin K-dependent proteins have been discovered both in mammalian cells and in invertebrate cells ^{27,28}. These Gla-proteins require Ca^{2+} either for their binding to phospholipid surfaces, the regulation of tissue mineralization, or for the regulation of cell growth. In all cases in which their function is known, the binding of Ca^{2+} to Gla-residues is essential for the activity of the various Gla-proteins (figure 4). Since also Gla-proteins of (a yet) unknown function have been discovered (e.g. the proline-rich Gla-proteins, PRGP's ²⁹) another Ca^{2+} -independent role for Gla-residues cannot be excluded.

	1	10	20	30	40
Prothrombin	A N T	F L Y Y V R K G N L Y R Y C V Y Y T C S Y Y Y A F Y A L Y S S T A T D V F			
Factor VII	A N A	F L Y Y L R P G S L Y R Y C K Y Y Q C S F Y Y A R Y I F K D A Y R T K L F			
Factor X	A N S	F L Y Y M K K G H L Y R Y C M Y Y T C S Y Y Y A R Y V F E D S D K T N Y F			
Protein C	A N S	F L Y Y L R H S S L Y R Y C I Y Y I C D F Y Y A K Y I F Q N V D D T L A F			
Protein S	A N S	L L Y Y T K Q G N L Y R Y C I Y Y L C N K Y Y A R Y V F T N D P Y T D Y F			
Gas 6	A F Q	V F Y Y A K Q G H L Y R E C V Y Y L C S R Y Y A R Y V F Y N D P Y T D Y F			
Protein Z	A G S Y L L Y Y L F Y G N L Y K Y C Y Y Y I C V Y Y A R Y V F Y N Y V T D Y F				
Factor IX	Y N S G K L Y Y F V Q G N L Y R Y C M Y Y K C S F Y Y A R Y V F Y N T Y R T T Y F				
MGP	W R A K V Q Y R I R Y R S K P V H Y L N R Y A C D D Y R L C Y R Y A M V Y G Y N A				
Osteocalcin	Y L Y Q W L G A P V P Y P D P L Y P R R Y V C Y L N P D C D E L A D H I G F Q E A				

Figure 4: The Gla-domains of all the known human vitamin K-dependent proteins. The sequence is in the one letter code; γ stands for Gla, highly homologous Gla residues are boxed, MGP stands for matrix Gla protein. No homology between MGP and/or Osteocalcin and the other vitamin K-dependent proteins can be detected.

However, since Gla has been discovered in both vertebrates and invertebrates, this amino acid obviously plays an important role in protein function.

The vitamin K-dependent carboxylation reaction

The vitamin K mediated conversion of Glu to Gla is one of the alleged post-translational conversions such as glycosylation, hydroxylation, phosphorylation, and disulfide bond formation, which take place during protein synthesis. These processes occur at the luminal side of the rough endoplasmic reticulum (RER), after which the processed proteins are transported through the luminal area of the RER via the Golgi apparatus to the extracellular environment. These type of proteins are generally referred to as secretory proteins. All known Gla-proteins are secretory proteins with the possible exception of carboxylase itself³⁰, or the putative Proline Rich Gla Proteins 1 and 2 (PRGP1 and PRGP2)²⁹. The PRGP cDNA's predict amino acid sequences that contain a potential transmembrane region and a proline rich cytoplasmic domain. Until now only

Figure 5: Biosynthetic pathway for vitamin K-dependent production of Gla. It has been hypothesized that a free cysteine residue present in the carboxylase converts KH₂ into a strong base, able to abstract a hydrogen from the γ-carbon of Glu³². In a next reaction step CO₂ is added to the γ-carbon of Glu to form Gla. The activated vitamin K alkoxide collapses into vitamin K epoxide and is converted back to KH₂ by the action of two vitamin K reductases which are sensitive to warfarin.

the mRNA's of these proteins have been demonstrated, but no functional protein has been detected or isolated yet. Moreover the γ -glutamyl carboxylase itself has been shown to be a transmembrane Gla-protein³⁰. Although vitamin K in the human food is mainly present in the quinone (K) form, the active cofactor for carboxylase is reduced vitamin K, vitamin K hydroquinone (KH₂). During the carboxylation reaction KH₂ is converted into vitamin K-epoxide (KO) in the same reaction in which Glu is converted to Gla in a 1:1 stoichiometry, and in the presence of both CO₂ and O₂. Carboxylation of Glu residues within the Gla-domain of vitamin K-dependent proteins is a processive mechanism³¹, which implies multiple carboxylations to occur within one molecule during a single association with the enzyme. A recently proposed mechanism for the carboxylation reaction is that of basicity enhancement by which oxygenation of vitamin KH₂ results in the generation of a strong active oxygenated base which then removes a proton from the γ -carbon of glutamic acid³². The active vitamin K intermediate, which is potentially toxic, collapses subsequently to vitamin KO and the glutamate carbanion undergoes carboxylation to yield γ -carboxyglutamic acid (figure 5)³³.

Because of its toxicity, it is undesirable to generate the vitamin K intermediate intracellularly. Therefore it is not present in the absence of substrate glutamate residues³⁴. In two following steps vitamin KO is reduced to vitamin K and vitamin KH₂ by the action of one or more dithiol dependent reductases (figure 6). This "recycling" mechanism explains why the daily requirement for vitamin K is low and thus why vitamin K deficiency is rarely seen in healthy subjects^{35,36}.

More recently, two components of multiprotein enzyme complex vitamin K epoxide reductase have been identified: microsomal epoxide hydrolase and a glutathione S-transferase super gene family member^{37,38}. The dithiol-dependent reduction of KO and K is not the only mechanism known to generate KH₂. Vitamin K-quinone (and not KO) may be reduced by DT-diaphorase, an NAD(P)H dehydrogenase, but since this pathway does not allow recycling of vitamin K, a blockade of the dithiol-dependent pathway leads to an at least 1000-fold increased vitamin K requirement³⁹. Therefore the NAD(P)H-dependent reductase does not play an important role in case of physiological concentrations of vitamin K *in vivo*³⁹. The dithiol-dependent pathway

Figure 6: Schematic representation of the vitamin K cycle. Vitamin K is reduced to KH₂ via the dithiol dependent step 1. Step 2 is the subsequent carboxylase / epoxidase reaction in which Glu is converted to Gla. Step 3 is the reduction of vitamin K epoxide to KH₂. Step 4 is required in case of anticoagulation (phenprocoumon) therapy or rodenticide poisoning, since steps 1 and 3 are blocked in this situation.

of vitamin K recycling is sensitive to coumarin anticoagulants such as acenocoumarol, warfarin and phenprocoumon⁴⁰. Treatment of patients suffering from thromboembolic disease with coumarin-type anticoagulants is based on the prevention of reduction of vitamin K and KO by blocking the dithiol-dependent reductases. In this way the vitamin K-dependent coagulation factors are synthesized in an inactive form and cannot contribute to blood coagulation⁴¹. Intoxication with coumarins as a result of ingestion of rat poison must be treated with high daily doses of vitamin K, and is invariably associated with the appearance of vitamin KO in plasma after the administration of vitamin K. In this case the NAD(P)H-dependent reductase is the sole enzyme generating vitamin KH₂ *in vivo* to overcome the intoxication.

Vitamin K-dependent proteins

During recent years a large variety of vitamin K dependent Gla proteins have been

discovered that are involved in a) haemostasis^{6,8,24,42-44}, b) mineralization processes^{45,46}, c) regulation of cellular proliferation^{11,47,48} and d) other processes. Below the proteins in these three categories will be discussed briefly.

A. Haemostasis

Haemostasis is a complex process which is triggered by the release of tissue factor (TF) after injury of a blood vessel and which results in the formation of a fibrin clot via a cascade of zymogen activations. In this cascade about 15 different proteins are involved, 7 of which are Gla-proteins. Ca^{2+} mediated binding to negatively charged phospholipid surfaces is essential for either the activation or the function of these proteins, or both.

Prothrombin (FII) was the first protein discovered to contain Gla-residues^{24,25,49}, soon followed by factors VII, IX and X^{42,43}. These proteins are all involved in the formation of thrombin and thus in the pro-coagulant properties of blood. Proteins C and S are also Gla-proteins but regulate blood coagulation by inhibiting it. Protein C is activated by thrombin to activated protein C (APC). APC on its turn inactivates both activated and non-activated factors V and VIII by proteolytic cleavage.

Protein S serves as a cofactor for the inactivation of factors Va and VIIIa by APC. The cofactor activity of protein S is regulated by C_4 -binding protein (C4BP)⁵⁰, a member of the classical complement pathway. Approximately 40% of the protein S is available for this cofactor function, whereas 60% of the protein is bound to C4BP and thus lacks cofactor activity⁵⁰. Moreover, it is demonstrated that protein S is synthesized by osteoblasts in bone⁵¹, and that protein S deficiency in children results in reduced bone mineral density and increased fracture risk of the vertebra⁵². Nakamura et al.⁵³ demonstrated that protein S enhances the bone resorbing activity of mature osteoclasts suggesting that protein S is a direct regulator of osteoclastic function.

Protein Z, the physiological role of which remained unknown until 1991, is involved in thrombin fixation. This was demonstrated by Hogg and Stenflo⁵⁴ who reported that protein Z binds to thrombin in a Ca^{2+} -independent way. Furthermore these authors showed that protein Z, when bound to phospholipid vesicles, bound to thrombin

and therefore could function as an intermediate to retain thrombin to the site of a vascular injury.

B. Mineralization processes

The discovery of Gla also led to the identification of vitamin K-dependent proteins not related to blood coagulation. These proteins were isolated from bone ⁷, atherosclerotic plaque ^{55,56}, renal calculi ⁵⁷ and kidney ^{58,59}, but were not sufficiently characterized in all cases. It is possible that most of these reports were referring to the same protein, later identified as matrix Gla-protein (MGP).

The first Gla-protein to be discovered in bone was osteocalcin or bone Gla-protein (BGP) ^{7,60}, the most abundant non-collagenous protein found in bone. Osteocalcin synthesis is induced in osteoblasts by the vitamin D metabolite 1,25-dihydroxy-D₃ (1,25(OH)₂D₃) ^{61,62}. Its function was demonstrated by Ducy et al ⁶³, who showed that osteocalcin deficient mice displayed an increased bone formation, and therefore the authors concluded that osteocalcin limits bone matrix formation without affecting resorption or mineralization. Thus, osteocalcin was identified as a negative regulator of bone formation.

The second Gla-protein isolated from bone was matrix Gla protein (MGP) ¹⁰. It did not take long before it was demonstrated that MGP was predominantly found in cartilage and in various soft tissues ⁶⁴. In developing bone it is associated mainly with the nonmineralized matrix, and therefore it appears earlier than osteocalcin ⁶⁵. Its function in mineralization processes was clearly demonstrated by Luo et al ⁴⁵, who reported that MGP deficient mice underwent spontaneous calcification of the arteries and cartilage. Absence of MGP also resulted in inappropriate calcification of the growth plate cartilage and caused short stature, osteopenia and fracture. Therefore the authors concluded that MGP is an inhibitor of soft tissue calcification. Price et al. recently demonstrated that the vitamin K antagonist warfarin was capable of inducing similar results as observed in the MGP deficient mice ⁶⁶. Since vitamin K cannot counteract the effect of warfarin in extrahepatic tissues, the synthesis of Gla-proteins can be blocked in these tissues by a combination of warfarin and vitamin K, without the risk of

bleeding⁶⁷. Using this regiment it could be shown that rats developed calcifications of the elastic lamellae in arteries and heart valves within 3-5 weeks.

C. Regulation of cell growth

Growth arrest specific gene 6 product (Gas6) is one of the more recently discovered vitamin K-dependent proteins but has only been investigated in cell culture systems thus far. Manfioletti et al. reported that it is expressed in many human and murine tissues and may be involved in the regulation of a protease cascade relevant in growth regulation¹¹. Gas6 has been demonstrated to be a ligand for several receptor tyrosine kinases^{68,69} a function for which Gla residues are essential⁷⁰. Furthermore, Nakano et al⁴⁷ showed that Gas6 specifically potentiates vascular smooth muscle cell (VSMC) proliferation mediated by Ca^{2+} -mobilizing receptors and prevents cell death if VSMC are starved. Since Gas6 is involved in the *in vitro* maintenance of growth arrested VSMC's and the proliferation of growing VSMC's this suggests that Gas6 may be essential for the development and maintenance of the arterial vessel wall and the process of atherosclerosis⁷⁰. Other authors reported Gas6 to be capable of inducing the re-entry of starved NIH3T3 cells into the cell cycle and thus preventing cell death by apoptosis⁷¹. Nakamura et al. more recently demonstrated that Gas6 enhanced bone resorbing activity of mature osteoclasts, and that this function could be inhibited by a tyrosine kinase inhibitor⁵³. These results indicate that Gas6 is also involved in bone resorption *in vivo*.

D. Other

The enzyme γ -glutamyl carboxylase itself was recently reported to a Gla-protein³⁰, containing 3 Gla residues per molecule carboxylase. The physiological importance of this remarkable observation is unclear as yet.

The cDNA's of the proline rich Gla proteins 1 and 2 (PRGP1 and PRGP2) were reported recently by Kulman et al²⁹. These proteins are putative since the corresponding proteins have not yet been found. cDNA's were synthesized from mRNA's using primers based on a DNA sequence from an expressed sequence

database that was discovered using the query sequence LEEXXXLXLERECXEEXCXEEARE. This sequence was derived via alignment of all known human Gla domain sequences. Both PRGP's contain potential transmembrane domains, which makes them distinct from the other Gla-proteins. Possibly, both PRGP's are members of a yet undiscovered family of transmembrane Gla-proteins.

The γ -glutamyl carboxylase

The enzyme γ -glutamyl carboxylase is an integral transmembrane protein of the RER and consists of 758 amino acids with a molecular weight of 94 kDa. The cDNA sequences for both human ⁷² and bovine ⁷³ carboxylase have been reported. Their sequences predict a hydrophobic region including a minimum of 5 and a maximum of 7 transmembrane domains surrounded by a hydrophilic stretch both at the N-terminal and the C-terminal region. The C-terminal hydrophilic region contains eight of the nine potential glycosylation sites ⁷².

γ -Glutamyl carboxylase occurs in liver ⁷⁴ as well as in a large variety of other tissues such as lung, kidney, testis ⁷⁴, spleen, pancreas, thyroid, vessel wall, bone (osteoblasts) ^{75,76}, tumor cells and skin ^{77,78}. Although not yet proven, it is generally accepted that the carboxylases from the various tissues are the product of the same gene, located on chromosome 2 at position p12 ⁷⁹. After its chromosomal location had been discovered, the complete genomic sequence including the transcription start site of the enzyme was reported ⁸⁰. Furthermore, a recent study showed that the γ -glutamyl carboxylase could carboxylate itself, both *in vitro* and *in vivo* in a reaction dependent on vitamin K ³⁰. Berkner demonstrated that carboxylase contains 3 moles of Gla per mole of carboxylase and suggested that carboxylase *in vitro* is involved in substrate turnover whereas *in vivo* these results suggested a potential role of Gla in carboxylase stability. Whether carboxylase is capable of autocarboxylation via an intramolecular process or is carboxylated by a different carboxylase molecule via an intermolecular reaction remains unclear, although the intramolecular mechanism is the most likely one. Price and Williamson observed a sequence within carboxylase sharing homology over

24 residues (495 -518) with the MGP region 14 - 37 which covers the internal recognition site for carboxylase⁸¹. These authors suggested that this sequence might block the active site of the enzyme and thereby play a regulatory role in preventing nonspecific protein carboxylation. However, it was shown by Berkner and Pudota that carboxylase is capable of carboxylating itself without the requirement of an exogenous propeptide containing substrate³⁰. This suggests strongly that the regulatory theory of Price and Williamson does not apply to the carboxylation of carboxylase itself.

The propeptide binding site present on carboxylase has been demonstrated by several authors using substrate- and propeptide-based affinity labeling reagents⁸²⁻⁸⁶. However, these authors report different locations of the propeptide binding site on the enzyme between residues 1 and 225⁸²⁻⁸⁵, between residues 438 and 758⁸⁶. Another group reports the position of the active site on carboxylase to be located between residues 350 and 508⁸⁷. The reason for these differences may be due to variations in affinity labelling experiments giving different results. Although it is not clear yet, the propeptide binding site and possibly the Glu binding site of carboxylase is located in the N-terminal third and central third of the enzyme³⁴.

Purification of γ -glutamyl carboxylase

Several attempts to purify the vitamin K-dependent carboxylase from bovine liver have been made. The first author describing a 400 fold purification of the carboxylase was Girardot⁸⁸. De Metz et al. used an immobilized factor X antibody, capable of binding carboxylase from coumarin treated animal liver via the complexed factor X precursor⁸⁹. This "solid phase carboxylase" retained its activity for FLEEL. Hubbard et al.⁹⁰ and Harbeck et al.⁹¹ reported similar results but unfortunately the specific activity of the solid phase carboxylase differed not substantially from the preparations by Girardot⁸⁸. Wu et al. were the first to report the purification of bovine carboxylase to near homogeneity⁹². These authors used a recombinant protein, produced in *Escherichia coli*, which consisted of the propeptide attached to the Gla-domain of the human factor IX precursor molecule (ProIX-59 Q/S)⁹³. This 59 amino acid long substrate displayed

Figure 7: Activity profile and reducing SDS-PAGE analysis (10 % silver-stained gel) of fractions from Affi-FIXQ/S chromatography elution. The fraction number is shown on the x axis, whereas the y axis represents the carboxylase activity in each lane. Carboxylase activity was determined by the $^{14}\text{CO}_2$ incorporation into FLEEL. Lane L is microsomal loading material; fractions 1-20 are flow-through (only one is shown); fractions 21-30 are wash; fractions 31-49 are Triton X-100 gradient; fractions 56-63 are CHAPS gradient; fractions 64-75 are CHAPS / proFIX19 double gradient; fractions 76-90 are 1 % CHAPS / 2 μM proFIX19 elution. Molecular mass are shown on the right. Figure from Wu et al, 1997 ⁹⁴.

a K_m for carboxylase in the submicromolar range. Affinity chromatography of ProlX-59 Q/S as a solid phase ligand resulted in the specific extraction of carboxylase from solubilized bovine liver microsomes. Next, the enzyme was eluted with propeptide of factor IX (proFIX19) resulting in a carboxylase preparation which had a 185-fold higher specific activity than that obtained with the best method previously described ⁹⁰. The molecular weight of the purified carboxylase was 94 kDa (figure 7). Previous attempts to purify carboxylase using only the propeptide coupled to a solid phase had resulted in the purification of a protein with a molecular weight of 77 kDa ⁹⁰. Subsequent experiments showed that the protein in case was not carboxylase, but BiP, one of the most abundant proteins present in the endoplasmic reticulum ⁹². It was suggested by Wu et al. that BiP preferentially binds small peptides (e.g. propeptide), since it was not present in the preparation by Wu et al. ⁹². Also no carboxylase activity for BiP was found.

The fact that the carboxylase purified by Wu et al. ^{92,94} was of bovine- in stead

of human origin was one of the disadvantages of this purification. Since research was mainly focussed on human carboxylase, a method to obtain sufficient carboxylase of human origin was desired. Another disadvantage was the presence of 2 μM propeptide in the preparation after the elution of carboxylase from the solid phase. Interactions between pro-containing substrates and the purified carboxylase *in vitro* are expected to be interfered by the propeptide present. As a consequence a propeptide-free purification protocol for human carboxylase was necessary. Several authors described the construction and *in vitro* expression of recombinant (human) carboxylase in various cultured cells^{95,96}. However, these protocols use free propeptide in the final elution step of the carboxylase, apparently to stabilize the carboxylase. Using a *Trichoplusia ni* High Five insect cell line, which overexpresses transiently infected human γ -glutamyl carboxylase cDNA, it was demonstrated by Stafford et al. that carboxylase can be purified without the need of a free propeptide. Transcription and subsequent translation of the cDNA resulted in a carboxylase with a covalently attached affinity tag at the C-terminus of the molecule. Impure carboxylase from solubilized insect cell microsomes is bound to an affinity resin in the presence of Ca^{2+} . Subsequently the resin is washed and the carboxylase is eluted from the resin using an ethylenedinitrilo tetraacetic acid (EDTA) concentration gradient. This purified carboxylase was fully active without the need of stabilizing propeptide present. The purification of human γ -glutamyl carboxylase from insect cells is described in more detail in chapter 4 of this thesis.

The vitamin K-dependent carboxylation reaction *in vitro*

Shah and Suttie⁹⁷ were the first authors to report a reaction in which postmitochondrial supernates from vitamin K deficient rats responded to the *in vitro* addition of vitamin K by producing prothrombin. They reported that this system was energy dependent, and that it was inhibited by antagonists of vitamin K but not by cycloheximide, a cytostatic, added to inhibit *de novo* synthesis of prothrombin. The authors concluded that the system represented a conversion of endogenous rat liver prothrombin precursor to prothrombin by modification of the precursor molecule through attachment of a calcium

binding group. The authors also claimed that this conversion involved carboxylation of glutamyl residues, rather than the synthesis of new prothrombin polypeptide. Subsequently, other investigators applied a similar system using microsomal preparations from both warfarin treated and normal rats and cows⁹⁸⁻¹⁰¹, in which $\text{NaH}^{14}\text{CO}_3$ was used to measure $^{14}\text{CO}_2$ incorporation into the endogenous precursor proteins present in the microsomal preparations. This reaction turned out to be dependent on O_2 , CO_2 , vitamin K, NAD(P)H and/or a reduced nucleotide-generating system. In the early experiments vitamin K-quinone was used as a cofactor for carboxylase. Under these conditions relatively low $^{14}\text{CO}_2$ incorporation rates were found. After adding NAD(P)H to the microsomes the incorporation rates doubled, suggesting that vitamin K had to be reduced before it could function as cofactor for carboxylase. Later it was demonstrated that NAD(P)H could be omitted by using the reducing agent dithiol threitol (DTT), which increased $^{14}\text{CO}_2$ incorporation fivefold, thus providing further evidence for the necessity of KH_2 in the carboxylation reaction^{100,101}. Finally, the *in vitro* carboxylation reaction was shown to be maximally stimulated by KH_2 even in the absence of reducing agents like DTT.

Triton X-100 was frequently used to solubilize the microsomal fraction but was later replaced by 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). This solubilized washed microsomal system contained all activities necessary for carboxylation *in vitro*.

Substrate specificity of γ -glutamyl carboxylase, function of the propeptide

The first *in vitro* substrates for γ -glutamyl carboxylase were based on homologous amino acid sequences of the Gla domains of mature vitamin K-dependent proteins. The peptides synthesized were Phe-Leu-Glu-Glu-Leu (FLEEL) and Phe-Leu-Glu-Glu-Val (FLEEV), similar to residues 5 to 9 of various descarboxy blood coagulation factors¹⁰². Suttie and Hageman used this sequence in particular although the Gla domain of bovine descarboxy-prothrombin consists of 10 Glu residues at positions 7, 8, 15, 17, 20, 21, 26, 27, 30 and 33⁴⁹. The reason to choose a short peptide was that the

published prothrombin sequence did not reveal a unique sequence which might act as a recognition signal for carboxylase, and the choice for FLEEL was mere coincidence¹⁰². FLEEL was carboxylated *in vitro* but displayed a low affinity for carboxylase, resulting in K_m values in the millimolar range. These results suggested that *in vivo* substrates for carboxylase must contain a high affinity recognition sequence outside the Gla-domain. Therefore, intact heat-decarboxylated coagulation factors such as prothrombin were used as a substrate, but again very high K_m values for carboxylase were found¹⁰³. Since the plasma concentrations of prothrombin *in vivo* are so low (in the *micro* to *nano* molar range), the K_m value of thermally decarboxylated prothrombin was expected to be in that range also. An exception was heat-decarboxylated bovine osteocalcin, which had low K_m values for carboxylase¹⁰⁴. Whether this high affinity was due to protein damage as a result of the thermal decarboxylation remained unclear. Later it was shown by Engelke et al¹⁰⁵ that heat decarboxylated MGP displayed similar substrate properties as heat-decarboxylated osteocalcin.

In 1987 several authors reported independently that a "propeptide" could be responsible for the high affinity of Gla-protein precursors for carboxylase¹⁰⁶⁻¹⁰⁹. *In vivo* the 18 amino acid residues of the propeptide are removed prior to secretion by a peptidase-cleavage between arginine -1 and tyrosine 1, to yield the mature protein sequence. A significant contribution to our insight in the posttranslational processing of Gla-proteins was made by analysis of three naturally occurring factor IX mutants; factor IX Cambridge¹¹⁰, factor IX Oxford-3¹¹¹, and factor IX San Dimas¹¹². The resulting defects are caused by point mutations in the gene which resulted in a serine to arginine substitution at position -1 in factor IX Cambridge and a glutamine to arginine substitution at position -4 in both factors IX Oxford-3 and IX San Dimas. As a consequence, the propeptide is not removed by protease cleavage and therefore the mutant proteins circulate in plasma with the propeptide still attached¹⁰⁶. N-terminal protein sequence analysis revealed the presence of residues -18 to -1 in the mutated molecules and thereby the length of the factor IX propeptide could be defined¹⁰⁶. Subsequent cDNA analysis of prothrombin^{113,114}, factor X^{115,116}, factor VII¹¹⁷, protein C¹¹⁸⁻¹²⁰, protein S^{121,122}, and osteocalcin^{123,124} revealed that the latter proteins, like factor

	-18	-16				-10			-6					-1
Prothrombin	H	V	F	L	A	P	Q	Q	A	R	S	L	L	Q - - - R V R R
Factor VII	R	V	F	V	T	Q	E	E	A	H	G	V	L	H - - - R R R R
Factor IX	T	V	F	L	D	H	E	N	A	N	K	I	L	N - - - R P K R
Factor X	S	L	F	I	R	R	E	Q	A	N	N	I	L	A - - - R V T R
Protein C	S	V	F	S	S	S	E	R	A	H	Q	V	L	R - - - I R K R
Protein S	A	N	F	L	S	K	Q	Q	A	S	Q	V	L	V - - - R K R R
Protein Z	S	L	F	L	P	A	S	K	A	N	D	V	L	V - - - R W K R
PRGP1*	R	V	F	L	T	G	E	K	A	N	S	I	L	K - - - R Y P R
PRGP2*	E	V	F	L	G	P	P	E	A	Q	S	F	L	S S H T R I P R
Matrix Gla Protein*	N	P	F	I	N	R	R	N	A	N	T	F	I	S - - - P Q Q R
Osteocalcin	A	A	F	V	S	K	Q	E	G	S	E	V	V	K - - - R P R R

Figure 8: The propeptide sequences of all the known vitamin K-dependent proteins (with the exception of Gas-6) in the one-letter code. The highly conserved amino acid residues are boxed in grey.

*Aligned in such a way to create maximal homology

IX, are synthesized in the form of precursor molecules. The precursor molecules contain a hydrophobic signal peptide and a propeptide (figure 8) ¹⁰⁶. Considerable sequence homology between the various vitamin K-dependent proteins was found in the amino terminal Gla domain, two epidermal growth factor (EGF) domains (protein C, factors VII, IX and X) ¹²⁵, a connecting region, containing an activation peptide, and a trypsin-like serine protease domain (except protein S) ¹⁰⁷. However, the mature form of osteocalcin has no homology with Gla-proteins involved in blood coagulation. The protein sequence of mature MGP revealed that it contains an internal peptide sequence sharing homology with the propeptide sequence found in the Gla-proteins ¹²⁶. Based on the results of the cDNA analysis of the Gla-proteins it was proposed that the propeptide designates specific precursor proteins for γ -carboxylation ^{110,111,120,121,124,127}. Moreover it was demonstrated by Price et al. that all Gla-proteins contain a highly conserved Glu-Xaa-Xaa-Xaa-Glu-Xaa-Cys (EXXXEXC) sequence structure ¹⁰⁹. These authors suggested that this sequence could function as an indicator for carboxylase to distinguish between the substrate and the carboxylated product ¹⁰⁹.

Increasing knowledge about structural requirements for carboxylatable substrates initiated the development of high affinity substrates for *in vitro* carboxylation. Cheung et al. were the first to synthesize the propeptide of human factor X and

determine its characteristics in combination with several short Glu-containing peptides (bEELOMe, FLEEL, bLEEL, ALEPPA) to carboxylase¹²⁸. Addition of 10 μM free factor X propeptide to rat liver carboxylating reaction mixture increased $^{14}\text{CO}_2$ incorporation in these peptides 12-fold and lowered the apparent K_m 8-fold. Free propeptide did not affect the K_m for vitamin KH_2 , O_2 or CO_2 ¹²⁸. These results confirmed the hypothesis that the propeptide is required for high affinity binding to carboxylase. Ulrich et al. were the first to report the synthesis and subsequent carboxylation of a high affinity *in vitro* substrate¹²⁹. They reported the synthesis of a peptide consisting of residues -18 to +10 of pro-prothrombin (ProPT-28). The K_m of this peptide was 4 μM . Subsequently, other groups reported the synthesis of recombinant propeptide containing substrates^{93,130-132}. These substrates also had high affinity for carboxylase with K_m values in the micromolar range. Mutations induced at various sites in the propeptide resulted in a decreased affinity of the propeptide for carboxylase^{93,132,133}. Recent results have shown that a covalently attached propeptide directs carboxylation of adjacent Glu-rich regions, even if these sequences are not structurally related to any Gla-domain^{74,130,134}. Since oxidation of KH_2 precedes the conversion of Glu to Gla on the substrate and this can not occur without the formation of KO, this suggests that epoxidation of vitamin K by carboxylase is stimulated by the propeptide³⁴. The propeptide can be defined by a Z-F-Z-X-X-X-X-A-X-X-Z motif from residues -17 to -6, in which Z is an aliphatic hydrophobic residue (Ile, Val, Leu), F stands for phenylalanine, A is alanine and X represents any amino acid. Although phenylalanine is preferred at position -16 of carboxylase substrates, other residues such as alanine, lysine, leucine and valine are also well carboxylated (e.g. Gas6 or ProPT-28 F/A). The propeptide region of the precursor proteins presumably dock with carboxylase, resulting in a conformational change of the enzyme and thus bringing the active site in close proximity to the Glu residues of the precursor proteins. The enzyme responsible for the removal of the propeptide from the Gla proteins is not known, although a role for Furin in this process has been suggested¹³⁵. There is, however, a large family of related enzymes, called proconvertases, involved in the cleavage of propeptides containing arginine or lysine residues at position -2 and arginine residues at position -1¹³⁶. Furie et al. hypothesized

very recently that any protein will undergo γ -carboxylation if : (I) the protein includes a propeptide which interacts with carboxylase; (II) the protein is transported through the RER during protein biosynthesis; (III) the cell has the carboxylase enzyme associated with the RER; (IV) there are Glu residues present within 40 residues of the propeptide; and (V) intracellular vitamin K is present ³⁴.

Fully carboxylated Gla proteins bind phospholipid membranes in the presence of Ca^{2+} . After the binding and subsequent carboxylation of the precursor Gla- proteins like proprothrombin the propeptide is still attached and the precursor protein is transported to the trans Golgi network. During this transport the proprothrombin does not bind to the endoplasmic reticular membranes although the Ca^{2+} concentration in the endoplasmic reticulum is much higher than in the cytoplasm. Presumably this is caused by the propeptide which is, in this phase of the processing, still attached and thus prevents proper folding of the Gla domain, and thereby inhibits the exposure of the phospholipid binding site ³⁴.

However, it became clear that descarboxy-osteocalcin, lacking a covalently attached propeptide, is an *in vitro* substrate for carboxylase capable of binding the enzyme with high affinity. From the results presented in this thesis it appears that descarboxy-osteocalcin binds carboxylase with high affinity at a site distinct from the propeptide binding site.

Osteocalcin; structure and function

Osteocalcin is exclusively synthesized by osteoblasts and odontoblasts ^{46,63,137,138}, and its synthesis is stimulated by 1,25-dihydroxy- D_3 ($1,25(\text{OH})_2\text{D}_3$) and vitamin A (as retinoic acid). It is a relatively small (5700 Da), Ca^{2+} -binding protein which consists of 49 amino acid residues. The molecule contains two helices, of which the N-terminal "Gla-helix" consists of residues 16-25, whereas a more C-terminal "Asp-Glu-helix" is composed of residues 30-41. Both helices are stabilized by a disulfide bond between residues Cys-23 and Cys-29. The hydrophilic "COOH-terminal beta sheet" extends from residues 42 to 48, and lies in proximity to the NH_2 -terminus thus covering the hydrophobic

backsides of the helices ¹³⁷. The helical conformation of the COOH terminal beta sheet is induced by Ca^{2+} -binding of its three Glu residues, for instance during the absorption of osteocalcin to hydroxyapatite (figure 9) ¹³⁹. The Glu residues are located at position 17, 21 and 24, but in man they are only partly carboxylated.

The extent of incomplete γ -carboxylation in osteocalcin depends strongly on the position of the glutamic acid residue, with 67 ± 14 % carboxylation at residue 17, 88 ± 9 % carboxylation at residue 21, and 93 ± 4 % carboxylation at residue 24 ¹⁴⁰. This incomplete carboxylation probably results in low affinity for hydroxyapatite which may explain why in human bone the concentration of osteocalcin is only 10 - 20 % of that in other species ¹⁴¹. Differences observed in the percentage of osteocalcin

Figure 10: Schematic structure representation of the native chicken osteocalcin molecule. X_{17} , X_{21} and X_{24} are the potentially carboxylated Glu residues. Important side chains are labelled in the one-letter code with a subscript indicating their sequence position. M_1 , M_2 and M_3 represent the probable metal binding sites. The aromatic hydrocarbons represent possible coordinating oxygen-containing side-chains. The dashed line indicates the disulfide bridge between residues C_{23} - C_{29} . Reproduced from Hauschka and Carr, 1982 ¹³⁹.

γ -carboxylation in bone samples may be caused by individual differences in the efficiency of osteocalcin γ -carboxylation during synthesis ¹⁴⁰. Osteocalcin was initially thought to function in bone mineralization, but experiments performed by Hauschka and Reid in chickens fed on a vitamin K-deficient diet in combination with vitamin K-antagonists showed that 50 % of the bone osteocalcin was lost, but no bone defects were visible ¹⁴². A more prolonged state of vitamin K-deficiency was induced in rats treated according to the warfarin + vitamin K regimen ¹⁴³. This resulted in a decrease of bone osteocalcin to not more than 2% of the normal concentration. After 8 months of treatment the animals displayed increased bone mass in combination with abnormal fusion of the growth-plate and absence of longitudinal bone growth. However, no distinction was made between osteocalcin, MGP or protein S in this experiment, thus the results may also be caused by a decrease in concentration of the latter two proteins. Similar results as found by Hauschka and Reid were obtained by Ducy et al. ⁶³, who used osteocalcin deficient mice and found that these animals were normal, viable and fertile. Furthermore these mice had no bone defects at birth and the ultrastructure of the extracellular matrix was normal. However, at 6 months of age increased bone density of the long bones was observed together with increased cortical thickness. Biomechanical measurements of the mutant bones revealed increased bone strength indicating increased bone formation although the number of osteoblasts was comparable to that in control mice. Therefore, Ducy et al. concluded that osteocalcin functions as a negative regulator of bone formation, by limiting bone matrix formation without affecting mineralization or resorption.

Molecular biology and recombinant DNA techniques in vitamin K research

To study the interactions that take place between carboxylase and its substrates, a large number of substrates have been developed during the past two decades. The first peptides synthesized were short tri- and pentapeptides. They were chosen because (I) they were structural analogs of the natural substrate, (II) they contained Glu residues which might act as CO₂ receptors and (III) their synthesis was relatively simple and

cheap. The main limiting step involving peptide synthesis was the length of the peptide to be synthesized. Longer peptides result in higher costs and more difficulties during synthesis, protecting, coupling, subsequent cleavage and final deprotection. The short pentapeptides were unsatisfactory when high affinity interactions were to be studied. Therefore the need for longer synthetic peptides rose, especially after the discovery of the propeptide. In 1988 Ulrich et al. were the first to report the synthesis of a high affinity substrate for carboxylase, ProPT-28¹²⁹. The synthesis of ProPT-28 was a major leap forward in the research on substrate properties of Gla- proteins. However, ProPT-28 contained only part of the Gla-domain of prothrombin, and thus could not be used for carboxylation studies on the entire Gla-domain and the effect that this domain might have on the interaction with carboxylase. Here the limitations of peptide synthesis became obvious and a different method of protein synthesis was needed.

In 1990 Wu et al. described the production and subsequent purification of ProIX-59 from *E. coli*⁹³. The reason for this approach is that *E. coli* does not possess any posttranslational processing machinery and therefore is unable to carboxylate protein sequences of overexpressed vitamin K-dependent precursor proteins. Therefore the cDNA of the desired precursor protein is ligated into an expression plasmid (figure 10), or vector, after which the construct is introduced (transformed) into competent *E. coli*. The bacterial membranes of these *E. coli* are treated with CaCl_2 so that uptake of the modified plasmids is facilitated. An expression vector is a plasmid which not only contains the gene of interest but also contains the necessary regulatory sequences by which expression of the gene can be controlled¹⁴⁴. Expression vectors furthermore contain an antibiotic resistance gene, which makes the bacteria integrating the vector able to grow on nutrients containing a specific antibiotic agent. This is an easy method to discriminate between *E. coli* that do or do not contain a vector. It is known that *E. coli* can produce large quantities of a desired recombinant protein after the Lac Z promotor, which is present on the plasmid, is induced with isopropyl β -D-thiogalactopyranoside (IPTG). Depending on the culture conditions, the *E. coli* strain used and the vector type, up to 12 mg of protein can be purified per liter of culture⁹³. An approach to produce recombinant descarboxy-osteocalcin using bacterial

expression as reviewed in this paragraph is discussed in more detail in chapter 2, paragraph 2.

Figure 11 Construction of an expression plasmid containing the complete propeptide and γ-carboxyglutamic acid (Gla) domain sequences of human factor IX. A 1.1-kilobase *Bgl*II-*Bam*HI fragment that contains the promoter sequence and most of the coding region of gene 10 from pAR3070 was ligated into the *Bam*HI site of pMc 254. Phagemid pMc10, which has the promoter of gene 10 oriented opposite to CAT and BLA gene, was chosen to express FIXGla (ProIX-59). A 272-bp *Bam*HI-EcoRI fragment that contains the propeptide and γ-carboxyglutamic acid domain sequences of human factor IX from a modified factor IX cDNA clone, pMa254IX, was first ligated to a linker containing an *Eco*RI site at its 5' end, 2 termination codons, and a *Bam*HI at its 3' terminus. The resulting 292-bp *Bam*HI fragment was then ligated to the *Bam*HI site of pMc10. Phagemid pMc292, which contains the promoter and most of the sequences coding for gene 10, FIXGla sequence, termination codons and f1 ori, was further modified for expressing FIXGla fusion protein by using site-specific *in vitro* mutagenesis. BLA, β-lactamase; CAT, chloramphenicol acetyltransferase; AP, ampicillin resistance; CM, chloramphenicol resistance; CIP, calf intestinal alkaline fosfatase. Reproduced from Wu et al., 1990⁹³.

Introduction to this thesis

Since native osteocalcin purified from bone and subsequently heat decarboxylated is the only known Gla-protein thus far capable of binding vitamin K-dependent γ -glutamyl carboxylase with high affinity, without the need for a propeptide, we focussed our attention on this unique protein.

The main goal of this thesis was to study enzyme substrate interactions between decarboxy-osteocalcin (d-OC) or propeptide-containing d-OC (Pro-d-OC) and several other recombinant or organically synthesized substrates and carboxylase. The initial decision was to generate d-OC, Pro-d-OC and several mutant forms of both d-OC and Pro-d-OC via recombinant DNA technology. This material could then also be used for other fields of research (e.g. as a protein standard for in vitro diagnostic testkits or as antigen for the generation of antibodies). The production of recombinant osteocalcin in bacteria could provide an unlimited source of the desired protein for the different applications. Several strategies have been developed to produce recombinant osteocalcin both in prokaryotic and eukaryotic cells (chapter 2).

Since the expression of recombinant osteocalcin in *E. coli* resulted in low yields (chapter 2), which were unsuitable for purification- and carboxylation purposes, and because synthesis of polypeptides up to 50 residues became available, we chose to generate both d-OC and carboxylated osteocalcin (OC) via chemical synthesis (chapter 4). Besides both full length d-OC and OC, several d-OC and OC derived short peptides were chemically synthesized (chapters 3, 4, 5 and 6).

The role of the propeptide and γ -carboxyglutamic acid domain of factor IX and the substrate properties of decarboxy-osteocalcin are discussed in chapters 3 and 4. In chapter 3, affinity purified bovine carboxylase is used to study enzyme substrate interactions. However, this carboxylase preparation contains at least 2 μ M of propeptide, due to the purification protocol. In the consecutive chapters recombinant human purified carboxylase expressed in insect cells was available. This carboxylase preparation is free of propeptide and thus became the enzyme of choice for our research regarding the substrate properties of osteocalcin. Because of the poor yields of bacterially synthesized osteocalcin, both organically synthesized full length

osteocalcin and descarboxy-osteocalcin have been used in the experiments described in chapters 3, 4, 5 and 6 as well as a number of osteocalcin derived peptides.

Since the osteocalcin propeptide is the only known human propeptide which possesses a glycine at position -10 instead of the highly conserved alanine, and because osteocalcin is very sensitive to anticoagulant therapy *in vivo*, we investigated the affinity of the osteocalcin propeptide and d-OC for carboxylase (chapter 4).

The results found in chapter 4 indicated a possible d-OC binding site on carboxylase, and therefore we studied this hypothesis using unique and highly specific active site inhibitors for carboxylase. In this chapter we demonstrate that our hypothesis concerning osteocalcin binding appears valid (chapter 5). If the binding of d-OC to carboxylase is a specific interaction between the two molecules, the question remains what the requirements for d-OC are to bind the enzyme. To investigate what specific N-terminal amino acid residues are necessary for the interaction we have studied the kinetic characteristics of several d-OC peptides with a decreasing length of the N-terminus, and a recombinant d-OC molecule lacking the last 10 C-terminal residues (chapter 6).

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Chapter 2

THE REQUIREMENT OF RECOMBINANT OSTEOCALCIN: POSSIBILITIES, LIMITATIONS AND *IN VITRO* CARBOXYLATION

Based on: Roger J.T.J. Houben, Berry A.M. Soute, Marjo H.J. Knapen & Cees Vermeer (1997);
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Roger J.T.J. Houben, Berry A.M. Soute & Cees Vermeer (1997);
Assay of vitamin K-dependent carboxylase activity in hepatic and extrahepatic tissues, *Methods Enzymol*, **282**: 358 - 368

Introduction to chapter 2

This more technical chapter describes three main topics all associated with osteocalcin. The requirement for osteocalcin as a standard for in vitro diagnostic tools and the strategy to be used to ensure optimal yields of osteocalcin is discussed in the first paragraph as a spin-off of this thesis.

The second paragraph focusses on the practical implementation of recombinant DNA technology as a tool to produce substantial amounts of osteocalcin. It also discusses the difficulties that were encountered with this technology. Moreover, a balanced discussion is made between the use of recombinant osteocalcin and synthetic osteocalcin for the continuation of this thesis. In the third paragraph the basic characteristics of bovine carboxylase from both hepatic and various extrahepatic tissues are described. The carboxylase preparations from washed microsomes and applications to investigate enzyme substrate interactions is explained. Although in this paragraph several substrates for the carboxylases are discussed, recombinant decarboxy-osteocalcin was not included because it was not available at the time of the study. Therefore, thermally decarboxylated native bovine osteocalcin was used in addition to a variety of both synthetic and recombinant substrates. Although the utilization of washed bovine microsomes as a source of carboxylase is discussed in this paragraph it will not be used in the consecutive chapters, since purified carboxylase became available.

Nevertheless, the methods described in paragraph 2.3 of this chapter are still valid and particularly useful to study the vitamin K reductases since these enzymes are not present in the purified carboxylase preparations.

Paragraph 2.1

THE REQUIREMENT FOR OSTEOCALCIN PROTEIN STANDARDS

Introduction

Osteocalcin (also known as Bone Gla Protein, BGP) is a relatively small protein containing 49 amino acid residues ^{1,2}. During its biosynthesis osteocalcin undergoes three posttranslational carboxylation reactions by which glutamate residues are converted into γ -carboxyglutamate (Gla). The conversion of a glutamate residue into Gla is accomplished by the endoplasmic vitamin K-dependent γ -glutamyl carboxylase, which recognizes its potential substrates by an 18-residues pro-sequence immediately preceding the amino terminus of the mature sequence ^{3,4}. Since osteocalcin is exclusively produced by osteoblasts (and odontoblasts), its serum concentration is a good marker for bone formation ⁵⁻⁷. A number of commercial kits are presently available for serum osteocalcin detection, but a drawback is that for a number of reasons well-defined reference material is lacking. This may be one of the reasons why the value obtained for the same serum sample may vary more than 10-fold, depending on the particular kit used ⁸⁻¹⁰. In this paragraph we describe a number of strategies which may be followed for preparing homogenous human osteocalcin, and we will comment on their potential advantages and disadvantages.

Vitamin K-status in elderly women

As early as in 1984 it was reported that patients with osteoporotic hip fractures had very low circulating vitamin K levels ¹¹. This finding was confirmed in a number of subsequent studies ¹²⁻¹⁴. From epidemiological studies it became clear that the nutritional intake of vitamin K decreased substantially at increasing age ¹⁵. Finally, using a test based on the differential affinity of carboxylated and undercarboxylated osteocalcin for hydroxyapatite, Knapen et al. demonstrated that vitamin K-deficiency in bone tissue is common among elderly women resulting in increased levels of circulating undercarboxylated osteocalcin ^{16,17}. In this paper we will refer to undercarboxylated osteocalcin as ucOC (= osteocalcin not bound to hydroxyapatite in the test mentioned above). Szulc et al. showed that serum ucOC is inversely correlated with bone mass ¹⁸ and with fracture risk ¹⁹.

Figure 1: Actual bone mineral density (BMD) as a function of the predicted BMD. Participants in this study were 33 apparently healthy women, 1-10 years postmenopausal (as decided from the date of their last menstruation). Bone densitometry of the right femur neck was performed with a lunar DPX-L densitometer, osteocalcin fractions were assessed as described in ¹⁷. Multiple linear regression analysis was used to determine associations between biochemical markers and BMD. Stepwise variable entry showed age and ucOC the only variables to contribute significantly to the bone mineral density in this age group.

We have confirmed these data in a group of 33 women who were 1-10 years postmenopausal. Multiple linear regression analysis showed age and ucOC to be two independent variables both of which were inversely correlated with bone mineral density. Based on the derived equation $BMD_{pred} = 2.60 - 0.023 \times age - 0.14 \times ucOC$ we have calculated the predicted BMD of the right femur neck, which was plotted against the observed BMD (figure 1). The fact that in this study a correlation coefficient of 0.82 was obtained, demonstrates the importance to measure independently both normal osteocalcin and ucOC. Two questions remain to be addressed: a) how do we explain that osteocalcin is the only known Gla-protein which, under normal physiological conditions, is partly synthesized in its descarboxy form, and b) how to ascertain a sufficient supply of homogenous reference material for both normal (3-Gla) and descarboxy-osteocalcin.

Role of pro-sequence in substrate recognition by carboxylase

In all Gla-proteins the presence of the pro-sequence is essential both for *in vivo* and *in vitro* carboxylation of protein substrates, and the role of individual amino acids in substrate recognition has been investigated in detail. As seen in figure 2, two amino acid residues are highly conserved in all Gla-proteins: Phenylalanine (Phe)⁻¹⁶ and Alanine (Ala)⁻¹⁰, and their presence at these positions has been proven critical for carboxylation. Osteocalcin is the only known Gla-protein in which no Ala is found at position -10. The effect of a mutation at this site was first shown in the pro-sequence of a patient who had a spontaneous mutation Ala⁻¹⁰ → Threonine (Thr)⁻¹⁰ in his coagulation factor IX precursor, which caused an extreme sensitivity to low doses of warfarin, leading to severe haemophilia B ²⁰. The mechanism underlying this phenomenon turned out to be the 30-fold reduced affinity of the precursor protein for γ -glutamylcarboxylase, and a similar decrease in affinity was observed for the exchange of Ala⁻¹⁰ by Glycine (Gly)⁻¹⁰. Since this is the regular sequence in human osteocalcin, it must be expected that the affinity of osteocalcin precursor proteins for carboxylase is well below that of other Gla-proteins. On the basis of these results it was suggested by Chu et al. that this reduced affinity may result in premature dissociation of the enzyme-substrate complex, and thus in incomplete carboxylation. Obviously, the result of a poor enzyme-substrate affinity will show most clearly during episodes of vitamin K-deficiency and the use of vitamin K-antagonists, i.e. when competition for the substrate binding site on carboxylase takes place. Indeed it was demonstrated that during very mild oral anticoagulant treatment the inhibitory effect on osteocalcin synthesis is almost maximal, whereas at the same time the carboxylation of prothrombin is hardly affected (M.H.J. Knapen et al, unpublished data).

The Requirement of Recombinant Osteocalcin

			-16						-10							-1	+1				
Prothrombin	H	V	F	L	A	P	Q	Q	A	R	S	L	L	Q	-	R	V	R	R	-	A
Clotting factor VII	R	V	F	V	T	Q	E	E	A	H	G	V	L	H	-	R	R	R	R	-	A
Clotting factor IX	T	V	F	L	D	H	E	N	A	N	K	I	L	N	-	R	P	K	R	-	Y
Clotting factor X	S	L	F	I	R	R	E	Q	A	N	N	I	L	A	-	R	V	T	R	-	A
Protein C	S	V	F	S	S	S	E	R	A	H	Q	V	L	R	-	I	R	K	R	-	A
Matrix Gla Protein	N	P	F	I	N	R	R	N	A	N	T	F	I	S	P	Q	Q	R	W	R	A
Protein S	A	N	F	L	S	K	Q	Q	A	S	Q	V	L	V	-	R	K	R	R	-	A
Gas 6	A	A	L	L	P	A	R	E	A	T	Q	F	L	R	P	R	Q	R	R	-	A
Osteocalcin	A	A	F	V	S	K	Q	E	G	S	E	V	V	K	-	R	P	R	R	-	Y

Figure 2: Amino acid sequences in propeptides of human Gla-proteins. The various amino acid residues are denominated according to the one-letter code. Matrix Gla protein has an internal pro-sequence, which is aligned in such a way to obtain maximal homology.

Three strategies for preparing human osteocalcin standards

For the calibration of osteocalcin test kits it will be essential that carboxylated osteocalcin (3 Gla residues per molecule) and descarboxy-osteocalcin (0 Gla residues per molecule) are available in sufficient amounts. In principle the preparation of these compounds may be performed in three different ways:

A. Isolation from human bone

Apart from disadvantages such as the difficult preparation procedure, availability of fresh human bone, and risk of spreading infectious diseases, a major drawback to this route is that human bone was shown to contain a mixture of osteocalcins of varying degrees of carboxylation, and that it will be hard to purify these fractions to homogeneity. Notably the Gla residue at position 17 is substantially undercarboxylated²¹, and it has been shown that this residue is critical for the calcium-dependent conformation of osteocalcin²².

B. Chemical synthesis

With the present technical facilities this route is feasible, and - though expensive today - it may turn out to be the cheapest way of producing sufficient amounts of standard in the near future. It should be kept in mind, however, that *in vivo* osteocalcin is synthesized in the form of a pre-pro-protein precursor the conformation of which may

be different from that of a chemically synthesized polypeptide, even after cleavage of the leader sequence.

C. Recombinant osteocalcin

For reasons indicated above it will be crucial to express recombinant human osteocalcin in a eukaryotic carboxylating cell line. Cell lines with a high potential of carboxylating recombinant proteins (e.g. coagulation factors) are presently available^{23,24}. By culturing the host cells in the presence of either vitamin K or warfarin, mature osteocalcin in its carboxylated or non-carboxylated form would be obtained respectively. To ensure maximal carboxylation of the recombinant osteocalcin, it seems indicated to prepare by site-directed mutagenesis the modified precursor form Gly⁻¹⁰ → Ala. Since osteocalcin is neither glycosylated nor phosphorylated *in vivo*, it is possible to produce descarboxy-osteocalcin using bacterial expression in *Escherichia coli*. This technique has the advantage that it may yield larger quantities of recombinant protein than eukaryotic cell cultures do, because prokaryotes (bacteria) can be grown faster at much higher densities. Another advantage of prokaryotic expression is that the produced osteocalcin is completely non-carboxylated whereas it remains uncertain whether this is the case in eukaryotic cells grown in the presence of warfarin.

From this evaluation it seems that the preparation of carboxylated and non-carboxylated recombinant osteocalcin is an important step in the standardization of the osteocalcin determination. Whether the recombinant product will be structurally similar to native osteocalcin remains to be seen, but the recent expression of fully carboxylated and functionally active recombinant coagulation factors has shown that the proper eukaryotic systems are available now to ensure the large scale production of recombinant Gla-proteins.

As mentioned above, expression of osteocalcin in *E.coli* is the technique of choice to obtain sufficient amounts of descarboxy-osteocalcin since bacteria lack posttranslational processing. In the next paragraph the construction and production of recombinant descarboxy-osteocalcin and descarboxy-pro-osteocalcin and its limitations

are discussed.

Paragraph 2.2

CLONING AND EXPRESSION OF OSTEOCALCIN USING *ESCHERICHIA COLI*
AS A HOST

Introduction

The main goal of our efforts was to prepare sufficient amounts of non-carboxylated pro-osteocalcin and osteocalcin, to identify sequence compositions present within osteocalcin necessary for the recognition and subsequent carboxylation by the vitamin K-dependent γ -glutamyl carboxylase. We therefore required sufficient osteocalcin of human origin in the non-carboxylated form.

Because prokaryotes do not possess vitamin K-dependent carboxylase activity, expression of the osteocalcin cDNA will result in descarboxy-(propeptide containing) protein. Furthermore it is possible to introduce mutations in the osteocalcin sequence through molecular biology and thus study the effect of these mutations on the substrate properties of osteocalcin.

The expression of osteocalcin and the additional problems and limitations that were encountered during this process are described in this paragraph.

Synthesis of human (pro)-osteocalcin cDNA

Human osteoblasts were obtained after bone biopsy and cultured in the presence of $1,25(\text{OH})_2$ -vitamin D_3 until the cells had reached confluency. Cells were grown in RPMI 1640 medium (Flow ICN) supplemented with 2 mM L-glutamate, 10 % newborn calf serum (Gibco BRL) and gentamycin (Boehringer) as an antibiotic. Cultures were maintained in a humidified incubator at 37 °C and 5% CO_2 . At confluency (approximately 3×10^7 cells per flask), cells were washed with PBS and harvested.

Cell suspensions (10^8 cells) were centrifuged at 1200 rpm for 5 minutes and the cell pellets were stored at -80 °C until use. Subsequently cells were thawed and treated with RNAzol B (Campo scientific) according to the manufacturer's protocol²⁵. Total RNA was stored at -20 °C in 75 % ethanol (v/v) until further use. For the reverse transcriptase (RT) reactions 24 μl total RNA, 10 μl 10x RT buffer (Gibco BRL), and 5 μl oligo(dT) primer (Gibco BRL) were mixed and incubated at 70 °C for 5 minutes. After rapid cooling on ice, 2 μl 25 mM dNTPs (Pharmacia), 5 μl 0.1 mM DTT (Gibco BRL), 1 μl Rnasin, and 3 μl 200 U/ μl M-MLV reverse transcriptase (Gibco BRL) were added in a

total volume of 50 μ l. The RT reactions were incubated at 37 °C for 1.5 hour and subsequently stored at -20 °C until further use (figure 1). The resulting cDNA's of human osteocalcin and propeptide-containing osteocalcin are indicated as OC and Pro-OC cDNA, respectively.

Figure 1: Principle of reverse transcriptase (RT) reaction on mRNA to create cDNA. Oligo dT stands for the primer which binds the poly (A) tract at the 3' terminus of mRNA's and primes the synthesis of the first strand of cDNA; dNTP's stands for deoxyribonucleoside triphosphates mix consisting of deoxyadenosine (A), deoxycytidine (C), deoxyguanosine (G) and deoxythymidine (T).
Picture by Henri H.M. Spronk.

Construction of expression vectors pQE30-OC and pQE30 pro-OC

For cloning and expression of OC and pro-OC cDNA the commercially available QIAexpress system was used (Qiagen Inc.). To facilitate protein purification by metal chelate affinity chromatography the recombinant protein was engineered to contain a six residues long histidine tail at the N-terminus. For in frame fusion with the start codon

of the 6-His region the expression vector pQE30 was linearized with SphI and HindIII. Subsequently, the linearized vector was purified by agarose gel electrophoresis according to standard procedures²⁶.

For cloning of the human OC and pro-OC cDNA (figure 2), 5'-SphI and 3'-HindIII digestion sites were introduced by PCR. Primers proOC-SphI and proOC-HindIII were used for amplification of pro-OC cDNA and primers OC-SphI- and OC-HindIII for OC cDNA amplification. Each PCR reaction consisted of 5 µl cDNA solution, 1 mM of each dNTP, 1 µg of each primer, 4 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 8.3 (RT), 0.01% gelatin, and 2.5 U Taq polymerase (Pharmacia) in a total volume of 50 µl. Each PCR cycle consisted of 1 minute at 95 °C, 2 minutes at 60 °C and 2 minutes at 72 °C. After 33 cycles the PCR was terminated by 10 minutes incubation at 72 °C. The amplified cDNA's of OC and pro-OC were both digested with SphI and HindIII and the resulting fragments were then separated by agarose gel electrophoresis and isolated by electrolution²⁶. The isolated fragments and linearized vector were then ligated in reactions containing 1 µl digested fragment, 1 µl digested pQE30 vector, 1x T4 DNA ligase buffer, 1 mM ATP, and 1 Weiss unit T4 DNA ligase (Pharmacia). Ligations were performed overnight at room temperature in a total volume of 10 µl. Recombinant plasmids, pQE30-OC and pQE30-pro-OC, were transformed into *E. coli* strain M15 (Nal^r Str^r rif^r, lac⁻ ara⁻ gal⁻ mtl⁻ F⁻ recA⁺ uvr⁺) containing the pREP4 vector (Qiagen Inc.). Stable transformants were selected for ampicillin and kanamycin resistance. Final examination for OC or pro-OC cDNA inserts of the ampicillin and kanamycin resistant *E. coli* was performed through digestion of plasmid constructs with SphI and HindIII.

Figure 2: Schematic representation of the cloning of OC / pro-OC into expression vector pQE 30. AmpR stands for the ampicillin resistance gene; 6His stands for the histidine purification tag. Picture by Henri H.M. Spronk.

Preparation of competent cells

The desired bacterial strain (M15 [pREP4], Qiagen Inc.) was spread on LB plates containing kanamycin and incubated overnight at 37 °C. A single colony was taken and used to inoculate 50 ml LB medium. The culture was shaken at 37 °C and 250 rpm until an OD_{550 nm} of 0.4 - 0.5 was reached. After cooling the culture down on ice for 10 minutes, cells were collected by centrifugation at 2500 x g for 12.5 minutes at 4 °C. The bacterial pellet was resuspended in 16 ml transformation buffer (10 mM K-MES pH 6.3, 50 mM MnCl₂, and 50 mM CaCl₂). After incubation on ice for 10 minutes, cells were collected by centrifugation at 2500 x g for 12.5 minutes. The cell pellet was resuspended in 4 ml transformation buffer containing 10 % (v/v) glycerol. Aliquots of 500 µl were frozen in liquid nitrogen and stored at -80 °C until further use.

Construction of expression vectors pQE40-OC and pQE40-pro-OC

For the production of OC and pro-OC fusion proteins in *E. coli*, the pQE40 vector (Qiagen Inc.) was chosen. It contains an expression cassette consisting of the phage T5 promotor fused to the mouse DHFRS protein. To facilitate protein purification by metal chelate affinity chromatography using Ni²⁺-NTA resin the recombinant protein was engineered to contain a six residues long histidine tail at the N-terminus. Furthermore, a sequence encoding a methionine residue was introduced N-terminal of both the OC and pro-OC sequences between the DHFR sequence and the first amino acid residue of the osteocalcin cDNA's (figure 3). The methionine was introduced to enable cyanogen bromide (CNBr) mediated cleavage of the fusion proteins after purification using metal chelate affinity chromatography, and thus facilitating removal of the fusion protein. For in frame fusion with the start codon of the 6-His region the expression vector pQE40 was linearized with SphI and HindIII. Subsequently, the same procedure was followed as for the pQE30-OC- and pQE30-pro-OC constructs, with this respect that the pQE30-OC- and pQE30 pro-OC constructs served as the source of OC and pro-OC cDNA.

Figure 3: Schematic representation of the pQE 40 vector including the (pro) OC insert

Construction of expression vectors pMALc-OC and pMALc-pro-OC

A 5'-end EcoRI and 3'-end HindIII restriction site was added to both OC and pro-OC cDNA's using PCR. This enabled introduction of the cDNA's downstream from the malE gene of *E. coli* present in the pMALc plasmid (New England Biolabs) (figure 4). The malE gene encodes a maltose-binding protein (MBP), resulting in expression of an MBP fusion protein. Moreover, a sequence encoding a methionine residue was introduced in the same way and for the same purpose as mentioned above. Production and subsequent purification of the fusion protein is obtained after induction of a strong "tac" promoter using IPTG resulting in expression of the cloned cDNA's and a one-step purification of the desired proteins using MBP's affinity for maltose.

Figure 4: Schematic representation of the pMALc vector including the (pro) OC cDNA.

DNA sequencing reactions

DNA sequence analyses were performed according to the cycle sequencing procedure developed by Perkin Elmer^{27,28}. In brief, approximately 0.2 µg of CsCl₂ purified DNA was added to a reaction mixture which included 8 µl Terminator Ready Reaction Mix (Perkin Elmer) and 5 pmol primer. The volume was adjusted to a total of 20 µl using distilled water. Each cycle consisted of an incubation for 30 seconds at 95 °C, 15 seconds at 60 °C and 4 minutes at 72 °C. After 25 cycles the cycle sequencing procedure was finished by cooling the reaction down to 4 °C. Subsequently, the extension products were precipitated using ethanol precipitation and purified by spin-column purification, using Sephadryl S200 columns (Pharmacia Biotech). Samples were dried under vacuum and resuspended in 25 µl of Template Suppression Reagent (Perkin Elmer). Prior to loading, samples were heated at 95 °C for 2 minutes and subsequently put on ice. Sequence analysis of the samples on the ABI Prism 310 Genetic Analyzer (Perkin Elmer) was performed according to the manufacturers manual²⁷.

Rapid screening of small-scale expression cultures

For production of recombinant proteins OC and pro-OC, cells (10 ml) were grown in Luria Bertani-(LB) medium until a bacterial density at $A_{600\text{ nm}}$ of 0.7 was reached. The expression of the recombinant gene was induced by the addition of isopropyl-b-D-thiogalactopyranoside (IPTG) to a final concentration of 2 mM. Cultivation was continued for 2, 3 or 4 hours. Cells of 1 ml cultures were harvested by centrifugation for 1 minute in a microcentrifuge (13 000 rpm).

A. pQE constructs

For OC and pro-OC from either pQE-30 or pQE-40 lysis of the cells was performed using 200 μ l Buffer B (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl pH 8.0). Cellular debris was pelleted by centrifugation for 10 minutes at 10 000xg at 4 °C and the supernatant was transferred to a fresh tube. To the supernatant 50 μ l of 50% slurry of Ni-NTA resin was added and incubated for 30 minutes at RT. After this incubation the suspension was transferred to a spin-column and the resin was washed 3 times with 400 μ l Buffer C (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl pH 6.3). Finally the recombinant protein was eluted with 50 μ l Buffer C containing 100 mM EDTA.

B. pMALc constructs

For OC and pro-OC fusion proteins from pMALc cells were lysed by overnight storage at -20 °C. Subsequently, the lysed cells are sonicated for 2 minutes on ice. Lysed and sonicated cells are centrifuged at 9000xg at 4 °C for 20 minutes after which the supernatant (crude extract) is decanted and stored on ice. If the crude extract is too viscous it can be diluted by adding 5 ml Column Buffer (200 mM NaCl, 20 mM Tris-HCl, pH7.4 and 1mM EDTA). To the crude extract ~200 μ l of amylose resin in Column Buffer was added and the mixture was incubated on ice for 15 minutes. After incubation, the resin was spun down in a microcentrifuge for 1 minute and the supernatant was removed and discarded. The pellet was then washed with 1 ml Column Buffer and again centrifuged for 1 minute in a microcentrifuge. Subsequently the resin was resuspended in 50 μ l SDS-PAGE sample buffer (15 % (v/v) β -mercaptoethanol, 15 %

(w/v) SDS, 1.5 % (w/v) bromophenol blue, 50 % (v/v) glycerol). Appropriate aliquots of purified expression samples in SDS-PAGE sample buffer were boiled for 1 minute after which the samples were loaded onto a 10% SDS-PAA-gel and electrophoresis was carried out under denaturing conditions according to Laemmli ²⁹.

Figure 5: 10 % reducing SDS gel. In lane 1 DHFR-pro-OC, in lane 2 DHFR-OC. Molecular mass is shown on the far left.

Western blot analysis of expressed fusion proteins

For Western blot analysis of recombinant expression proteins osteocalcin (OC) and pro-osteocalcin (pro-OC), small scale expression cultures were purified as described previously. 20 μ l aliquots of either Ni^{2+} -NTA resin or amylose resin purified expression samples were boiled for 1 minute in 5 μ l of SDS-PAGE sample buffer and the samples were loaded onto a 10% SDS-PAGE gel. Electrophoresis was carried out under denaturing conditions according to Laemmli ²⁹. After electrophoresis samples were transferred to 0.2 μ m nitrocellulose (Schleicher & Schuell) by electroblotting as described by Towbin et al.³⁰. Blots were washed for 5 minutes in PBST (phosphate

buffered saline (PBS) supplemented with 0.1 % (v/v) Tween20) followed by blocking of the nonspecific binding sites by incubation for 1 hour in PBST-buffer supplemented with 3 % (w/v) bovine serum albumin (BSA). Incubation of the blots was performed with a 1:1000 dilution of the appropriate antiserum (anti 1-19 osteocalcin monoclonal antibody, Hoffmann-La Roche) for 1 hour. A secondary monoclonal peroxidase conjugated rabbit anti-mouse antibody (DAKO) was used in a 1:1000 dilution. The blot was incubated for 1 hour. After extensive washing of the blots with PBS, the osteocalcin positive bands on the blot were stained by a reaction with 0.05 % (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB) (FLUKA) and 0.025 % (v/v) H_2O_2 in PBS. The staining process was stopped by washing the blots in water (figure 6).

Figure 6: Western blot of DHFR-pro-OC (lane 1) and DHFR-OC (lane 2). Molecular mass is shown on the far right.

Results

Initially, OC and pro-OC cDNA's were cloned into the expression vector pQE30 to obtain optimal production of the recombinant proteins in *E. coli*. After stable transformation into *E. coli* strain M15 [pREP4], expression was induced with 2 mM IPTG. Two and four hours after induction, Ni-NTA-resin purified bacterial culture extracts were analysed using SDS-PAGE. No expression products of either OC or pro-

OC were detectable after staining the SDS-PAA gels with Coomassie Brilliant Blue (CBB) (data not shown). Subsequently, the expression of a DHFRS-6His construct from pQE16 (Qiagen) was performed as a control, together with the pQE30-OC and pQE30-pro-OC constructs. After two and four hours post-induction an expression product became visible in the control with an apparent molecular weight of 25 kD, indicating expression of DHFR³¹. In both pQE-30-OC and pQE30-pro-OC constructs no band of either OC or pro-OC was visible on SDS-PAA gels after CBB staining. Also analysis of the expressed proteins on Western blot did not reveal any expression of a product that was recognized by anti 1-19 osteocalcin antibodies.

The absence of expression products of both pQE30-OC and pQE30-pro-OC was probably caused by the low molecular weights of the two constructs, 5900 and 7800 Dalton respectively, which makes them susceptible to proteolysis in *E. coli*. Therefore, the cDNA constructs of both proteins were ligated into the pQE40 expression vector in such a way that both proteins became N-terminally fused to the C-terminal end of mouse DHFRS protein and thus become more stable and less susceptible to proteolysis during expression. Ni-NTA-resin purified extracts from two hours induced and non-induced bacterial cultures containing the expression vector pQE40-OC and pQE40-pro-OC were analysed on SDS-PAGE. The same results as for the two pQE30 constructs were obtained. The positive control showed an expression product, whereas the two pQE40 constructs remained negative. After DNA sequence analysis of pQE40-OC and pQE40-pro-OC, it became clear that both constructs were ligated in the correct reading frame.

Theoretically, expression of the constructs was expected. Low expression levels are sometimes the result of poor plasmid maintenance in the cells. Ampicillin is a labile antibiotic, and is rapidly consumed in growing cultures. Stability of the plasmid maintenance can be improved by doubling the ampicillin concentration to 200 µg/ml. Therefore, cells were grown in the presence of 200 µg/ml ampicillin until an OD_{600 nm} of 0.7 was reached and then induced with IPTG. Ni²⁺-NTA-resin purified extracts from two hours induced and non-induced cultures were analysed on SDS-PAGE. In both pQE40-OC and pQE40-pro-OC constructs expression products were obtained after 2 hours of

induction. In case of the 6His-DHFR-OC the apparant molecular weight was ~29 kDa whereas the 6His-DHFR-pro-OC construct had an apparant molecular weight of approximately 31 kDa.

On Western blot using a primary antibody against 1-19 osteocalcin, expression products could be detected. As a control 6His-DHFR from the empty pQE 40 vector was negative for anti 1-19 osteocalcin antibodies and positive when an anti 6His monoclonal antibody was used. 6His-DHFR could therefore be detected at ~25 kDa. Both the osteocalcin expression products were also immunoreactive to anti-6His monoclonal antibody, and could be detected at the same molecular weight as when the anti 1-19 osteocalcin monoclonal antibody was used.

The amount of each expression product was determined with a protein analysis according to Sedmak et al.³². From 200 μ l Ni^{2+} -NTA-resin purified expression culture, derived from 1 ml cell culture, 50 μ l was used for the measurement of total protein concentration. Subsequently the total protein concentration was corrected for the protein content of a induced negative control, which contained only the pREP4 vector. The amount of full length recombinant protein was estimated on the basis of the CBB-gel (Figure 8) and expressed as percentage of total protein on gel. This percentage was used to calculate the concentration full length recombinant protein. 3 Hours expression of the pQE40 constructs in *E. coli* strain M15[pREP4] resulted in approximately 2 μ g/ml of recombinant human osteocalcin. After 3 hours 1 ml of bacterial suspension was taken from each expression culture, centrifuged and the bacterial pellet was resuspended in 250 μ l Triton/1x PBS. After sonication, the osteocalcin concentration in 50 μ l of suspension was analyzed using the ELSA-OSTEO kit, according to the manufacturers protocol³³. In the expression culture of M15[pREP4] pQE40 (negative control for osteocalcin) no detectable amount of osteocalcin could be measured. In expression cultures from three different pQE40-OC and pQE40-pro-OC clones, an osteocalcin concentration of approximately 40 ng/ml was detected. These low concentrations were in contrast with the intensity of the DHFR-OC and DHFR-pro-OC bands visible on CBB stained SDS-PAA gels and after Western blotting using anti 1-19 osteocalcin antibodies. A possible explanation for the low detection of the DHFR fusion

proteins may be their poor recognition by the antibodies or the poor solubility of the fusion proteins.

The solubility of the DHFR-osteocalcin constructs turned out to be a major problem. Although expression levels were optimized and larger quantities of the osteocalcin fusion proteins could be produced and purified using Ni^{2+} -NTA-resin the proteins still were solubilized in 8M of urea. Dialysis of these protein solutions resulted in massive precipitations, and no proteins could be detected in the supernatant. With these results, 2 possibilities were considered; (i) to produce large amounts of purified insoluble DHFR-osteocalcin fusion protein, lyophilize it and subject the protein to CNBr-mediated cleavage, that might result in a soluble osteocalcin protein (either OC or pro-OC) or (ii) create a new fusion protein, in this case using MBP, with increased solubility and test this fusion protein directly or subject it to the same procedure as mentioned above using CNBr mediated cleavage. In the latter case only MBP-pro-OC, which could function as a propeptide-containing substrate for carboxylase, was produced. A methionine residue was introduced at the C-terminus of MBP for cleavage with CNBr. After construction of the MBP-pro-OC vector, it was introduced in a JM109 *E. coli* strain and multiplied. Isolation of the plasmid and subsequent DNA sequencing revealed that the pro-OC construct was in the proper reading frame. Expression cultures were subjected to SDS-PAGE and Western blotting and showed that indeed MBP-pro-OC was produced. Also it appeared that the protein was at least partly water-soluble, which facilitated its purification and fractionation. A drawback of the procedure was that both MBP alone and the MBP-pro-OC fusion protein had molecular weights of ~51 kDa. This was due to the fact that at introduction of the cDNA coding for pro-OC in the pMALc vector, a sequence of similar length was removed. Hence, gel electrophoretic techniques could not reveal the ratio between unchanged MBP and the chimeric MBP-pro-OC in our preparations. Fractionation of the crude cell extract on amylose resin and subsequent analysis on SDS-PAGE showed a prominent band at 51 kDa which was not recognized, however, by monoclonal antibodies against human osteocalcin (Western blot). This suggests that the expression of the chimeric fusion protein had been extremely low.

Since neither DHFR-pro-OC nor MBP-pro-OC exhibited substrate activity for vitamin K-dependent carboxylase, we have tried to liberate the pro-OC moiety from the respective fusion proteins. At equal substrate concentrations (100 ng / ml $\approx$ 1.5 nM, as determined by ELISA) the $^{14}\text{CO}_2$ incorporation was 8.4 nmol / hr for pro-OC originating from MBP-pro-OC and 14.4 nmol / hr for pro-OC from DHFR-pro-OC. Because of the poor yield of both the pro-OC proteins, which resulted in substrate concentrations far below the expected K_m ($\sim$ 1 μM), and the difficult purification of both preparations, it was decided to discontinue this line of substrate production, and to focus on substrates produced by organic synthesis. Polypeptides which had become available by then were: full length osteocalcin and descarboxy-osteocalcin, as well as a number of osteocalcin derived peptides.

Discussion

A plasmid expression vector has been constructed that directs synthesis in *E. coli* of human osteocalcin proteins containing an additional stretch of six histidine residues at its C-terminus. This enabled its single-step purification by chelate affinity chromatography. Initial expression experiments of human MGP and osteocalcin using the pQE30 expression vector and *E. coli* gave no detectable expression as analysed by SDS-PAGE. This was probably a result of the small size of both proteins. Stable expression in *E. coli* of small proteins is known to be difficult because they cannot fold correctly and are often subject to proteolytic degradation. Therefore, recombinant human osteocalcin was expressed as part of a DHFR fusion protein. The protein was fused to the C-terminus of mouse DHFR (6-His DHFR) in the vector pQE40, and expressed in *E. coli*. Isolation of expression products through the N-terminal 6-His tag by using Ni^{2+} -NTA-resin resulted in a product > 95 % pure when analysed by SDS-PAGE. Unfortunately this protein was highly insoluble.

Four main reasons for low expression are: presence of hydrophobic regions, toxic gene products, unstable proteins, or poor plasmid maintenance in the *E. coli* strain used. To prevent poor plasmid maintenance, the cultures were grown successfully in the

presence of 200 µg/ml of ampicillin. This leaves hydrophobic regions and toxicity of gene products as major causes for low expression. The toxicity of expressed proteins can be overcome by expressing proteins in freshly transformed cells. Normally the presence of very few transformants in comparison to the parent vector is an indication for toxicity of a gene product. The resulting transformants that do arise have often deletions and mutations. To overcome this problem transformants should be grown in media with high levels of antibiotics and 2 % glucose. Since the amount of transformants obtained from both the parent vector and the construct vectors were closely similar, the possibility of toxic gene products was excluded. Growing the cell culture in media containing 200 µg/ml ampicillin turned out to be the only condition at which expression was established. Under these conditions poor plasmid maintenance was bypassed.

The presence of hydrophobic regions in recombinant proteins often have a toxic effect on the host cells, probably due to association of the protein with vital membrane systems. Examples of such hydrophobic regions are signal peptides and transmembrane sequences. Using the method developed by Kyte and Doolittle³⁴ the hydrophobicity of both DHFR-OC and DHFR-pro-OC was determined, and it was found that none of the recombinant proteins had large hydrophobic stretches.

Identification of transmembrane regions was based on the method of Goldman, Engleman, and Steitz³⁵. The values used are the sum of the hydrophobic and hydrophilic components of each amino acid. The hydrophobic components are derived from the free energy of transfer of water-oil for the amino acid side chains. The hydrophilic component is derived from the free energy for the insertion of charged groups into a membrane bi-layer, along with the free energy of the hydrogen bonding with water and the carbonyl groups of the protein background. The method is based on a sliding window of 20 to identify possible transmembrane regions. In the graphs thus obtained hydrophobicity is plotted as positive and hydrophilicity as negative. For both recombinant proteins no transmembrane regions were identified (data not shown). Thus, the low expression levels are not the result of hydrophobic regions such as transmembrane sequences.

The detectable osteocalcin concentration in expression cultures of pQE40-OC, pQE40-pro-OC and MBP-pro-OC and the absence of osteocalcin in samples of pQE40 is a good indication that the expressed protein is indeed recombinant osteocalcin. Only through protein-sequencing the exact identity of the protein can be established. Since the ELSA-OSTEO kit measures only 1-49 human osteocalcin and human osteocalcin peptide 1-43, the detectable concentrations of approximately 40 ng/ml should be interpreted with care. The expressed recombinant human osteocalcin is part of a fusion protein and/or contains the pro-peptide. Conformational differences between the recombinant and mature human protein can result in poor antibody recognition. Compared to the total protein concentration of 2 µg/ml full length construct, the detected 40 ng/ml osteocalcin is 50 fold lower.

Conclusions

Recombinant DNA technology as a tool to produce a desired protein is an elegant technique but certainly has its limitations. The yields depend on the type of proteins to be expressed. Furthermore, there is a great choice in systems to express recombinant proteins. Since the production of recombinant osteocalcin in our hands was hardly cost-effective, the decision was made to obtain osteocalcin using a different technique.

As mentioned earlier in this chapter, there are other possibilities to obtain a specific protein, for instance by organic chemical synthesis. This field of research develops very rapidly, and what was impossible to synthesize a few years ago, is now routinely produced. Therefore we decided to have osteocalcin synthesized, both in its mature, carboxylated- as well as in its descarboxylated form. The synthetic peptide consisted of amino acid residues 1 to 49, which comprised of the entire mature sequence without the presence of a propeptide. One gram of both proteins was synthesized with a purity > 99 %. Both proteins were water soluble and could therefore be used for *in vitro* vitamin K-dependent carboxylation.

In the next paragraph, the possibilities of vitamin K-dependent *in vitro* carboxylation in various tissue types and the use of different substrates therein is discussed.

Paragraph 2.3

VARIOUS *IN VITRO* VITAMIN K-DEPENDENT GAMMA-CARBOXYLATING
TISSUES

Introduction

Vitamin K-dependent carboxylase is present in a wide variety of mammalian cells and tissues, where it is involved in the production of different kinds of Gla-proteins. In some cases these Gla-proteins are unique products of one cell type; examples of this category are prothrombin and osteocalcin which are exclusively synthesized by liver (hepatocytes) and bone (osteoblasts), respectively. Other Gla-proteins, like protein S and matrix Gla-protein, are expressed at low levels in many different extrahepatic tissues. Presently, there is a discrepancy between the tissue carboxylase content and the production of known Gla-proteins, such that it is to be expected that new Gla-proteins will be discovered. One way of identifying these new proteins is to induce the accumulation of their intracellular precursors by treatment of experimental animals with vitamin K-antagonists. These precursor proteins may then be specifically labeled by *in vitro* carboxylation using $^{14}\text{CO}_2$, and subsequently purified and characterized using denaturing techniques. Reliable procedures for isolating and testing the vitamin K-dependent systems from various tissues is a first requirement for these investigations.

The vitamin K-dependent carboxylase is a typical integral membrane protein, localized in the endoplasmic reticulum. After tissue homogenization, its activity can only be preserved by leaving the carboxylase within the microsomal membrane remnants, or by incorporating the enzyme into well-defined detergent micelles. Although there are no indications that carboxylases from different tissues are different gene products, accompanying proteins may affect the substrate specificity or affinity for vitamin K of carboxylase. Besides carboxylases in various states of purification, research of this kind also requires the availability of potential substrates and substrate analogs, differing from each other in their primary structure and their affinity for carboxylase. Substrates for carboxylase may vary from simple, synthetic peptides (e.g. Phe-Leu-Glu-Glu-Leu, FLEEL) to polypeptides of more than 60 amino acid residues, which are generally produced by molecular biology techniques. The cofactors required are either vitamin K hydroquinone (for assaying the carboxylase exclusively) or one of the oxidized forms (vitamin K quinone or epoxide). In the latter case, the combined action of carboxylase

and vitamin K reductase would be measured. In this paper the preparation procedures of enzymes, substrates and cofactors will be detailed.

Preparation procedure for carboxylase from different tissues

The best sources of vitamin K-dependent carboxylase are soft organ tissues, and the preparation procedures are very similar. Here we will describe the preparation of carboxylase from bovine liver, lung, kidney and testis, but the same procedure may be used for other tissues as well. Tissue homogenates may be frozen in liquid nitrogen and stored at -80 °C for several years without loss of carboxylase activity. For experiments in which a pretreatment of the animals precedes their use as organ donors, it may be practical to work with small animals like rats. In those cases in which the availability of the same (frozen) material over a long period is preferred, the cow is the experimental animal of choice. Also if large amounts of tissue are required (e.g. for purifications) the cheapest and most practical tissue donor is the cow.

Washed microsomes

Bovine liver, testis, kidney and lung are collected at the abbatoir, immediately after slaughter. The different tissues are cooled on ice and sliced in small pieces, following removal of the tougher parts like membranes, veins, and connective tissue. All subsequent steps are performed at 4 °C unless stated otherwise. In a typical procedure, the sliced tissue is added to 2 volumes of buffer A (100 mM NaCl, 50 mM Tris-HCl, pH 7.5) and minced in a blender. The mixed slurry is then homogenized in a Potter-Elvehjem tube (equipped with a teflon pestle) at 300 rpm with 2 strokes (up and down) of the pestle. At this stage the solution thus obtained may be either frozen, or used immediately for the preparation of microsomes (see below). In the latter case, 500 ml of tissue homogenate are centrifuged for 15 min at 10,000 g, after which the red supernatant fluid (post-mitochondrial fraction) is collected and centrifuged for 1 h at 105,000 g. The supernatant fluid and a loose interface are discarded, and the pellets

are transferred to the Potter tube, homogenized in 450 ml of buffer A, and centrifuged again for 1 h at 105,000 g (first washing step). Washing with buffer A is repeated until a colorless supernatant fluid is obtained (in total at least 3 washing cycles are required), and is followed by a washing step with buffer B (1 M NaCl, 50 mM Tris-HCl, pH 7.5). Finally the pellet is resuspended in buffer C (0.5 M NaCl, 25 mM Tris-HCl, pH 7.5), at a final protein concentration of approximately 40 mg/ml. Washed microsomes thus obtained may be either subsampled and stored at -80 °C or used for further purification.

Partly purified carboxylase

During purification, carboxylase is increasingly sensitive to proteases. We therefore recommend the use of a protease inhibitor cocktail in all further steps described below. Unless mentioned otherwise, all buffers used in our purification procedure contain the following protease inhibitors: benzamidine (1 mM), aprotinin (0.3 μ M), phenylmethylsulphonylfluoride (PMSF, 12 μ M), dithiothreitol (DTT, 2 mM) and EDTA (2 mM). Washed microsomes are supplemented with an equal amount of solubilization buffer (2% w/v CHAPS, 2 M NaCl), stirred for 30 min, and centrifuged for 2 h at 105,000 x g. The supernatant fluid (containing most of the carboxylase activity) is transferred into a pre-cooled flask to which ammonium sulphate is added with constant stirring to a final concentration of 55% saturation. The solution is kept at 4 °C for at least 30 min and subsequently centrifuged for 30 min at 20,000 x g. The resulting "floating pellet" contains the major part of the carboxylase activity and is dissolved in buffer C to a final protein concentration of 18 mg/ml. Aliquots of 1 ml may be frozen in liquid nitrogen and stored at -80 °C for several years. The preparation thus obtained is called "partly purified carboxylase" and was used in the experiments described below. Carboxylase has been identified in most soft tissues, except in brain and muscle, and the procedure described here can be used for nearly all tissues except those which are hard or tough (bone, cartilage, tendon and vessel wall). Partly purified carboxylase can also serve as the starting material for further purification of the enzyme. A protocol for the affinity purification of hepatic carboxylase to homogeneity will be described elsewhere in this

volume. It should be noted, however, that for the affinity purification of extrahepatic carboxylases this protocol requires substantial adaptations; for instance the concentrations of salt and detergent concentrations must be optimized for each type of tissue.

Substrates for carboxylase

In vivo substrates for carboxylase invariably contain the so-called "pro-sequence", which serves as a recognition site for carboxylase. In most substrates the pro-sequence is located in the leader peptide, immediately preceding the NH₂-terminus in the mature protein⁴. Shortly before cellular excretion the pro-sequence is removed from the precursor protein by limited proteolytic degradation. Only in the case of matrix Gla-protein (MGP) does the pro-sequence form an integral part of the mature protein³⁶. For *in vitro* carboxylation one may rely on endogenous precursor proteins which accumulate in the tissues during vitamin K-deficiency or warfarin-treatment of the donor animal³⁷. In general this leads to a rapid carboxylation reaction which will come to an abrupt stop when the substrate is depleted. The carboxylation reaction continues for a prolonged period of time when reaction mixtures are supplemented with exogenous substrates. These substrates may be prepared by thermal decarboxylation of isolated Gla-proteins, by peptide synthesis or by the production of recombinant Gla-proteins in carboxylase-deficient systems.

Decarboxylation of Gla-proteins

In general decarboxylated Gla-proteins will not contain the pro-sequence, which is the main reason why they are poor substrates for carboxylase. Unfortunately the only exception (MGP) is highly insoluble and difficult to isolate². Therefore, this technique can only be recommended for one other protein, i.e. osteocalcin (also known as bone Gla-protein, BGP). As can be seen in many reviews the pro-sequences of all known Gla-proteins have a number of strictly conserved amino acid residues, e.g. Ala¹⁰ and

Phe⁻¹⁶, which implies that these residues are probably critical for substrate recognition by carboxylase. Osteocalcin forms an exception to this rule, because the Ala⁻¹⁰ has been replaced by Gly. Substitution of either Ala⁻¹⁰ or Phe⁻¹⁶ by other amino acid residues strongly impaired substrate carboxylation of blood coagulation factors³⁸. Hence it might be expected that the pro-sequence of osteocalcin has a relatively low affinity for carboxylase. This could be the reason why the osteocalcin molecule itself has developed in such a way that the mature sequence contributes significantly to the recognition by carboxylase. Whatever the reason may be, decarboxylated osteocalcin is a fairly good and easily prepared substrate for *in vitro* carboxylation³⁹. Bovine tibia may be obtained from the abattoir, cleaned, and defatted in acetone before they are ground in a bone mill. Further removal of traces of fat may be accomplished in a second acetone wash, after which the powder is extracted in three subsequent steps of 24 h each, with a solution containing 1 M EDTA, 0.2 M KCl, 10 mM benzamidine, 5 mM ϵ -aminocaproic acid, 1 mM p-hydroxymercuribenzoate, 2 mg/L aprotinin, pH 8.0. In a typical preparation procedure one may use 500 g of bone powder and 3 x 2 litres of extraction buffer. After each extraction step the residue is removed by centrifugation (5,000 x g), and the supernatant is diluted 20 times with distilled water. Then, pre-swollen QAE-Sephadex slurry (in 20 mM Tris-HCl, 0.1 M NaCl, pH 8.0; 10 ml per litre of diluted extract) is added followed by 2 h of stirring; the Sephadex is isolated by filtration over nylon cloth, and eluted with 1 M NaCl. Osteocalcin is purified from the solution obtained by size exclusion chromatography on Sephadex G-75, and by high performance liquid chromatography using a Mono-Q column. Fractions may be tested using a commercial test kit with cross-reactivity for bovine osteocalcin (e.g. Incstar). For decarboxylation the peak fractions are pooled, dialysed against phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, pH 7.4) and brought to pH 1 with 0.1 M HCl. This preparation is lyophilized to dryness, and subsequently heated to 105 °C overnight, under vacuum. Optimal results are obtained if during the entire heating period there is constant suction using a powerful vacuum pump. The preparation thus obtained is reconstituted with water to its original volume, and the pH is adjusted to 7.4. The precise concentration of osteocalcin must be determined by at least two

independent methods (e.g. protein measurement and a specific osteocalcin assay).

Peptide synthesis

It is surprising how little structural elements are required to permit the carboxylation of a peptide substrate. Of course, the predominant requirement is the presence of a glutamate residue. Glu derivatives like Boc-Glu methylester (Boc-Glu-Me) and short peptides like Phe-Leu-Glu-Glu-Leu (FLEEL) are commercially available (Bachem, Switzerland), and others (Boc-Glu-Glu-Val⁴⁰) may be synthesized. These small substrates do not contain the pro-sequence, but since they can be added in high concentration, they allow for relatively high carboxylase activities. Different chain lengths of poly-Glu may also serve as a substrate for carboxylase⁴¹. A common characteristic of all these substrates is that their apparent K_m (K_m^{app}) is rather high (in the millimolar range). With the present technology it is possible also to synthesize substrates containing the pro-sequence of one of the coagulation factors, for instance the substrate known as proPT-28 containing the amino acid residues -18 through +10 of the human prothrombin precursor⁴².

Molecular Biology

Since prokaryotes do not contain the complicated machinery for posttranslational processing of proteins, expression of cDNAs from all Gla-proteins in prokaryotic systems will result in non-carboxylated proteins still containing the pro-sequence. This has been reported for osteocalcin⁴³ as well as for coagulation factors and their Gla-domains. In particular we draw attention to the peptide consisting of the pro-sequence and the first 41 residues of human coagulation factor IX. The preparation of this polypeptide (designated as proIX-59) and its crucial importance for the purification of carboxylase have first been described by Wu et al.^{10,11}. These authors reported the construction of a cDNA coding for a chimeric protein composed of proIX-59, linked to a phage T7 capsid protein⁴⁴. The recombinant protein product accumulated in inclusion

bodies, which were harvested and subjected to CNBr-mediated cleavage to liberate the recombinant proIX-59. After an anion exchange purification step, proIX-59 formed an excellent carboxylase substrate, all 12 glutamate residues of which may be carboxylated after prolonged incubation ⁴⁴. Even more important was the observation that proIX-59 - in contrast to proPT-28 - could be used as an affinity ligand for purifying carboxylase ⁴⁵.

Coenzymes for carboxylase

Vitamin K hydroquinone (KH₂) is the active coenzyme for carboxylase, and it can be prepared by incubating 2.5 mM of a detergent-solubilized vitamin K (e.g. Konakion™, or Aquamephyton™) in 150 mM dithiothreitol, pH 8.5, at 37 °C overnight in a light-protected tube. The resulting KH₂ is colourless and gives the best results in CO₂ incorporation studies. In non-purified systems, vitamin K quinone (K) and vitamin K 2,3-epoxide (KO) may be used as coenzymes for carboxylase as well. In these cases, however, the coenzymes have to be reduced by the enzyme KO-reductase, which is present in washed microsomes from all tissues, and also in partly purified carboxylase from some sites (e.g. liver, testis). So if the carboxylase reaction is performed with either K or KO instead of KH₂ the sequential activities of carboxylase and KO-reductase will be tested. By comparing these data with those obtained for the KH₂-initiated reaction, an impression may be obtained concerning the KO-reductase content of the preparation. KO is prepared by dissolving 20 mg of phylloquinone in 5 ml of isopropanol / hexane in a ratio of 2:1 (v/v), to which 0.1 ml of 0.5 M NaOH in 0.2 M Na₂CO₃ and 0.3 ml of 30% H₂O₂ are added ⁴⁶. After an overnight incubation in a light-protected tube at 37 °C the mixture is supplemented with 3 ml of water, vortex-mixed for 1 min, and the hexane phase is collected. The latter is washed twice with 5 ml of water and evaporated to dryness with gently heating under a constant stream of nitrogen. The residue is dissolved in ethanol to a concentration of 10 mg/ml.

Optimal conditions for *in vitro* carboxylation of endogenous and exogenous substrates

Endogenous substrates are protein precursors which have accumulated during *in vivo* treatment of the donor animals with vitamin K-antagonists (warfarin, brodifacoum). At least part of these precursor proteins remain complexed to carboxylase during the purification procedure, and may be carboxylated *in vitro* under the conditions described below. In general the supply of endogenous substrate is rapidly exhausted, and the addition of an exogenous substrate (e.g. the pentapeptide FLEEL) substantially increases the total amount of CO₂ fixed. After the carboxylation reaction is completed, the (pro-containing) endogenous substrate can be separated from the short peptide substrate by trichloroacetic acid (TCA) precipitation (see below). An important difference between substrates which do and those which do not contain the pro-sequence is that the carboxylation of the latter ones is greatly enhanced (10-20 fold) by the presence of 1 M (NH₄)₂SO₄. The mechanism behind this stimulatory effect is not quite clear, but kinetic analysis has shown that the high salt concentration affected the V_{max}, and not the K_m^{app} ⁴⁷. No such an effect was observed for the carboxylation of endogenous precursor proteins, or for pro-containing substrates like proPT-28 and proIX-59. Another point to realise is that if radiolabeled bicarbonate is the only source of ¹⁴CO₂, the concentration of bicarbonate is the rate-limiting step in the carboxylation. The carboxylation rate will substantially increase by adding 5 mM of non-labeled NH₄HCO₃, but obviously the amount of incorporated label will decrease. Carboxylase is not very stable at 37 °C, which is why most investigators work at 20 °C or lower. Maximal substrate carboxylation may even be obtained in overnight incubations at 10 °C ⁴⁸.

To allow the carboxylation reaction to proceed, the following ingredients should be pipetted in the order indicated to give 0.125 ml reaction volumes of the following composition: 0.45 mg of microsomal proteins, a suitable substrate (either 4 mM of a substrate devoid of pro-peptide, or 20 μM of a pro-containing one), 4 mM dithiothreitol (DTT), 1 M (NH₄)₂SO₄, 0.5 M NaCl, 25 mM Tris-HCl, and 5 μCi (185 kBq) of NaH¹⁴CO₃.

Reactions are initiated by the addition of 5 μ l (220 μ M) of KH_2 , K, or KO. Incubation is performed for 30 min at 20 $^{\circ}\text{C}$, and the reaction is stopped by adding 0.1 ml of the reaction mixture to 0.8 ml of 5% (w/v) trichloroacetic acid (TCA) in a glass vial, containing anti-bumping granules. The vials are boiled for a short time (2 min) on a hot plate to remove the last traces of unbound $^{14}\text{CO}_2$, after which scintillation liquid is added for radiolabel counting.

Optimal conditions for measuring KO-reductase in microsomal fractions

During the carboxylation reaction, KH_2 is oxidized into KO, which may be recycled via the action of the enzyme KO-reductase. Several procedures for the assessment of KO-reductase have been described, the data shown below were produced using the method described by Thijssen ⁴⁹. Reaction mixtures (0.25 ml) contained 0.9 mg of microsomal proteins, 1 M $(\text{NH}_4)_2\text{SO}_4$, 0.5 M NaCl, 4 mM dithiothreitol (DTT) and 25 mM Tris-HCl, pH 7.5. After a pre-incubation of 2 min at 20 $^{\circ}\text{C}$, reactions were started by adding 10 μ l of 220 μ M KO in ethanol. After incubation periods of 0, 5, 10 and 20 min, 50 μ l aliquots were taken and extracted with 1 ml isopropanol / hexane (2:1, v/v) containing 5 μ g D- α -tocopherol as an internal standard. After the addition of 1 ml of water, the mixtures were vortexed and 0.2 ml of the hexane phase was taken and evaporated to dryness under a gentle stream of N_2 at room temperature. The residue was dissolved in 50 μ l isopropanol of which 20 μ l was used for HPLC analysis¹⁵. The enzyme activity was deduced from the initial reaction rate during the first 10 minutes of incubation.

Comparison of enzymatic activities of hepatic and extrahepatic carboxylases

The enzymes of the vitamin K cycle obtained from different tissues may be compared by a) measuring the carboxylase and KO-reductase per mg of protein, and b) by assessing their respective kinetic constants. In these measurements, it is important that substrates and cofactors are present in excess and that the rate of product formation

is assessed under linear reaction rate conditions. Carboxylase activity is assessed by initiating the carboxylation reaction with KH_2 . A test for the simultaneous activities of carboxylase and KO-reductase is performed by initiating the carboxylase reaction with KO. If KO-stimulated carboxylase is less than 25% of the KH_2 -driven reaction, this is indicative of a relative KO-reductase deficiency of the preparation. This can be assessed in a more direct way by measuring the conversion of vitamin K-epoxide into the corresponding quinone. In table 1 we have summarized these three enzymatic activities for the four partly purified carboxylase preparations presented in this paper. It is clear that partly purified carboxylase was obtained from all four tissues and that the activities of the extra-hepatic preparations ranged from 23 - 84% of that obtained from the liver. KO-reductase was present in washed microsomes from all tissues but, on fractionation, the enzyme activity was lost in the preparations from kidney and lung.

Table 1

Partly Purified Carboxylase From Different Origins: Comparison Of Tissue Content ^a

Origin of carboxylase	KH_2 carboxylase (pmol CO_2 fixed)	KO carboxylase (pmol CO_2 fixed)	KO reductase (pmol K formed)
Liver	0.93	0.24	69
Testis	0.78	0.19	496
Kidney	0.36	0	0
Lung	0.22	0	0

^a Carboxylase activity is expressed as picomoles CO_2 incorporated per milligram of microsomal protein and per minute of incubation at 20°C, using the pentapeptide FLEEL as a substrate. KH_2 carboxylase means that the reaction was initiated with vitamin K hydroquinone, KO carboxylase stands for the carboxylation reaction initiated with vitamin K epoxide. KO reductase is expressed as picomoles vitamin K quinone formed per milligram of microsomal protein and per minute of incubation at 20°C, using vitamin K epoxide as a substrate.

This loss was due to inactivation (probably because of the high detergent concentration) rather than to separation in a different fraction. Partly purified carboxylase from liver and testis contained high levels of KO-reductase, and it appears that the testis especially is the tissue of choice for the purification of KO-reductase.

Another striking point is that there is no apparent stoichiometry between carboxylase and reductase: in terms of picomoles of product formed, the enzymatic activity of reductase exceeds that of carboxylase by 75 (liver) to 635 (testis) fold. The reason for this large excess of reductase is not known, but obviously it will result in a rapid reduction of any KO formed.

Different carboxylase preparations can also be compared by measuring their kinetic constants using various substrates. In table 2 we show the results for 6 different substrates tested in the four partly purified carboxylase preparations.

Table 2
Partly Purified Carboxylases From Different Origins: Comparison of
Substrate Specificity ^a

Substrate used	Origin of carboxylase							
	Liver		Testis		Kidney		Lung	
	K_m^{app}	V_{max}	K_m^{app}	V_{max}	K_m^{app}	V_{max}	K_m^{app}	V_{max}
FLEEL	2.2 mM	1.4	2.7 mM	1.0	2.1 mM	0.6	2.2 mM	0.4
Boc-EEV	1.6 mM	1.1	1.3 mM	0.9	1.2 mM	0.5	1.3 mM	0.2
Boc-Glu-Me	2.4 mM	1.0	2.5 mM	0.8	2.7 mM	0.4	2.7 mM	0.2
D-Osteocalcin	8.2 μ M	0.19	6.8 μ M	0.11	7.2 μ M	0.08	6.3 μ M	0.04
ProPT(Glu) ₁₀	4.1 μ M	0.21	4.9 μ M	0.12	3.5 μ M	0.06	2.7 μ M	0.05
ProPT-28	2.1 μ M	0.24	3.5 μ M	0.14	2.7 μ M	0.07	3.0 μ M	0.03
ProIX-59	0.8 μ M	0.25	1.1 μ M	0.13	1.2 μ M	0.08	1.5 μ M	0.03

^a K_m^{app} for the first three substrates is expressed in millimolar, for the last three substrates in micromolar; V_{max} is expressed in pmol CO₂ incorporated per minute and per milligram of microsomal protein at 20°C; proPT-28 stands for the prothrombin precursor sequence -18 to +10; ProIX-59 stands for the factor IX precursor sequence -18 to +41; ProPT(Glu)₁₀ stands for the prothrombin propeptide with 10 glutamate residues at its carboxy-terminal site.

Peptide substrates lacking the pro-sequence have K_m^{app} values in the millimolar range, except for decarboxylated osteocalcin the mature sequence of which seems to

contribute substantially to the enzyme-substrate recognition. The reason for this unique property is presently unknown. K_m^{app} values for pro-containing substrates are at least three orders of magnitude lower than those for the short peptides. The fact that there is very little difference between a pro-sequence connected to a sequence derived from a clotting factor, and that linked to poly-L-glutamate demonstrates that the pro-sequences in the clotting factor precursors are the major (if not the only) structural requirement for enzyme-substrate interaction. Strikingly, there is a large difference between the carboxylation of peptide substrates in the presence of non-covalently bound propeptide, and the carboxylation of polypeptides containing both the pro-sequence as well as the carboxylatable glutamate residues. As was reported by Ulrich et al.⁴², the K_m^{app} for small substrates like FLEEL and PT/1-10 (descarboxyprothrombin residues 1-10) decreased by a factor of three with the addition of non-bound pro-peptide, but it was at least a thousand times lower if the pro-peptide was covalently attached. Similarly, pro-containing substrates decreased the K_m^{app} of carboxylase for its cofactor KH_2 about 20-fold, whereas the combination of pro-peptide + peptide substrate did not⁵⁰. Thus, the covalent attachment of the pro-peptide and the substrate glutamate residues is a critical requirement for efficient carboxylation of the substrate.

The fact that the kinetic characteristics of carboxylases from various tissues are very similar is consistent with the idea that all are products of the same gene. It is to be expected, therefore, that the various extrahepatic Gla-proteins rather than the extrahepatic carboxylases may be a subject of interest in forthcoming years. Warfarin treatment of animals, followed by endogenous substrate labeling in the *in vitro* carboxylase reaction is a promising technique to recognize these proteins during their purification and characterization, even if they would have lost all functional activity.

Conclusions

The methods to obtain carboxylase from hepatic and extra-hepatic tissue microsomes described in this paragraph are basic but still valid. Several authors have already described the purification of the enzyme from bovine liver and mammalian cell lines⁵¹⁻⁵³.

Stafford et al. reported a method to purify endogenous substrate-free human γ -glutamyl carboxylase expressed in an insect cell line (D.W. Stafford, unpublished results), thus the use of washed microsomes in vitamin K research may be questioned. However, from data obtained with either carboxylase from washed microsomes, purified bovine carboxylase or purified human carboxylase it is evident that the results are similar, if the K_m values of the substrates used are compared. The V_{max} values may vary for a certain substrate if different preparations of carboxylase are used, depending on the enzyme concentration of the preparation, especially if washed microsomes are used as the source for carboxylase. To obtain accurate data on substrate properties for carboxylase, the propeptide- and endogenous substrate free human carboxylase from insect cells is the best choice. However, to obtain this form of carboxylase, sophisticated molecular biology techniques have to be available in combination with facilities for eukaryotic cell cultures. Moreover, the purification of the enzyme is laborious and time consuming. Therefore, washed microsomes are still a usefull source for carboxylase data on enzyme- and substrate properties. The method is simple, does not require specific techniques and can be performed in one day. Moreover it should be kept in mind that the purified carboxylase preparations possess neither vitamin KO- nor vitamin K reductase activity. Since the reductase enzyme has not yet been purified, the washed microsomal system forms the only possibility to investigate the combined function of carboxylase and reductase.

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Chapter 3

THE ROLE OF THE PROPEPTIDE AND GAMMA-GLUTAMIC ACID DOMAIN OF FACTOR IX FOR *IN VITRO* CARBOXYLATION BY THE VITAMIN K-DEPENDENT CARBOXYLASE

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Abstract

The vitamin K-dependent γ -glutamyl carboxylase catalyzes the processive carboxylation of specific glutamates in a number of proteins related to blood coagulation and bone. To address the independent importance of the propeptide, γ -carboxyglutamic acid (gla) domain and elements beyond the gla-domain of factor IX in vitamin K-dependent carboxylation, we have examined the kinetics of carboxylation of peptides containing: 1) propeptide and gla-domain, 2) the gla-domain alone, 3) uncarboxylated osteocalcin, 4) propeptide followed by the entire uncarboxylated factor IX molecule, and 5) the factor IX propeptide followed by a non-gla-domain sequence. Our studies indicate that peptides with a covalently linked propeptide have K_m values similar to the physiological substrate of the carboxylase. In contrast, the gla-domain of factor IX has a ≥ 230 - fold higher K_m for the carboxylase than the corresponding peptide with a covalently linked propeptide. This contrasts with osteocalcin, another vitamin K-dependent protein, which appears not to require a covalently linked propeptide for high affinity binding to the carboxylase. Analysis of the carboxylation products of a propeptide/non-gla-domain substrate indicate that it is carboxylated multiple times in a processive manner. These studies show that the perceived binding affinity of the carboxylase substrate and processivity is conferred by the propeptide without requiring the conserved gla-domain sequences and that factor IX and osteocalcin may have distinct mechanisms of interacting with the carboxylase.

Introduction

The vitamin K-dependent γ -glutamyl carboxylase is an integral membrane protein that is found in the rough endoplasmic reticulum and has been purified from bovine liver ¹, cloned and sequenced ^{2,3}. In addition to its carboxylase activity, the purified vitamin K-dependent carboxylase also possesses the vitamin K epoxidase activity ⁴. The vitamin K-dependent carboxylase catalyzes the post-translational modification of specific glutamic acid residues to γ -carboxyglutamic acid (Gla) in a number of proteins essential for blood clotting as well as for the bone-related proteins: osteocalcin and matrix Gla protein (MGP) ⁵⁻⁸. Vitamin K-dependent proteins may be even more prevalent than previously thought as evidenced by the discovery of growth-arrest protein Gas-6 ⁹ and the very recent identification of two putative vitamin K-dependent membrane proteins PRGP1 and PRGP2 ¹⁰.

The amino-terminal domain of the mature vitamin K-dependent blood proteins where multiple glutamic acid residues are converted to γ -carboxyglutamic acid is referred to as the gla-domain. The gla-domains of the known vitamin K-dependent blood proteins share extensive sequence identity. In addition, all of the known vitamin K-dependent proteins contain a highly conserved propeptide region that is proteolytically removed to form the mature protein (except for MGP where the propeptide-like sequence is found within the mature form of the protein) ¹¹. Expression of vitamin K-dependent proteins in mammalian cell culture demonstrates that deletion of the propeptide region from factor IX ¹² or protein C ¹³ abolishes carboxylation, while specific amino acid substitutions within the eighteen amino acid propeptide sequence affect carboxylation ^{12,14,15}. Furthermore, naturally occurring mutations in the factor IX propeptide region have been found in warfarin sensitive hemophilia B patients ^{16,17}. Synthetic peptides spanning the propeptide and first 10 residues of the gla-domain of factor IX or prothrombin have an apparent affinity for the carboxylase that is three orders of magnitude lower than the small glutamate substrate FLEEL ^{18,19}. These studies indicate that the propeptide plays an essential role in the carboxylase's ability to recognize its substrate.

There is also ample evidence that the interaction of the carboxylase with the propeptide activates the enzyme. Evidence for propeptide activation of the enzyme is seen in studies which suggest that the propeptide lowers the apparent K_m for glutamate²⁰, lowers the apparent K_m for vitamin K²¹, increases formation of the carbanion intermediate²² and activates the epoxidase activity of the carboxylase²³. Therefore the propeptide has a dual role as a primary recognition element for the carboxylase as well as an allosteric activator of enzyme activity.

Most studies of the carboxylase have utilized small glutamic acid containing substrates, such as the pentapeptide FLEEL or peptides containing the propeptide and two potential carboxylation sites. Although these studies have provided important information about the carboxylase, elucidation of carboxylase kinetics and mechanism requires the use of peptides that are multiply carboxylated and that more closely resemble the native substrates of the enzyme. Previous work from our laboratory has shown that the FIXproGLA41 peptide, which contains the factor FIX propeptide followed by the first 41 amino acids of the gla-domain of factor IX, possesses a number of qualities expected of *in vivo* substrates of the vitamin K-dependent carboxylase. Specifically this peptide contains all twelve glutamates normally carboxylated in factor IX, has a physiologically reasonable affinity for the carboxylase, can be fully carboxylated²⁴, and is processively carboxylated²⁵. Therefore, the propeptide and the gla-domain alone are sufficient to direct processive carboxylation. In contrast to numerous studies which indicate that an amino-terminal propeptide extension is important for recognition by the carboxylase, studies with thermally decarboxylated osteocalcin indicate that a covalently attached propeptide is not necessary for binding of the carboxylase²⁶⁻²⁸. Osteocalcin is a 49 amino acid vitamin K-dependent protein that contains three gla residues and is expressed with an eighteen amino acid propeptide extension analogous to the blood vitamin K-dependent proteins. The mature osteocalcin shares little sequence identity with the gla-domains of the blood vitamin K-dependent proteins, except for a conserved sequence identified by Price¹¹. Therefore, for osteocalcin, essential elements other than the propeptide appear to be involved in substrate recognition.

In this paper the relative contribution of the propeptide and gla-domain of factor IX to vitamin K-dependent carboxylation has been examined by *in vitro* kinetic studies with purified components. These studies show that the perceived binding affinity of the carboxylase substrate and processivity is conferred by the propeptide without requiring the conserved gla-domain sequences and that factor IX and osteocalcin may have distinct mechanisms of interacting with the carboxylase.

Materials and Methods

Reagents

All chemicals were reagent grade. Vitamin K₁ (phylloquinone) was from Abbott Laboratories, FLEEL was purchased from Bachem, Phosphatidylcholine, Type X-E from dried egg yolk is from Sigma. NaH¹⁴CO₃ with specific activity of 56.4 mCi/mmol was from ICN Pharmaceuticals.

Proteins

Bovine carboxylase was purified as previously described, concentrated by chromatography on SP-Sepharose (Pharmacia) and its concentration was estimated using the specific activity of the purified protein according to Wu et al (1). The proteins proFIX and proFIX(GLA) expressed in chinese hamster ovary cells were kindly provided by Mary Switzer of the Purification Process Development Department at Genetics Institute Inc. (Andover, MA). Human plasma factor IX was purchased from Haematological Technologies (Essex Jct, VT). The factor IX propeptide (proFIX19) was synthesized by Dr Frank Church (University of North Carolina at Chapel Hill). The chemically synthesized mature form of human osteocalcin (amino acids 1 to 49) was provided by Dr. Berry A.M. Soute (Maastricht University, The Netherlands). Oligonucleotides for cloning and mutagenesis were from Oligos Etc. Inc., (Guilford, CT)

Construction of expression vectors

The multifunctional expression vector phagemid pMc292 described previously²⁴ was used for both mutagenesis and expression of foreign peptides in *E. coli*. This phagemid contains an insert that codes for the propeptide and first 41 amino acids of the gla-domain of human factor IX. The methionine at amino acid 19 is mutated to isoleucine as found in mouse factor IX. Arginines at -4 and -1 are mutated to asparagine and serine respectively. Initially a vector was constructed that contained the gene10 sequence followed by the factor IX propeptide/gla-domain/egf1/egf2 domains in the pMc292 phagemid. This was done using oligonucleotides 5'-T GAG AGA GAA TGC ATA GAA AAG TGT-3' and 5'-GGA GGA TCC TAT GCT GGT TCA CAG GAC T-3' to

amplify a fragment from the human factor IX cDNA that engineers a Nsi I site at the 5' end, a BamHI site at 3' end and codes for amino acid residues 20-127 of human factor IX. This fragment was digested with Nsi I/BamHI and ligated into the Nsi I/BamHI digested pMc292 phagemid. The resulting vector is referred to as pMc292(IX-18 to 127) and was used for further mutagenesis and cloning.

In vitro mutagenesis

All *in vitro* mutagenesis were performed by the Uracil method²⁹ using single stranded DNA prepared using M13KO7³⁰. The FIXproGLA46 construct was generated by inserting a stop codon after amino acid 46 using oligonucleotide 5'-CAG TAT GTT TAA GGA TCC CAG TGT GAG. The FIXGLA46 construct was created by using oligonucleotide 5'ATT ATC GCT AAG TAC GCC ATG TAT AAT TCA GGT AAA CTC GAG-3' and the original pMc292 phagemid to loop out from Alanine at -18 to the Serine at -1. The FIXproNon-GLA construct was generated by using oligonucleotide 5'-TCT TGA ATC AGC CAA AGA GCG GAA CCT TGA GAG AGA ATG TAG AGG AGA AAA GTG TA-3' which loops out from tyrosine at position 1 to the middle of the codon at position 12 which codes for glycine. The oligonucleotide also replaces a stop codon that would occur 8 amino acids after the end of the propeptide with a glutamate and changes a lysine at position 9 to glutamate. This introduces a frameshift that results in a construct that codes for the factor IX propeptide attached to a 45 amino acid non-gla-domain sequence.

Expression, purification and characterization of peptides

The various peptides were prepared as described previously²⁴ with the following modifications. The inclusion bodies were isolated from either a 4L culture or a 5 L culture prepared with a BioFlo IIc fermentor (New Brunswick Scientific). Peptides which contained a tryptophan residue (FIXproGLA46 and FIXGLA46) were digested with cyanogen bromide in 72% trifluoroacetic acid in place of trichloroacetic acid to minimize oxidation of the tryptophan residues. After cyanogen bromide cleavage, the dried down peptides were resuspended in 50 ml of 8 M Guanidine-HCl. The peptides were

sulfonated by the addition of 0.15 g sodium tetrathionate, 0.3 g sodium sulfite and adjustment of the pH to 9.0 with ammonium hydroxide and incubating at room temperature for 16 hr. The sulfonated peptides were then dialyzed against three changes of 25 mM MOPS pH 8.0, 10 mM NaCl to precipitate the gene 10 fragments while the target peptides remained in solution. The gene 10 fragments were removed by centrifugation at 12,000 g for 15 min. The supernatant was loaded on a DEAE-Sepharose CL-6B (Sigma) column and the target peptide was eluted by the addition of 25 mM MOPS pH 8.0/ 250 mM NaCl. Sulfonation was reversed by incubating the eluted peptides at room temperature for three hours with 100 mM dithiothreitol. The peptides were further purified by reversed-phase HPLC using a Rainin 0.46 x 50 mm C18 column (83-203-F5). Peptides were eluted using a linear 10 minute gradient (1ml/min) of 0% Buffer B (0.1% TFA) to 100% Buffer B (90% Acetonitrile/0.1% TFA). The peptides were dried by rotary evaporation.

Confirmation of peptides by electrospray ionization mass spectroscopy

The molecular mass of each peptide was determined using a Fisons-VG Quattro BQ triple quadrupole mass spectrometer equipped with a pneumatically assisted electrostatic ion source operating at atmospheric pressure. Samples were reconstituted in 50% aqueous acetonitrile containing formic acid (1% v/v) and were introduced by loop injection into a stream of 50% aqueous acetonitrile flowing at 6 μ L/min. Mass spectra were acquired in the multichannel analyser (MCA) mode from m/e 700-1400 with a scan time of 10 seconds. The mass scale was calibrated with horse heart myoglobin (M_r 16951.48) with a resolution corresponding to a peak width at half height of 1.0 Da for m/e 893. The mass spectra were transformed to a molecular mass scale using software supplied by the manufacturer.

Kinetics of carboxylation of propeptide containing peptides

Reactions (125 μ L) were conducted at 17^o C for 8 hr with indicated amounts of purified bovine carboxylase and 25 mM MOPS pH 7.4, 0.5 M NaCl, 0.16% CHAPS, 0.16 % phosphatidyl choline, 222 μ M vitamin K hydroquinone, 6 mM DTT and 5 μ Ci of

$\text{NaH}^{14}\text{CO}_3$ (specific activity, 54 mCi/mmol; ICN Corp). A final concentration of 60 nM free factor IX propeptide from the purified carboxylase is also present. Reactions were quenched by the addition of 75 μL of 1 N NaOH and transferred to vials containing 1 ml of 5% trichloroacetic acid. Samples were gently boiled to remove unincorporated $^{14}\text{CO}_2$ and total incorporation was determined by scintillation counting. Previous studies on the kinetics of $^{14}\text{CO}_2$ incorporation into representative peptides of the vitamin K-dependent substrates have employed short incubation times (< 1 hr) to measure carboxylation. A previous study along with this work indicate that the time course of carboxylation of peptides with an attached propeptide demonstrates a biphasic burst with an initial rapid pre-steady state phase followed by a slower linear steady state phase²⁵. Therefore, to measure the steady state kinetics of $^{14}\text{CO}_2$ incorporation, we used prolonged incubation times such that we were measuring the steady state rather than the pre-steady state rate. Inhibition experiments were done under the same reaction conditions and incubation times as above. For inhibition studies with proFIX(GLA), control experiments were done in the absence of substrate to account for traces of uncarboxylated or partially carboxylated proFIX in the proFIX(GLA) preparation. The $^{14}\text{CO}_2$ incorporation in the absence of substrate was small (< 8%) compared to IX -18 to 46 carboxylation and was subtracted from the activity in the presence of substrate.

Determination of the extent of FIXproNon-GLA carboxylation of by mass spectrometry

The number of carboxylations of the FIXproNon-GLA peptide was determined by a similar method to that used previously to demonstrate processivity in FIXproGLA41 carboxylation²⁵. Briefly, a 5 ml carboxylation reaction containing 20 nM purified bovine carboxylase, 25 mM MOPS pH 7.4, 0.15 M NaCl, 0.16% CHAPS, 0.16 % phosphatidyl choline, 120 μM vitamin K hydroquinone, 200 μCi of $\text{NaH}^{14}\text{CO}_3$ (specific activity, 54 mCi/mmol; ICN Corp) and 2.5 μM FIXproNon-GLA peptide were incubated at 17° C and were supplemented with an additional 120 μM vitamin K hydroquinone after 12 hours. After 17 hr the reaction was quenched by acidification with acetic acid to remove free $^{14}\text{CO}_2$ and neutralized with sodium hydroxide. The reaction was diluted 20-fold,

buffered to pH 5.0 and bound to 1 ml of SP-sepharose and eluted with 0.5 M NaCl. The labeled material was loaded on a 0.46 x 50 mm Rainin C18 HPLC column and eluted at a flow rate of 1 ml/min with a linear gradient from 0% acetonitrile/0.1% trifluoroacetic acid to 100% acetonitrile/0.1% trifluoroacetic acid. Fractions along the elution gradient were collected and those containing $^{14}\text{CO}_2$ incorporation above background were pooled and dried by rotary evaporation. The dried down sample was resuspended in 20 μL of 10 mM ammonium hydroxide. Samples were then analyzed by online mass spectrometry as described previously ²⁵.

Kinetics of carboxylation of gla-domains

Reactions were performed at 17^o C for 1 hr with 4 nM purified bovine carboxylase, 25 mM MOPS pH 7.4, 0.5 M NaCl, 0.16% CHAPS, 0.16 % phosphatidyl choline, 0.6 M ammonium sulfate, 20 μM proFIX19, 222 μM vitamin K hydroquinone, 6 mM DTT and 5 μCi of $\text{NaH}^{14}\text{CO}_3$ (specific activity, 54 mCi/mmol; ICN Corp). Unlike the time course of propeptide containing peptides, the time course of carboxylation of the gla-domains did not demonstrate a burst but rather was linear during the 1 hr reaction course.

Results

Expression, purification and identification of peptides

All peptides were expressed in *E. Coli*, purified by high pressure liquid chromatography, and verified by ion spray mass spectrometry. FIXpro-NonGLA is expressed as a 63 amino acid peptide, but cleavage by cyanogen bromide removes 11 amino acids from its C-terminus. The resulting peptide is 52 amino acids with an approximately 1:1 mixture of the homoserine or homoserine lactone residue at the C-terminus. (Table 1). The experimental molecular mass for each of the peptides is as follows: FIXproGLA46 is 7645.7 ± 0.4 daltons (predicted: 7646.4), FIXGLA46 is 5625.31 ± 1.96 daltons (predicted: 5626.1) and FIXproNon-GLA 5938.7 ± 0.29 daltons (predicted: 5938.6) for the homoserine form and 5920.49 ± 0.57 (predicted 5920.6) for the homoserine lactone form.

Table 1
Sequences of Factor IX and Osteocalcin-Based Peptides^a

FIXproGLA46	<u>AVFLDHENANKILNQPKS-</u>	YNSGKLEEFVQGNLERECIEEKCSFEEAREVFENTERTEFWKQYV
FIXGLA46		YNSGKLEEFVQGNLERECIEEKCSFEEAREVFENTERTEFWKQYV
Osteocalcin		YLYQWLGA ^P VPYPDPLEPRREVCELNPDCELA ^D HIGFQEAYRRFYGPV
FIXproNonGLA	<u>AVFLDHENANKILNQPKS-</u>	GTLENVEEKSVVLKKHEKFLKTLKEQLNFGSShS

^a The propeptide sequence is underlined. Known carboxylated glutamates in factor IX and osteocalcin and potential carboxylation sites in FIXproNonGla are indicated in boldface type. hS stands for homoserine / homoserine lactone.

Inhibition of carboxylation by free propeptide and proFIX(GLA)

To ascertain the importance of the propeptide in substrate recognition by the carboxylase, we examined the effect of free propeptide (proFIX19) on carboxylation of a propeptide/gla-domain (FIXproGLA41) peptide. As seen in figure 1, proFIX19 acts as a competitive inhibitor of FIXproGLA41 carboxylation with a $K_i = 0.22 \mu\text{M} \pm 0.10 \mu\text{M}$. Competitive inhibition indicates that binding of free propeptide to the propeptide binding

site of the carboxylase prevents association of the FIXproGLA41 substrate with the carboxylase. This is consistent with an initial interaction of the carboxylase with its substrate through the propeptide sequence rather than the gla-domain or glutamic acid residues themselves. In addition, we find that carboxylated factor IX with the propeptide still attached (proFIX(GLA)), is a product inhibitor of a propeptide/gla-domain peptide carboxylation with a K_i $0.40 \mu\text{M} \pm 0.07$, similar to that observed for free propeptide. In contrast, plasma factor IX, which does not contain the amino-terminal propeptide extension, is unable to inhibit carboxylation at the concentrations tested (Figure 2). Therefore, product inhibition by proFIX(GLA) is conferred by the propeptide and is a result of preventing access of the substrate to the propeptide binding site.

Figure 1: Free Propeptide is a Competitive Inhibitor of FIXproGLA41 Carboxylation. The effect of free propeptide (proFIX19) on carboxylation was examined with $1 \mu\text{M}$ ($\blacktriangle$) and $5 \mu\text{M}$ ($\blacksquare$) FIXproGLA41 at 17°C for 8 hr with $\sim 10 \text{ nM}$ purified bovine carboxylase. Data were fit by non-linear regression and lines were drawn according to the equation for competitive inhibition (35) using $V_{\text{max}} = 124 \pm 15 \text{ nM/hr}$, $K_m = 0.10 \pm 0.05 \mu\text{M}$ and $K_i = 0.22 \pm .14 \mu\text{M}$. Inset: Lineweaver-Burke plot of the effect of $0.068 \mu\text{M}$ ($\blacktriangle$), $0.32 \mu\text{M}$ ($\bullet$), $0.57 \mu\text{M}$ ($\blacksquare$) and $1.1 \mu\text{M}$ ($\blacklozenge$) proFIX19 on carboxylation of FIXproGLA41. Lines were drawn using the equation for linear competitive inhibition with $V_{\text{max}} = 171 \pm 16 \text{ nM/hr}$, $K_m = 0.16 \pm 0.05 \mu\text{M}$ and $K_i = 0.19 \pm 0.04 \mu\text{M}$.

Figure 2: Fully Carboxylated Factor IX with the Propeptide Still Attached Inhibits FIXproGLA46 Carboxylation. The effect of free propeptide ($\blacktriangle$), proFIX(GLA) ($\blacksquare$), and carboxylated FIX ($\bullet$) on FIXproGLA46 carboxylation was examined at 17°C for 8 hr with 10 nM purified bovine carboxylase. Indicated data points are the average of at least two independent experiments and the error bars indicate the standard deviation. Lines were drawn with the equation for competitive inhibition with $K_m = 0.21 \pm 0.14$ and $K_i = 0.29 \mu\text{M} \pm 0.03$ (propeptide) or $0.40 \mu\text{M} \pm 0.07$ (proFIX(GLA)).

Table 2

Kinetic Constants for $^{14}\text{CO}_2$ Incorporation for Peptide Substrates
with Purified Bovine Carboxylase

Substrate ^a	$K_m^{\text{app}} \pm \text{SD}^b$ (μM)	$V_{\text{max}} \pm \text{SD}^b$ (nM/h)
FIXproGLA41	0.19 ± 0.10	173 $\pm$ 45.3
FIXproGLA46	0.27 ± 0.09	191 $\pm$ 28.5
ProFIX	0.36 ± 0.04	169 $\pm$ 15.0
FIXproNonGLA	0.46 ± 0.18	63 $\pm$ 27.6

^a Reactions were performed as detailed in methods using at least 10 concentrations of each substrate. Kinetic parameters were determined by fitting the data to the Michaelis-Menten equation by non-linear regression.

^b Values are $\pm$ standard deviation, obtained from at least three independent determinations.

Kinetics of factor IX based peptides with purified bovine carboxylase

As shown in table 2, peptides with an attached propeptide: FIXproGLA41, FIXproGLA46 and proFIX demonstrate similar apparent K_m values (0.2 to 0.4 μM) which are ~ 6000 fold lower than the K_m observed for the pentapeptide substrate FLEEL. The proFIX peptide is the full length uncarboxylated factor IX with the propeptide still attached and should be analogous to the native *in vivo* substrate of the carboxylase. Therefore the similar K_m values and carboxylation rates of the propeptide/gla-domain peptides to the full length factor IX molecule suggest that in our *in vitro* system these peptides are good models for the physiological substrate of the carboxylase and elements beyond the propeptide and gla-domain are not required for carboxylation.

To examine whether the propeptide alone is adequate to direct carboxylation of a glutamate-containing substrate, we expressed in *E. Coli* a peptide which contains the FIX propeptide sequence followed by a non-gla-domain sequence. This sequence (Table 1), FIXpro-NonGLA, although containing five glutamic-acid residues, lacks the highly conserved sequences characteristic of the gla-domains of the vitamin K-dependent blood proteins. As can be seen in table 2, FIXpro-NonGLA is carboxylated with a K_m of 0.46 μM $\pm$ 0.18 which is similar to the range observed for the factor IX

propeptide/gla-domain peptides. The observed V_{max} for the FIXpro-NonGLA peptide is approximately three-fold lower than that observed for the FIX propeptide/gla-domain peptides.

Extent of carboxylation of the FIXpro-NonGLA peptide

To ascertain if the propeptide alone is able to direct multiple carboxylations of an attached substrate containing glutamates, we determined the extent of carboxylation of the FIXpro-NonGLA peptide. FIXpro-NonGLA was carboxylated in a preparative reaction with a large substrate excess over the enzyme, extracted by batch absorption, and elution from SP-sepharose. The peptide was isolated by reverse phase HPLC and all the fractions containing ^{14}C label were pooled. Online mass spectrometry of the labeled fraction is presented in figure 3 and shows a distribution of carboxylation states from 0 to 4 carboxylations. The uncarboxylated FIXpro-NonGLA is present as two roughly equal mass forms corresponding to the homoserine lactone at the C-terminal residue (5920.6 daltons) or the free homoserine form (5938.5 daltons). Each carboxylation increases the mass 45.4 ± 0.2 daltons as demonstrated previously²⁵. Doublets corresponding to each carboxylation state of both the homoserine lactone and homoserine isomer are observed. We were unable to identify peak(s) conforming to the expected mass of the 5 carboxylation state. The major product of the reaction is the uncarboxylated form consisting of >75% of the total peptide. The one, two, and three carboxylation states are present at ~5% each and the four carboxylation states comprises 10% of the total peptide.

The observed distribution of carboxylation states is consistent with a processive mechanism in which multiple carboxylations are catalyzed in a single binding event. The majority of the FIXpro-NonGLA peptide is uncarboxylated, while the fraction of peptide that is carboxylated appears to be multiply carboxylated with the 4 carboxylation state being the major form. Although other less likely formal mechanisms could give rise to the carboxylation profile we observe, the FIXpro-NonGLA carboxylation profile is consistent with a processive mechanism. This profile is also consistent with that shown in a previous study which demonstrated that the

FIXproGLA41 peptide is processively carboxylated²⁵. Therefore, the propeptide alone is able to confer processive carboxylation of an attached substrate that lacks the highly conserved gla-domain sequences found in the vitamin K-dependent blood coagulation proteins.

Figure 3: Mass Spectroscopic Analysis of Preparative FIXproNonGLA Carboxylation Reaction. Labeled fractions from preparative reaction and purified by reverse phase HPLC were analyzed by online mass-spectrometry as described in methods. The numbers above each peak indicate the number of carboxylations required to yield the observed molecular mass of the homoserine lactone (a) and homoserine form (b). The relative intensity for the uncarboxylated homoserine lactone form (0a) was 89% and the homoserine form (0b) was 100%.

Kinetics of Carboxylation of gla-domains of vitamin K-dependent proteins

In contrast to the peptides with an attached propeptide, we find that the gla-domain of factor IX (FIXGLA46) alone is a poor substrate for the carboxylase. For example, in the presence of 20 μ M of factor IX free propeptide, we estimate its K_m to be $140 \pm 32 \mu$ M

(Table 3) as compared to 0.2 μM for FIXproGLA46. Because of solubility problems, the highest concentration of substrate that could be achieved was 250 μM which is two-fold greater than its apparent K_m .

Table 3

Kinetic Constants for $^{14}\text{CO}_2$ Incorporation for Gla-domain Peptide Substrates
with Purified Bovine Carboxylase

Substrate ^a	$K_m^{\text{app}} \pm \text{SD}^b$ (μM)	$V_{\text{max}} \pm \text{SD}^b$ (nM/h)
FLEEL	1190 $\pm$ 300	14700 $\pm$ 1810
Osteocalcin	0.608 $\pm$ 0.07	3610 $\pm$ 82.6
FIXGLA46	$\geq 140 \pm 32$	3030 $\pm$ 202

^a Reactions were performed as detailed under Experimental Procedures using at least 8 concentrations of each substrate. Kinetic parameters were determined by fitting the data to the Michaelis-Menten equation by non-linear regression. The highest concentration of substrate used for FIXGLA46 was 250 μM , so the value obtained is a lower estimate, therefore the real value may be higher.

^b Values are $\pm$ standard deviation, obtained from at least three independent determinations.

Therefore, the actual K_m may be somewhat higher. Nevertheless, the apparent affinity of the FIXGLA46 peptide is clearly weaker (~500 fold) than that observed for the corresponding peptide with an attached propeptide (FIXproGLA46). We also compared the carboxylation of the FIXGLA46 peptide to that of the pentapeptide substrate FLEEL and the uncarboxylated mature form of osteocalcin. The apparent K_m for the small glutamate substrate FLEEL is 1190 $\mu\text{M} \pm 300 \mu\text{M}$, similar to previously reported values using purified carboxylase (4). The uncarboxylated osteocalcin has a $K_m = 0.61 \mu\text{M} \pm 0.07 \mu\text{M}$ (Table 3), nearly identical to the value obtained by Benton et al.²⁸. Therefore the apparent K_m for the FIXGLA46 peptide is at least 230-fold higher than observed for uncarboxylated osteocalcin and is closer to the range observed for small glutamate substrates.

Discussion

Our studies indicate that the propeptide is the initial binding site, confers substrate affinity, and is sufficient to direct multiple carboxylations in a single binding event (i.e. processive). In contrast to osteocalcin, an attached propeptide is required for efficient association of the gla-domain of factor IX with the carboxylase. Evidence that the initial interaction between the enzyme and substrate is mediated by the propeptide is provided by inhibition studies which indicate that free propeptide is a competitive inhibitor of FIXproGLA41 carboxylation. In addition, carboxylated full length factor IX with the propeptide still attached inhibits carboxylation of FIXproGLA41 with a K_i similar to free propeptide. Therefore the carboxylation state of the gla-domain do not appear to affect binding of the substrate to the carboxylase through the propeptide. Further evidence for the importance of the propeptide is provided by experiments which show that substrates with a covalently-linked propeptide have similar apparent affinities for the carboxylase. The K_m values are in the 0.2 to 0.5 μM range whether the propeptide is followed by just the gla-domain (FIXproGLA41), the gla-domain and hydrophobic stack domain (FIXproGLA46), the rest of the factor IX molecule (proFIX), or a non-gla-domain sequence containing glutamic acid residues (FIXproNon-GLA). Therefore, regions beyond the propeptide do not affect the apparent binding affinity of the carboxylase for its substrate.

An amino-terminal propeptide alone is also able to direct processive carboxylations of an attached glutamate containing region without the requirement for the highly conserved sequences present in the gla-domains of the vitamin K-dependent blood proteins. This is demonstrated by the carboxylation studies of the FIXproNon-GLA peptide, which contains the factor IX propeptide but not the highly conserved gla-domain sequences characteristic of the vitamin K-dependent proteins. The pattern of carboxylation of FIXproNon-GLA peptide is consistent with the processive mechanism previously observed for the factor IX propeptide/gla-domain peptide ²⁵. *In vivo*, processivity would be expected to be highly efficient, with molecules undergoing carboxylation being fully carboxylated. With FIXproNon-GLA, we find that 40% of the peptide that undergoes carboxylation is fully carboxylated, similar to the fraction fully

carboxylated with the FIXproGLA41²⁵. Thus, the FIXproNon-GLA peptide is processively carboxylated in a manner similar to the factor IX propeptide gla-domain peptide, indicating that the gla-domain sequences are not required for processivity. Therefore the binding affinity of the propeptide and the propeptide's ability to activate the carboxylase are sufficient to allow multiple carboxylations of factor IX during one binding event.

The reduced rate of carboxylation of FIXproNon-GLA is also likely a consequence of a processive mechanism of carboxylation. For processivity to occur, the rate of release of the substrate must be slower than the rate of carboxylations. For example, if propeptide dissociation were rate limiting, the observed rate of carboxylation would be proportional to the number of glutamates that can be carboxylated in a single enzyme turnover. Thus, a substrate with fewer than twelve carboxylation sites would undergo fewer carboxylations in a single enzyme turnover, and would appear to be carboxylated at a proportionally slower rate. This is consistent with our observations of the ~3-fold lower $V_{\max}$ for FIXproNon-GLA peptide compared to FIXproGLA41. Recently, it has been shown that a chimera containing the prothrombin propeptide and a normally uncarboxylated glutamate rich region of thrombin is efficiently carboxylated in mammalian cell culture³¹. This finding, along with our studies, indicates that both *in vitro* and *in vivo*, the propeptide alone can direct multiple carboxylations in an attached region containing glutamates.

The sequences of the gla-domains of the vitamin K-dependent proteins are highly conserved, therefore it seems plausible that they play an important role in carboxylation. For example, a conserved sequence (Glu-X-X-X-Glu-X-Cys) has been identified within the gla-domains of all known vitamin K-dependent proteins¹¹. In addition, the widely used substrate FLEEL was derived from a subset of gla-domain sequences. Although decarboxylated prothrombin was reported to be a poor substrate for the carboxylase³², several studies have shown that osteocalcin without an attached propeptide is an excellent substrate^{26,27}. Moreover, in the presence of free factor X propeptide, osteocalcin with a covalently linked propeptide has only a modestly lower

K_m than osteocalcin without an attached propeptide²⁸. Therefore we examined whether the gla-domain of factor IX has a similar recognition site for the carboxylase.

Our results indicate that the gla-domain alone of factor IX has a poor apparent affinity for carboxylase and that an attached propeptide is required for high affinity binding of factor IX. The K_m for the FIXproGLA46 is 500-fold lower than observed for the gla-domain alone (FIXGLA46), whereas the K_m for osteocalcin without an attached propeptide is in the same range as covalently linked propeptide substrates. The propeptide of osteocalcin contains a glycine at the highly conserved -10 position, which is alanine in all other known vitamin K-dependent proteins. It has been shown that substitution of glycine for alanine at the -10 position increases the K_m of the FIXproGLA41 peptide 30-fold¹⁶; therefore the osteocalcin propeptide may possess a significantly weaker affinity for the carboxylase than the factor IX propeptide. Consequently, osteocalcin and factor IX may have evolved different binding sites which confer substrate recognition and may be carboxylated by distinct mechanisms. Further studies will be required to verify this distinction and understand its physiological significance.

Binding of physiologically relevant substrates for the vitamin K-dependent carboxylase proceeds through a multi-step pathway. With factor IX, the initial binding step is through the propeptide which localizes the glutamates-to-be-carboxylated in the vicinity of the active site. Subsequent interactions are then required between the glutamates-to-be-carboxylated and the active site. Our studies with FIXproNon-GLA indicate that the position of the glutamates or the gla-domain structure is not required for multiple processive carboxylations, but we can not rule out the possibility that the characteristic gla-domain structure may direct the specificity or enhance the efficiency of carboxylation. The gla-domain sequences may play a role in determining which glutamates are carboxylated. For example, residues immediately surrounding a glutamate-to-be-carboxylated may influence its affinity for the active site once it is localized there by binding at the propeptide. Evidence for this is seen in the effect of adjacent amino acids on the activities of small glutamate containing peptides³³ and with partially or uncarboxylated glutamates in osteocalcin and matrix gla protein³⁴. The

domain structures of all the vitamin K-dependent blood proteins are highly similar; therefore, even in the uncarboxylated partially unfolded state, these domains may possess secondary structure that localizes all the glutamates-to-be-carboxylated proximal to the active site. Spatial orientation and physical distance of glutamates-to-be-carboxylated from the active site may also play a pivotal role in whether a particular glutamate is modified. With FIXproNon-GLA, only four of the five glutamates are carboxylated, therefore absence of structure that localizes one of the glutamates proximal to the active site or adjacent amino acids that influence a particular glutamate's affinity for the active site may prevent the fifth modification. The glutamates in the gla-domains of the vitamin K-dependent proteins may be ideally positioned for modification or carboxylation could induce a structural change which enhances subsequent modifications (i.e. cooperativity). Alternatively, native substrates may have an active release mechanism in which the accumulation of negative charge or carboxylation itself could cause a structural change in the gla-domain that signals that carboxylation is complete and directs product release. In such circumstances, the carboxylated product would be expected to have a weaker affinity for the enzyme than free propeptide or the uncarboxylated substrate. This is not observed with proFIX(GLA) in our *in vitro* system, which suggests that completion of carboxylation itself is not a release mechanism. We can not rule out the possibility that other accessory proteins absent from our *in vitro* assays may be critical for signaling the completion of carboxylation *in vivo*.

In conclusion, this work demonstrates that with an *in vitro* system using purified components, peptides containing the propeptide and gla-domain are good models for the physiological substrate in carboxylation by the vitamin K-dependent carboxylase. The initial interaction of the vitamin K-dependent carboxylase with its substrate is through the propeptide which confers the perceived affinity of the enzyme for its substrate without apparent influence of the gla-domain or regions beyond the gla-domain. Binding through the propeptide is also adequate to direct multiple carboxylations in a single binding event without the requirement for the highly conserved sequences in the gla-domains of the vitamin K-dependent proteins. Further

studies will be required to understand steps beyond the initial interaction of the propeptide with the carboxylase such as the interaction of the glutamates with the active site and product release and dissociation steps in the mechanism of efficient and processive carboxylation by the vitamin K-dependent carboxylase.

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Chapter 4

OSTEOCALCIN BINDS TIGHTLY TO THE GAMMA-GLUTAMYL CARBOXYLASE AT A SITE DISTINCT FROM THAT OF THE OTHER KNOWN VITAMIN K-DEPENDENT PROTEINS

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Abstract

Vitamin K-dependent proteins contain a pro-peptide which is required for recognition by the enzyme γ -glutamylcarboxylase. Substrates used for *in vitro* carboxylation studies lacking a pro-sequence are characterized by K_m values in the millimolar range, whereas the K_m for peptides containing a pro-sequence is 3-4 orders of magnitude lower. Here we report that descarboxy-osteocalcin is an exception in this respect. Using descarboxy-osteocalcin in purified propeptide-free recombinant carboxylase, the K_m was found to be 1.8 μM . Furthermore, osteocalcin was an inhibitor of descarboxy-osteocalcin carboxylation with a K_i of 76 μM . In contrast to the other vitamin K-dependent proteins, free propeptides do not inhibit descarboxy-osteocalcin carboxylation. Moreover, propeptide-containing substrates were neither inhibited by osteocalcin nor by its propeptide. From our studies we conclude that descarboxy-osteocalcin must have an internal recognition sequence which binds to γ -glutamylcarboxylase at a site different from the propeptide-recognition site.

Introduction

Osteocalcin, also called bone 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 weight of 5700 Da ^{3,4}. During its biosynthesis osteocalcin undergoes a series of post translational modifications including the conversion of three glutamate residues (Glu) into γ -carboxyglutamate (Gla) residues. In this process vitamin K serves as a co-enzyme 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 (osteocalcin and matrix Gla protein, MGP) ^{8,9}, and the growth arrest-specific gene 6 protein (Gas6) ¹⁰.

Except MGP, which has an internal recognition site for carboxylase, all Gla-proteins characterized thus far are synthesized as preproteins 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 a 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. Using *in vitro* carboxylating systems it was shown that small synthetic peptides lacking the propeptide (e.g. the pentapeptide 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 pro-sequence of either prothrombin or factor IX were 3-4 orders of magnitude lower ¹⁷. Also, the binding of pro-containing substrates lead to a 10-20 fold decrease of the K_m for the cofactor vitamin K hydroquinone, suggesting an allosteric effect of the pro-domain upon 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 (<10-fold) of the K_m

for small peptide substrates ¹⁹.

During recent years it has been shown that heat-decarboxylated bovine osteocalcin may serve as a substrate for bovine liver carboxylase ^{11,20}. The protein is exceptional because - despite its lack of a pro-domain - reported K_m values are comparable with those of pro-containing substrates ^{21,22}. Because heat decarboxylation may cause unwanted and undefined damage to the protein molecule, we have prepared synthetic human osteocalcin, both in its carboxylated and non-carboxylated (descarboxy) form. The preparations will be designated here as OC and d-OC, respectively. In this paper we have compared the substrate properties of d-OC with those of well-known other substrates for carboxylase, and we have tested the extent to which OC is capable of inhibiting the carboxylation of various substrates. Finally we have investigated whether the low K_m values for d-OC are the result of an unusual high affinity of its Gla-domain for the carboxylase active site or, alternatively, of an internal docking sequence located elsewhere in the molecule.

Materials and Methods

Reagents

All chemicals are of analytical grade or better. Vitamin K₁ (Konakion[®], mixed - micelles) was purchased from Hoffmann-La Roche (Basel, Switzerland), NaH¹⁴CO₃ with specific activity of 50 mCi/mmol was from New England Nuclear (Boston, MA) and CHAPS was from Sigma (St. Louis, MO). The insect cell expression vector (pVL1392) was purchased from Pharmingen (San Diego, CA) and the baculovirus viral DNA (BacVector 3000) was purchased from Novagen (Madison, WI). The Sf9 cells (*Spodoptera frugiperda*) were obtained from the Lineburger Cancer Center at the University of North Carolina-Chapel Hill, and the High Five cells (*Trichoplusia ni*) were a gift from Dr. Thomas Kost of Glaxo-Wellcome (Research Triangle Park, NC). The HPC4 antibody affinity resin was provided by Dr. Charles T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). The FLAG antibody and FLAG peptide standards were purchased from Sigma (St. Louis, MO).

Peptides

OC¹⁻¹⁶, OC¹³⁻²⁵, OC²²⁻³¹, OC²⁹⁻⁴³, d-OC¹³⁻²⁵ and d-OC²²⁻³¹ as well as Pro^{FIX} and Pro^{OC} were synthesized by Pepscan Systems (Lelystad, The Netherlands). Both FLEEL and bEELoMe were purchased from Bachem (Bubendorf, Switzerland). Recombinant ProIX-59 Q/S was produced and purified as described previously¹⁷. ProPT(Glu)₁₀ and 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.

Table 1

Sequences of Various Peptide Substrates and Inhibitors

[illegible]

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.

Synthesis of OC and d-OC

Mature 49 amino acid osteocalcin (OC) and descarboxy-osteocalcin (d-OC) (table 1) were synthesized by solid-phase peptide synthesis using amino acids with Fmoc-protected α -amino groups on a 431A peptide synthesizer (Perkin Elmer, Foster City, CA) using standard FastMoc programs. The side chain protecting groups used were trityl for Asn, Cys, Gln and His, *tert*-butyl for Tyr, 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl for Arg and *tert*-butyl ester for Asp, Glu and Glu. All reagents and amino acids, except for the protected amino acid Glu (Bachem, Switzerland), were peptide synthesis grade from Perkin Elmer. After each coupling step, the free amino termini of non reacted peptides were capped by acetic anhydride to avoid 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 μ l ethanedithiol, 500 μ l thioanisole, 500 μ l bidistilled 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 *tert*-butyl ether. The peptide was washed with methyl *tert*-butyl ether, dissolved in water and dried. The amino acid sequence of the synthetic proteins was confirmed by Edman degradation on a 477A/120A protein sequencer (Perkin Elmer, Foster City, CA).

Mass spectrometry of OC and d-OC

To verify purity and the full carboxylation at positions 17, 21 and 24 of the synthetic OC and the purity of d-OC, electron spray ionization (ESI) mass spectrometry (MS) was performed on a Micromass Platform II (Micromass UK Ltd., Altrincham, Cheshire, UK) single quadrupole bench-top 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 to 3000 in 2 seconds 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 peptides. Instrumental control, data acquisition and data processing were carried out using the MassLynx software package (version

2.3). The observed average m/z of OC of 5930.85 amu was compared with the calculated MH^+ average mass of 5931.55 amu. Also the observed average m/z of d-OC of 5800.05 amu was compared with its calculated MH^+ average mass of 5799.45 amu. The calculated masses of both OC and d-OC (with a free N-terminus and a carboxyl group at the C-terminus) did not differ significantly from the measured masses and we therefore conclude (I) that the OC is pure and fully carboxylated and (II) that d-OC is pure and not fragmented.

Expression of r-carboxylase in Trichoplusia ni High-Five insect cells

The cDNA encoding the human vitamin K-dependent carboxylase was sub-cloned into the EcoRI site of the pVL1392 vector. The sequence coding for the FLAG antibody epitope (DYKDDDDK) was introduced at the N-terminal end of the carboxylase and a HPC4 antibody tag (EDQVDPRLIDGK)²³ was added at the C-terminal end. The engineered vector was cotransfected with baculovirus BacVector 3000 triple cut virus DNA into Sf9 cells. Recombinant virus was isolated by plaque purification, amplified, and titered by plaque assay according to the manufacturer's instructions (Pharmingen, San Diego). Expression of carboxylase was done by infection of $\sim 2 \times 10^6$ /ml High Five Cells with the recombinant virus at a multiplicity of infection of ~ 1 . Cells were collected after 48 h by centrifugation and 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 cocktail²⁴ consisting of EDTA (2 mM), DTT (2 mM), Phe-Pro-Arg-chloromethyl ketone (0.125 $\mu\text{g/ml}$), Phe-Phe-Arg-chloromethyl ketone (0.125 $\mu\text{g/ml}$), leupeptin (0.5 $\mu\text{g/ml}$), pepstatin (0.7 $\mu\text{g/ml}$), PMSF (34 $\mu\text{g/ml}$), aprotinin (2 $\mu\text{g/ml}$) and 10% glycerol and resuspended in 50 ml of buffer A. The sample was homogenized by 5 strokes with a dounce homogenizer and then sonicated with twenty 5 second pulses and an interval time of 25 seconds using a 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 hr. The microsomal pellet was resuspended in buffer A with a final protein concentration of 20 mg/ml, 1x protease inhibitor cocktail and 10% glycerol, aliquoted and stored at - 80 °C until further use.

Purification of γ -carboxylase using 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 pH 7.4, 0.15 M NaCl, 1% CHAPS/ 0.2% phosphatidylcholine, 10% glycerol and 1x protease inhibitor cocktail) at 4°C for 1 hr. The solubilized microsomes were centrifuged at 105000g for 1 hr and the pellet was discarded. A total of 10 ml of HPC4 resin was equilibrated with wash buffer (20 mM TRIS (pH 7.4), 150 mM NaCl, 0.5% CHAPS/0.2% phosphatidylcholine, 1x protease inhibitor cocktail) and added to the solubilization supernatant along with a final concentration of 5 mM CaCl_2 and 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, aliquoted and stored at -80 °C. Enzyme concentration was estimated from dot blots of carboxylase 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 -glutamyl carboxylase, 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2 % CHAPS, 0.2 % phosphatidyl choline, 5 μCi $\text{NaH}^{14}\text{CO}_3$, 222 μM of vitamin K hydroquinone (KH_2) and 5 mM DTT. Substrates were added as indicated. The mixture was incubated at 20 °C during 30 minutes in sealed tubes. Kinetic analysis was performed on subsamples drawn at $t=0$, $t=15$, and $t=30$ min. All reactions were stopped by adding 0.8 ml of 5 % trichloroacetic acid to 100 μl of reaction mixture and unbound $^{14}\text{CO}_2$ was removed by boiling for 3 minutes. After cooling down 5 ml of Formula 989 (Packard Bioscience, Groningen, The Netherlands) was added. Finally samples were counted using a Wallac 1414 WinSpectral liquid scintillation counter (EG&G Wallac, Turku, Finland). Data are expressed as pmol $^{14}\text{CO}_2$ incorporated per minute.

Results

Kinetic constants for d-OC carboxylation

Kinetic constants of various osteocalcin-derived substrates were compared with other well known substrates for carboxylase (table 2). Substrates containing a propeptide sequence were characterized by K_m values ranging between 0.4 and 5 μM , whereas the K_m for pro-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-OC¹³⁻²⁵ was a poor substrate for carboxylase with a K_m 3 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 enabling its efficient carboxylation. Since this recognition site was not present in d-OC¹³⁻²⁵ (containing the entire osteocalcin Gla-domain) it seems that a sequence outside its Gla-domain contributes to the recognition of mature osteocalcin by carboxylase. We have also tested d-OC²²⁻³¹ for its ability to serve as a substrate for carboxylase.

Table 2

Kinetic Constants for Different Peptide Substrates and the Cofactor KH_2 During Carboxylation

Substrate ^a	$K_m \pm \text{SD}^b$	$V_{\text{max}} \pm \text{SD}^b$ ($\text{pmol } ^{14}\text{CO}_2 \cdot \text{hr}^{-1}$)	$K_m \text{ for } \text{KH}_2 \pm \text{SD}^b$ (μM)
ProIX-59 Q/S	$0.51 \pm 0.09 \mu\text{M}$	90 ± 13	5 ± 0.5
ProPT-28 (F/A)	$5.35 \pm 0.77 \mu\text{M}$	183 ± 18	3 ± 0.4
Pro(Glu) ₁₀	$0.56 \pm 0.18 \mu\text{M}$	119 ± 15	9 ± 1.1
d-OC	$1.71 \pm 0.35 \mu\text{M}$	287 ± 17	13 ± 0.9
d-OC ¹³⁻²⁵	$9.1 \pm 2.05 \text{ mM}$	470 ± 57	61 ± 3.4
Boc-EEL-OMe	$2.0 \pm 0.1 \text{ mM}$	182 ± 11	39 ± 2.3
FLEEL	$2.4 \pm 0.2 \text{ mM}$	401 ± 32	34 ± 1.7

^a Reactions were performed as detailed under Experimental procedures. Kinetic parameters were determined by fitting the data to the Michaelis-Menten equation.

^b Values are means $\pm$ standard deviation, obtained from at least 3 independent determinations.

However, this peptide was not measurably carboxylated, nor was carboxylation observed when OC¹⁻¹⁶ was added (data 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 upon binding of propeptide-containing substrates¹⁸. 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 which is 4 times higher than that in the case of ProIX-59 Q/S carboxylation, but almost 5 times lower than that of d-OC¹³⁻²⁵ carboxylation.

Inhibitory effect of OC on d-OC and bEELoMe carboxylation

Because of the high degree of sequence homology of d-OC and OC, it seemed at least feasible that both molecules have affinity for carboxylase. In that case OC would be expected to act as an inhibitor of d-OC carboxylation.

Figure 1: Effect of OC on the carboxylation of different substrates. All incubations were at 20 °C for 30 min. All values are means from at least three independent measurements, and are expressed as a percentage of the non-inhibited reaction rate. Explanation of symbols: (□), carboxylation of 2 μM d-OC; (●), of 1 μM of ProIX-59 Q/S; (▲), of 5 mM bEELoMe at increasing amounts of OC. If error bars are not visible they lie within the size of the symbol used.

Therefore, we have investigated the potential inhibitory effect of OC on the carboxylation of various substrates. As is shown in figure 1, the carboxylation of 1 μM of the propeptide-containing ProIX-59 Q/S was not inhibited, even at 200 fold molar excess of OC. d-OC carboxylation was inhibited with a K_i of 76 μM (about 45 times the K_m for d-OC), and bEELoMe with a K_i of 104 μM (about 0.05 times 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 homology to a similar extent. In an attempt to locate more precisely the sequence responsible for the high affinity of d-OC for carboxylase, we have tested the potential inhibitory effects of a number of peptides derived from OC: OC¹⁻¹⁶, OC¹³⁻²⁵, OC²²⁻³¹, and OC²⁹⁻⁴³. However, neither alone nor in combination any of these peptides inhibited d-OC carboxylation.

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

As was previously shown²⁵, the presence of free propeptide stimulates *in vitro* substrate carboxylation by lowering the K_m for substrates derived from blood clotting factors. We have investigated whether propeptides have a similar effect on substrates derived from osteocalcin. Also, we have tested to what extent free propeptides may act as competitive inhibitors for pro-containing substrates during substrate recognition by carboxylase. Propeptides used for these studies were those derived from human factor IX (Pro^{FIX}) and human osteocalcin (Pro^{OC}), and we have 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 pro-containing ProIX-59 Q/S, and that it mildly stimulated the propeptide-deficient bEELoMe. Its effects on osteocalcin-derived substrates were negligible. In contrast, Pro^{OC} did not affect the carboxylation of either substrate. At increasing Pro^{FIX} concentrations, a dose-dependent inhibition with a K_i of 0.8 μM of ProIX-59 Q/S was observed (fig. 2), whereas even at 200 μM of Pro^{FIX} the carboxylation of d-OC remained unaffected. On the other hand, Pro^{OC} did not inhibit either substrate even

at very high concentrations. In our opinion it seems unlikely that an attached propeptide lowers the K_m for osteocalcin even further. Data reported by Benton et al. showed only a 3-fold difference between propeptide- lacking and propeptide-containing descarboxy osteocalcin (0.72 μM versus 0.24 μM respectively) ²⁰. Comparison of these data with ours is hampered by the fact that the authors have studied the kinetic parameters in purified bovine carboxylase which contained 100 μM of free human factor X propeptide.

Table 3

Effect of Propeptides on K_m^{app} for Various Substrates in the Vitamin K-Dependent Carboxylation Reaction

Substrate ^a	$K_m \pm \text{SD}^b$	$K_m \pm \text{SD}^b$	$K_m \pm \text{SD}^b$
	No Addition	+ 20 μM Pro ^{FIX}	+ 20 μM Pro ^{OC}
d - OC	1.7 $\pm$ 0.35 μM	1.9 $\pm$ 0.24 μM	1.8 $\pm$ 0.37 μM
d - OC ¹³⁻²⁵	9.1 $\pm$ 2.05 mM	4 $\pm$ 0.90 mM	10 $\pm$ 1.90 mM
ProIX-59 Q/S	0.5 $\pm$ 0.10 μM	2.6 $\pm$ 0.35 μM	0.4 $\pm$ 0.26 μM
bEELoMe	2.1 $\pm$ 0.10 mM	0.6 $\pm$ 0.40 mM	2.2 $\pm$ 0.80 mM

^a Reactions were performed as detailed under Experimental procedures. Kinetic parameters were determined by fitting the data to the Michaelis-Menten equation.

^b Values are means $\pm$ standard deviation, obtained from at least 3 independent determinations.

Note that values for d-OC and ProIX-59 Q/S are in μM , those for d-OC¹³⁻²⁵ and bEELoMe are expressed in mM. For abbreviations see legend to table 1.

From these data we conclude (I) that the affinity of carboxylase for Pro^{OC} must be substantially lower than that of Pro^{FIX}, and (II) that carboxylase must have a high affinity site for d-OC which differs from the propeptide recognition site.

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 from at least three independent measurements, and are expressed as a percentage of the non-inhibited reaction rate.

Discussion

Decarboxylated bovine osteocalcin has been reported to serve as a substrate for crude microsomal carboxylase^{11,20,26} as well as for affinity-purified carboxylase²⁷. Although osteocalcin lacks an N-terminal propeptide, reported K_m values were in the micromolar range, which is characteristic for substrates containing the pro-sequence. Whereas bovine liver microsomal carboxylase may be contaminated with pro-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 osteocalcin carboxylation, and 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 pro-containing proteins. Synthetic osteocalcin and derived peptides were the substrates of choice in this system rather than thermally decarboxylated natural osteocalcin, because the heat treatment may induce structural changes or (partial) degradation.

Using this system we have demonstrated for the first time that, in the absence of propeptide, d-OC is a good substrate for carboxylase, with a K_m of 1.7 μM . This is in the range of substrates containing the pro-sequence (0.5 - 5.3 μM), and widely different from substrates lacking a pro-sequence (2 - 9 mM). The question remains which structural determinants of osteocalcin contribute to its recognition by carboxylase. It was shown that OC acts as an inhibitor of d-OC carboxylation, suggesting that elements of recognition are shared by both substrate and product. Shorter sequences in- or outside the Gla-domain were either (in their non-carboxylated form) poor substrates for carboxylase, or (in their carboxylated form) not inhibitors of d-OC carboxylation. Moreover, OC failed to inhibit pro-containing substrates such as ProIX-59 Q/S. Competitive inhibition by free propeptide was demonstrated between Pro^{IX} and ProIX-59 Q/S, but neither Pro^{IX} nor Pro^{OC} inhibited d-OC carboxylation. The fact that Pro^{OC} did not inhibit ProIX-59 Q/S carboxylation is consistent with its reported low affinity for carboxylase²⁸. Our data suggest that at least two different docking sites are located on carboxylase: one for pro-containing substrates, and a second one for d-OC. Whereas there seems to be no clear role in enzyme-substrate recognition for the pro-sequence in osteocalcin precursor proteins, osteocalcin itself may contain an internal recognition site for carboxylase. This is also seen in matrix Gla-protein (MGP), another Gla-protein synthesized in bone. Because osteocalcin and MGP have significant sequence similarity, it has been suggested that both proteins have developed from a common ancestor protein^{29,30}. However no sequence homology between the internal propeptide of MGP and mature osteocalcin is seen and therefore we suggest the presence of a different binding site on carboxylase.

Besides serving as an element of recognition for the docking site on carboxylase, it has been previously shown that the pro-sequence in pro-containing substrates possess the remarkable property of decreasing the K_m for KH₂ by 10-20

fold¹⁸. An explanation for this phenomenon may be found in postulating an allosteric conformational change of carboxylase upon binding of the pro-sequence. This effect is also evident from table 2, with K_m values for KH_2 ranging from 3-9 μM for carboxylation of pro-containing substrates, and from 34-61 μM for carboxylation of short peptides. The K_m of 13 μM for KH_2 at d-OC carboxylation is close to the range observed for carboxylation of pro-containing substrates, and demonstrates that the presence of the pro-sequence is not an absolute requirement to increase the affinity between carboxylase and its cofactor KH_2 . It cannot be excluded at this time that the pro-sequence in osteocalcin precursors is to induce a more pronounced interaction between carboxylase and KH_2 thus lowering the K_m for KH_2 to the same values as those observed for the other pro-containing substrates. Definite proof of this hypothesis might be obtained from a pro-sequence containing osteocalcin molecule. However, the long-chain synthesis by Fmoc chemistry with high efficiency is limited to about 50 residues in our hands. Only a few successful long-chain syntheses have been reported uptill now using the linear Fmoc protocol³¹⁻³⁴, indicating the problems accompanying the synthesis of these long peptide chains (> 50 residues).

In conclusion, we have demonstrated that descarboxy-osteocalcin binds with high affinity to carboxylase, and that the osteocalcin pro-sequence does not play a noticeable role in the enzyme-substrate interaction. Moreover, the data presented strongly suggest the presence of two substrate docking sites on carboxylase: one recognizing the pro-sequence in clotting factor-type substrates, and a second one with high affinity for osteocalcin precursors.

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Chapter 5

OSTEOCALCIN SPECIFIC BINDING SITE ON THE VITAMIN K-DEPENDENT GAMMA-GLUTAMYL CARBOXYLASE

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Abstract

Two different sites on vitamin K-dependent carboxylase are involved in enzyme-substrate interaction: the propeptide binding site required for high affinity substrate binding, and the active site for glutamate carboxylation. Synthetic descarboxy-osteocalcin is a low K_m substrate for the vitamin K -dependent γ -glutamyl carboxylase, but unique since it does not make use of the propeptide binding site on γ -glutamylcarboxylase. With the aid of a stereospecific substrate analog we have demonstrated that the high affinity of descarboxy-osteocalcin for carboxylase can neither be explained by a direct interaction with the active site nor with the propeptide binding site on carboxylase. On the basis of these data we postulate the presence of a second high affinity substrate binding site on γ -glutamylcarboxylase capable of specifically binding descarboxy-osteocalcin.

Introduction

In vivo, all known proteins containing γ -carboxyglutamic acid (Gla) residues are synthesized in a precursor form ¹ in which a propeptide sequence preceding the Gla domain serves as a high affinity recognition site for the vitamin K-dependent γ -glutamyl carboxylase ²⁻⁴. This concept implies that at least two separate sites on carboxylase must be involved in substrate interaction: a docking site for substrate recognition and binding, as well as the active site at which glutamic acid (Glu) residues are converted into Gla residues ².

Small synthetic Glu-containing peptides lacking this propeptide sequence (e.g. Phe-Leu-Glu-Glu-Leu; FLEEL) do not bind to the docking site but diffuse directly into the active site and are poor substrates with K_m values in the millimolar range ^{5,6}. The two-site model for carboxylase turned out to be insufficient describing all enzyme-substrate interactions because descarboxy-osteocalcin (d-OC) binds to carboxylase with high affinity without the requirement of a pro-sequence. In previous experiments we have shown that d-OC does not bind to the propeptide binding site on carboxylase, and that its propeptide does not contribute to the binding of d-OC to carboxylase. Based on these observations we have postulated a second substrate docking site on carboxylase ⁷. It could not be excluded, however, that the active site itself functions as the high affinity docking site for d-OC.

To further investigate this potential osteocalcin binding site on carboxylase we have compared the kinetic characteristics of a tripeptide substrate, a pro-containing substrate and d-OC in the absence and presence of the methylated tripeptide (S-MeTPT), a stereospecific substrate analog, acting as an active site inhibitor ⁸.

Materials and Methods

Reagents

All chemicals were of analytical grade or better. Vitamin K₁ (Konakion®, mixed-micelles) was purchased from Hoffmann - La Roche (Basel, Switzerland), NaH¹⁴CO₃ with a specific activity of 50 mCi/mmol was from NEN (Boston, MA) and CHAPS {3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate} was from Sigma (St. Louis, MO).

Peptides and proteins

d-OC was synthesized by Dr. P Proost (The Rega Institute, Dept. of Molecular Immunology, Catholic University of Leuven, Belgium), according to Wuyts et. al. ⁹, and a kind gift of Dr. R.H.M. Ebberink (Perkin - Elmer Netherlands Ltd, Nieuwerkerk aan de IJssel, The Netherlands). The tripeptide boc-Glu-Glu-Leu-O-Me (bEELOMe) was from Bachem (Bubendorf, Switzerland). Boc-(2S,4S)-4-methylglutamic acid was synthesized according to Righini-Tapie and Azerad ¹⁰, and incorporated into Boc-(2S,4S)-4-MeGlu-Glu-Val-(S-MeTPT) via a sequential method using selectively protected γ -benzyl, α -N-hydroxysuccinimide esters ^{11,12}. ProIX-59 Q/S, a 59 amino acid peptide containing the human factor IX propeptide and the first 41 residues of factor IX Gla domain (sequence -18 to 41) with an Arg to Gln mutation at the -4 position and an Arg to Ser mutation at position -1 was produced and purified as described previously ⁴. The propeptides (sequence -18 to -1) of both the human precursor molecules of osteocalcin (Pro^{OC}) and blood coagulation factor IX (Pro^{FIX}) were purchased from Pepscan Systems (Lelystad, The Netherlands).

Carboxylase assay

Expression of r-carboxylase in *Trichoplusia ni* High-Five insect cells the preparation of microsomes and the purification of r-carboxylase using HPC4 antibody resin was performed as described ⁷. Standard reaction mixtures were used as described ⁷; 25 nmol of HPC4 purified γ -glutamyl carboxylase was used. Kinetic parameters were determined by fitting to the Michaelis-Menten equation for linear regression ¹³.

Results

Propeptide binding site inhibition

First, we have examined the importance of the propeptide sequence in substrate recognition by testing the effect of free factor IX propeptide (Pro^{FIX}) on the carboxylation of recombinant ProIX-59 Q/S. As was reported previously, ProIX-59 Q/S has a high affinity for carboxylase which makes it an efficient substrate for *in vitro* vitamin K-dependent γ -carboxylation ⁴. As shown in figure 1a, ProIX-59 Q/S carboxylation is competitively inhibited by Pro^{FIX} with a K_i of $0.97 \pm 0.19 \mu\text{M}$, which is consistent with binding of substrate and inhibitor at the same site on carboxylase. These data also suggest an initial interaction of the propeptide sequence with the carboxylase propeptide binding site rather than with the active site.

Active site inhibition

The kinetic characteristics of active site inhibition by S-MeTPT were assessed using the homologous tripeptide substrate bEELOMe. This small substrate lacks a propeptide sequence and, therefore, has a K_m in the millimolar range. S-MeTPT is known to be an effective active site inhibitor and, as shown in figure 1b, the carboxylation of bEELOMe is competitively inhibited. These data illustrate that both inhibitor and substrate act directly on the active site of carboxylase. In contrast, the inhibition of the ProIX-59 Q/S carboxylation by S-MeTPT was found to be non-competitive (figure 1c). The non-competitive nature of the active site inhibition is consistent with a critical role for the propeptide binding site, rather than for the active site, during the formation of the enzyme-substrate complex.

Finally, we have examined the inhibition of d-OC carboxylation by S-MeTPT (figure 1d) and it was found that also d-OC was non-competitively inhibited.

The various kinetic characteristics are summarized in table 1.

Table 1

Kinetic Parameters for $^{14}\text{CO}_2$ Incorporation for bEELOMe, ProlX-59 Q/S and d-OC in the Absence or Presence of S-MeTPT

Substrate ^(a)	$K_m \pm \text{SD}^{(b)}$	$V_{\max} \pm \text{SD}^{(b)}$	$K_i \pm \text{SD}^{(c)}$ (pmol $^{14}\text{CO}_2$ incorporation * min ⁻¹)
bEELOMe	1.20 $\pm$ 0.3 mM	20.4 $\pm$ 1.05	289 $\pm$ 35 μM
ProlX-59 Q/S	0.15 $\pm$ 0.09 μM	3.3 $\pm$ 0.15	493 $\pm$ 66 μM
d-OC	2.58 $\pm$ 0.74 μM	23.7 $\pm$ 1.44	491 $\pm$ 30 μM

^(a) Reactions were performed as detailed in methods using 4 concentrations of each substrate in absence of inhibitor. ^(b) Values are $\pm$ standard deviation, obtained from at least three independent determinations in absence of inhibitor. ^(c) Values are $\pm$ standard deviation, obtained from at least three independent determinations in presence of inhibitor.

Fig. 1 A

Fig. 1 B

Fig. 1 C

Fig. 1 D

Figure 1: Lineweaver-Burk diagrams of substrate carboxylation. **(A):** Inhibition of ProIX-59 Q/S by increasing amounts of free propeptide of the precursor molecule of human blood coagulation factor IX (Pro^{FIX}). Symbols used: ($\square$), 0 μM Pro^{FIX} ; ($\circ$), 2 μM Pro^{FIX} ; ($\blacksquare$), 5 μM Pro^{FIX} ; ($\bullet$), 10 μM Pro^{FIX} ; (Δ), 20 μM Pro^{FIX} . **(B):** Inhibition of bEELOMe by increasing concentrations of S-MeTPT. **(C):** Inhibition of ProIX-59 Q/S by increasing amounts of S-MeTPT. **(D):** Inhibition of d-OC by increasing amounts of S-MeTPT. Symbols used: ($\square$), 0 μM S-MeTPT; ($\circ$), 100 μM S-MeTPT; ($\blacksquare$), 200 μM S-MeTPT; ($\bullet$), 400 μM S-MeTPT.

Discussion

The propeptide sequences which can be found in all precursors of Gla-containing proteins show substantial sequence similarity; notably the aminoacid residues at -16, -10, -4 and -1 are highly conserved. Osteocalcin however, is the only known Gla-protein of which the propeptide sequence contains a glycine residue at the -10 position instead of the highly conserved alanine residue present in all other propeptides. Chu et al. have shown that an Ala⁻¹⁰ → Thr mutation in the propeptide of human coagulation factor IX leads to a 30-fold decreased affinity of the protein precursor for carboxylase *in vitro*, and hence, to a strongly increased sensitivity for warfarin *in vivo*¹⁴. Using substrates derived of ProIX-59 Q/S it was shown that an Ala⁻¹⁰ → Gly mutation in the propeptide induced a similar effect *in vitro*.

These data may explain why in previous experiments the propeptide of osteocalcin did not seem to play a role in the binding of d-OC to carboxylase⁷.

In our attempts to characterize the differences between the binding to carboxylase of propeptide-containing substrates and of d-OC we have used inhibitors interacting with the propeptide binding site or with the active site of carboxylase. It was demonstrated that the carboxylation of ProIX-59 Q/S, and more generally the carboxylation of propeptide containing substrates, is competitively inhibited by Pro^{FIX}. Since the primary structure of d-OC lacks a propeptide and has no marked sequence similarity with any of the known propeptides, it seems unlikely that the propeptide binding site on carboxylase plays a major role in the binding to d-OC. The question remains, therefore, how to explain the high affinity of d-OC for carboxylase.

In agreement with previous data from Azerad et al.¹⁵, we found that the carboxylation of the short peptide substrate bEELOMe was competitively inhibited by S-MeTPT. In contrast, inhibition of both ProIX-59 Q/S and d-OC carboxylation by S-MeTPT was non-competitive, suggesting that interaction with the active site is necessary for carboxylation, but does not play a major role in the high enzyme-substrate affinity. We therefore postulate the presence of an internal high affinity binding sequence within the mature sequence of d-OC which binds neither to the propeptide binding site nor to the active site on γ -glutamylcarboxylase. This implies

that, besides its active site, carboxylase contains at least two distinct sites for binding of different substrate types (e.g. propeptide-containing substrates and d-OC). Both the location of the d-OC binding site on carboxylase as well as the position of the high affinity binding site within d-OC remain to be established.

Though no clear function can be attributed to the osteocalcin propeptide yet, the question remains why this sequence is present during osteocalcin biosynthesis. Speculating about its possible function we would like to point out that by the binding of covalently attached propeptides, a substantial decrease in the K_m for vitamin K hydroquinone is induced ¹⁶. Obviously this decreases the requirement for vitamin K, the dietary intake of which seems to be close to the published RDA values ¹⁷. Also, the substantial under-carboxylation generally observed in human osteocalcin and its strong response to vitamin K-supplementation demonstrates that human daily vitamin K intake hardly covers the requirement for osteocalcin carboxylation ¹⁸. It is possible therefore, that propeptide of osteocalcin is necessary for decreasing the K_m for the carboxylase cofactor vitamin K hydroquinone. Whether this is really the case remains to be investigated.

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Chapter 6

THE COMPOSITION OF THE VITAMIN K-DEPENDENT GAMMA-GLUTAMYL CARBOXYLASE BINDING DOMAIN ON OSTEOCALCIN

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Abstract

Descarboxy-osteocalcin (d-OC) possesses a high affinity recognition site for the vitamin K-dependent enzyme γ -glutamyl carboxylase. This recognition site is distinct from the conserved propeptide which serves as a carboxylase binding site in other precursor proteins. However, the exact location and composition of the γ -glutamyl carboxylase recognition domain on d-OC has remained unclear until now. With the use of various synthetic peptides derived from the N-terminal part of d-OC we report here that the main recognition sequence present on d-OC resides between residues 1 and 12, at the N-terminal site of the γ -carboxy glutamic acid (Gla) domain. By shortening the N-terminal part of d-OC¹⁻²⁵ using deletions of amino acids 1 and 2 (d-OC³⁻²⁵), 1 to 4 (d-OC⁵⁻²⁵), 1 to 6 (d-OC⁷⁻²⁵), 1 to 9 (d-OC¹⁰⁻²⁵) and 1 to 12 (d-OC¹³⁻²⁵), the K_m values for γ -glutamylcarboxylase gradually increased. Besides the residues 1 to 12, a second recognition unit is located between residues 26 to 39, C-terminal of the Gla -domain which substantially contributes to the efficiency of carboxylation. This is the first time that a vitamin K-dependent substrate is reported of which the carboxylase binding domain is interrupted by the Gla-domain.

Introduction

Descarboxy- osteocalcin (d-OC¹⁻⁴⁹) is the only known vitamin K-dependent protein that binds the enzyme γ -glutamyl carboxylase with high affinity without the presence requirement of a covalently attached propeptide¹⁻⁵. It also became clear that d-OC¹⁻⁴⁹ initially binds to carboxylase at a site distinct from both the propeptide binding site⁶ and the active site (Chapter 5, this thesis). However, the question remained which structural determinants of d-OC¹⁻⁴⁹ contribute to its high affinity for carboxylase. Although *in vivo* the precursor molecule of osteocalcin is covalently attached to its propeptide, it has been demonstrated that this propeptide does not possess measurable affinity for carboxylase and therefore probably does not improve the enzyme-substrate interaction⁶. Whether this is a consequence of the presence of a glycine residue at the -10 position of the propeptide instead of the highly conserved alanine residue, seen in all other propeptides of the vitamin K-dependent proteins, remains to be examined. Chu et al. reported a factor IX precursor molecule which displayed a 30 fold reduced affinity for carboxylase as a result of an alanine to threonine mutation at the -10 position of the propeptide⁷. A similar decrease was also observed if an alanine to glycine mutation was introduced at the same position in the propeptide of the factor IX precursor molecule⁷. Moreover, within the osteocalcin propeptide a valine residue is present at the -6 position of the propeptide, whereas most other propeptides possess a leucine at this position. Whether these differences contribute to the poor affinity of the osteocalcin propeptide is still unclear.

Within the mature amino acid sequence of osteocalcin no homology with a propeptide structure can be found, in contrast to Matrix Gla Protein (MGP), which is the only vitamin K-dependent protein that has developed a propeptide within its mature amino acid sequence⁸. Stanley et al. already postulated that for osteocalcin essential elements other than the propeptide appear to be involved in substrate recognition². This is in contrast with the postulation of Price et al., who speculated about the EXXXEXC unit which could allow the carboxylase molecule to discriminate between substrate and product and which might be involved in substrate recognition and product dissociation⁸. From previous experiments it became evident that the highly conserved EXXXEXC

consensus sequence present in the Gla domain of all known vitamin K-dependent proteins did not contribute to a high affinity binding in case of the d-OC¹³⁻²⁵ peptide⁶.

To investigate which domain within the d-OC¹⁻⁴⁹ protein is responsible for the high affinity binding to carboxylase, the peptides d-OC¹⁻²⁵, d-OC³⁻²⁵, d-OC⁵⁻²⁵, d-OC⁷⁻²⁵, d-OC¹⁰⁻²⁵ were synthesized and their K_m values were compared with those of d-OC¹⁻⁴⁹, d-OC¹³⁻²⁵, and two recombinant glutathion- S-transferase (GST) fused human descarboxy-osteocalcin molecules, GST-rhOC¹⁻⁴⁹ and GST-rhOC¹⁻³⁹.

Materials and Methods

Reagents

All chemicals were of analytical grade or better. Vitamin K₁ (Konakion[®], mixed-micelles) was purchased from Hoffmann - La Roche (Basel, Switzerland), NaH¹⁴CO₃ with a specific activity of 50 mCi/mmol was from NEN (Boston, MA) and CHAPS {3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate} was from Sigma (St. Louis, MO).

Proteins and peptides

Recombinant glutathion S-transferase (GST) fused human descarboxy-osteocalcin (GST-rhOC¹⁻⁴⁹) and a GST-fused truncated form of human descarboxy-osteocalcin lacking 10 COOH-terminal amino acid residues, (GST-rhOC¹⁻³⁹) were produced and purified according to Käkönen et al.⁹ and Hellman et al.¹⁰, respectively. d-OC¹⁻⁴⁹ was synthesized by Dr. P Proost (The Rega Institute, Department of Molecular Immunology, Catholic University of Leuven, Belgium), according to Wuyts et al.¹¹. d-OC¹³⁻²⁵ was purchased from Pepscan Systems (Lelystad, The Netherlands).

The peptides d-OC¹⁻²⁵, d-OC³⁻²⁵, d-OC⁵⁻²⁵, d-OC⁷⁻²⁵ and d-OC¹⁰⁻²⁵ were synthesized on an Applied Biosystems 433A Peptide Synthesizer (Foster City, California, USA) using the FastMoc[®] protocol¹² on 0.25 mmol scale. Peptide syntheses were carried out on TentaGel[®] S RAM resin (1 g, 0.24 mmol) functionalized with a 4-[(2',4'-dimethoxyphenyl)aminomethyl]phenoxyacetamido moiety (Rink amide linker¹³) (Rapp Polymere, Tübingen, Germany) to obtain C-terminal amides. N^α 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids with the following side chain protection were used: *tert*.-butoxycarbonyl (Boc) for Trp¹⁴, *tert*-butyl (tBu) for Tyr, *tert*-butyl ester (OtBu) for Asp and Glu, trityl (Trt) for Gln and Cys and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for Arg¹⁵. Coupling of N-Fmoc amino acids (1 mmol, 4 equiv) were performed with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/N-hydroxybenzotriazole (HBTU/HOBt)¹⁶ in the presence of 2 equiv N,N-diisopropylethylamine (DIPEA) in N-methylpyrrolidinone (NMP) for 45 min. Fmoc removal was carried out with 25% piperidine in NMP for 10 min. The

peptides were cleaved from the resin and deprotected by treatment with trifluoroacetic acid (TFA)/H₂O/1,2-ethanedithiol 76:4:20 v/v/v at room temperature for 3 hrs¹⁷. The peptides were precipitated with diethyl ether/hexane 1:1 v/v at -20°C. The precipitates were decanted and subsequently washed with cold diethyl ether/hexane 1:1 v/v (three times) and finally lyophilized from *tert.*-butanol/H₂O 1:1 v/v.

Peptide purification

Preparative HPLC runs were performed on a Gilson HPLC workstation (Middleton, Wisconsin, USA) using Adsorbosphere XL C18 (Alltech Corp., Deerfield, Illinois, USA), consisting of two 306 pumps, a 811C dynamic mixer, a 806 manometric module, a 119 UV/VIS detector a 402 syringe pump and a 233XL on-line column switching autoinjector (fractioncollector). The HPLC runs were controlled by Gilson Unipoint™ System Software (version 1.65). Preparative HPLC was performed on Adsorbosphere XL C18 (Alltech Corp., Deerfield, Illinois, USA), 10 µm particle size, 300 Å pore size (l: 250 mm, i.d.: 22 mm). Crude lyophilized peptides (30-100 mg) were dissolved in 0.1% TFA in H₂O and loaded onto the HPLC column. The peptides were eluted with a flow rate of 11.5 mL/min using a linear gradient of buffer B (100% in 60 min) (buffer A: 0.1% TFA in H₂O, buffer B: 0.085% TFA in CH₃CN/H₂O 95:5 v/v). After purification the peptides were lyophilized from demineralized water and stored at -20°C prior to biological testing.

Peptide characterization

Peptide purity was analyzed by analytical HPLC on an Adsorbosphere XL C18, 5 µm particle size, 300 Å pore size (l: 250 mm, i.d.: 4.6 mm) at a flow rate of 1 mL/min using a linear gradient of buffer B (100% in 30 min) from 100% buffer A (buffer A: 0.1% TFA in H₂O; buffer B: 0.085% TFA in CH₃CN/H₂O 95:5 v/v). Purity was determined to be 95% or higher. The peptides were characterized by Electrospray Ionization Mass Spectrometry (ESI-MS) on a Micromass Platform II (Micromass UK Ltd., Altrincham, United Kingdom) single quadrupole bench-top mass spectrometer operating in a positive ionization mode. Instrumental control, data acquisition and data processing

were carried out using the MassLynx software package (version 2.3). The observed average m/z was compared with the calculated $(M + H)^+$ average mass. An overview of the mass comparison is given in table 1.

Table 1
Comparison of Obtained and Calculated Molecular Masses
of the Synthesized d-OC Peptides

Osteocalcin	Peptide Amino Acid Composition	Molecular Mass $(M + H)^+$	
		Obtained	Calculated
d-OC 1-25	Ac-YLYQWLGA ¹ VPYPDPLEPRREVCEL-NH ₂	3046.20	3046.53
d-OC 3-25	Ac-YQWLGA ¹ VPYPDPLEPRREVCEL-NH ₂	2769.60	2770.19
d-OC 5-25	Ac-WLGA ¹ VPYPDPLEPRREVCEL-NH ₂	2478.35	2478.88
d-OC 7-25	Ac-GA ¹ VPYPDPLEPRREVCEL-NH ₂	2179.45	2179.51
d-OC 10-25	Ac-VPYPDPLEPRREVCEL-NH ₂	1953.30	1952.99

Carboxylase assay

Expression of r-carboxylase in *Trichoplusia ni* High-Five insect cells the preparation of microsomes and the purification of r-carboxylase using HPC4 antibody resin was performed as described ⁶. Standard reaction mixtures were used as described ⁶; 25 nmol of HPC4 purified γ -glutamyl carboxylase was used.

Results and Discussion

In a purified recombinant human γ -glutamyl carboxylase system devoid of any endogenous substrate, we have determined the K_m - and V_{max} values of various d-OC peptides and proteins. As can be seen in table 2, N-terminal deletion of amino acids of the d-OC ¹⁻²⁵ peptide results in an increase in the K_m values of these peptides. Experiments in which we compared d-OC ¹⁻⁴⁹ with chimeric proteins in which the N-terminus of d-OC was fused with GST, showed that recognition of d-OC by carboxylase was not influenced by the relatively large GST (Mw 26 kDa). This is important because in proteins containing the pro-sequence only the first n N-terminal glutamate residues (where n ranges from 5 to 12) are carboxylated. The newly discovered "OC-type" carboxylation recognition sequence (CRS) makes clear that -although no such protein has been discovered at this time- also a Gla-domain in the center or at the C-terminus of a protein is feasible. Another remarkable point was that the CRS in OC is formed by sequences located both at the N-terminal and C-terminal side of the Gla-motif. This became clear from comparison of the characteristics of d-OC ¹⁻⁴⁹ and d-OC ¹⁻²⁵. Since deletion of the last 10 carboxy-terminal residues had no effect, the contribution to carboxylase recognition must also be associated with residues 26-39.

Supposedly, the binding of the d-OC peptides to carboxylase results in a processive mechanism of carboxylation, similar to carboxylation of propeptide-containing substrates, although d-OC ¹⁻⁴⁹ lacks a propeptide. The rate of release of the substrate must be slower than the rate of carboxylation ². This process is described for the propeptide-containing substrate FIXQ/S, a recombinant substrate based on the amino acids -18 to 41 of the human factor IX precursor molecule ¹⁸, identical with ProlX-59 Q/S. FIXQ/S binds carboxylase and is released after all 12 glutamate residues are converted. Therefore, the rate of carboxylation is proportional to the number of glutamate residues which can be carboxylated during a single enzyme turnover. Thus, the V_{max} is rate-limited by the substrate bound. This process results in V_{max} values for pro-containing substrates that are 5 to 10-fold lower than the V_{max} values of FLEEL or d-OC ¹³⁻²⁵ as can be seen in table 3. This mechanism furthermore depends on the

differences in affinity of the propeptides. It is known that the affinity of the factor IX propeptide is 10-fold higher than of the prothrombin propeptide ¹⁹.

Table 2

Amino acid sequences overview of the d-OC peptides and proteins in combination with their kinetic parameters.

Position:	1	5	10	15	20	25	30	35	40	45	49	$K_m \pm S.D.^{(a)}$ (μM)	$V_{max} \pm S.D.^{(a)}$ ($\mu mol^{14} CO_2 \cdot s^{-1}$)
d-OC ¹⁻⁴⁹	YLYQWL	GAPV	PYPD	PLEPR	REV	CELNP	DCDEL	ADHIG	FQEA	YRRF	YGPV	3.14 $\pm$ 0.23	0.38 $\pm$ 0.020
GST-rhOC ¹⁻⁴⁹	GST-	YLYQWL	GAPV	PYPD	PLEPR	REV	CELNP	DCDEL	ADHIG	FQEA	YRRF	YGPV	3.06 $\pm$ 0.47
GST-rhOC ¹⁻³⁹	GST-	YLYQWL	GAPV	PYPD	PLEPR	REV	CELNP	DCDEL	ADHIG	FQ		3.08 $\pm$ 0.59	0.36 $\pm$ 0.022
d-OC ¹⁻²⁵	YLYQWL	GAPV	PYPD	PLEPR	REV	CEL						29 $\pm$ 8.90	0.26 $\pm$ 0.019
d-OC ³⁻²⁵	YQWL	GAPV	PYPD	PLEPR	REV	CEL						114 $\pm$ 25	0.24 $\pm$ 0.020
d-OC ⁵⁻²⁵	WL	GAPV	PYPD	PLEPR	REV	CEL						125 $\pm$ 21	0.24 $\pm$ 0.018
d-OC ⁷⁻²⁵			GAPV	PYPD	PLEPR	REV	CEL					568 $\pm$ 45	0.19 $\pm$ 0.015
d-OC ¹⁰⁻²⁵				VPYP	DPLEPR	REV	CEL					767 $\pm$ 53	0.16 $\pm$ 0.011
d-OC ¹³⁻²⁵					PDP	LEPR	REV	CEL				10000 $\pm$ 2250	0.70 $\pm$ 0.052

^(a) Values are $\pm$ standard deviations, obtained from at least three independent determinations

E: glutamic acid residues that become carboxylated

This difference may explain why the $V_{\max}$ of ProPT-28, containing only 2 glutamate residues is still 2-fold higher than the $V_{\max}$ of ProIX-59 Q/S.

Using the formula

$$E.C. = \frac{V_{\max}}{K_m * \text{Glu}} \cdot [E]$$

it is possible to calculate the efficiency of carboxylation for different substrates, in which E.C. stands for efficiency of carboxylation, [E] is the carboxylase concentration in the reaction mixture (pmol/ml), $V_{\max}$ is the maximal amount of $^{14}\text{CO}_2$ incorporated (pmol/s), and $K_m * \text{Glu}$ is the K_m adjusted for the theoretical number of the potentially carboxylated glutamate residues per substrate molecule.

Table 3
Carboxylation Efficiency of Various *In Vitro* Substrates

Substrate ^(a)	K_m (μM)	$V_{\max}$ (pmol $^{14}\text{CO}_2$ incorporated * s ⁻¹)	Carboxylation Efficiency (mol ⁻¹ * s ⁻¹)
d-OC ¹⁻⁴⁹	3.14	0.38	1.61
GST-rhOC ¹⁻³⁹	3.08	0.37	1.61
GST-rhOC ¹⁻⁴⁹	3.00	0.35	1.55
Pro(Glu) ₁₀	0.56	0.13	0.93
ProPT-28 F/A	5.35	0.22	0.82
ProIX-59 Q/S	0.51	0.10	0.65
d-OC ¹⁻²⁵	29	0.27	0.13
d-OC ³⁻²⁵	114	0.25	0.029
d-OC ⁵⁻²⁵	125	0.25	0.027
d-OC ⁷⁻²⁵	568	0.20	0.0047
FLEEL	2400	0.47	0.0039
d-OC ¹⁰⁻²⁵	767	0.15	0.0026
bEELOMe	2000	0.22	0.0022
d-OC ¹³⁻²⁵	10000	0.70	0.0009

^(a) See chapter 4 for a description of the propeptide-containing substrates.

As is shown in table 3 the efficiency of carboxylation can be divided in three groups: low efficiency ($0.0009\text{--}0.0047\text{ mol}^{-1}\cdot\text{s}^{-1}$) for the short peptides including FLEEL, d-OC¹³⁻²⁵, d-OC¹⁰⁻²⁵ and d-OC⁷⁻²⁵, intermediate efficiency ($0.027\text{--}0.13\text{ mol}^{-1}\cdot\text{s}^{-1}$) for d-OC⁵⁻²⁵ through d-OC¹⁻²⁵, and high efficiency ($0.65\text{--}1.61\text{ mol}^{-1}\cdot\text{s}^{-1}$) for the pro-containing substrates and full length osteocalcin. The efficiency of the propeptide-containing substrates is in the same range, independent of the amount of glutamate residues, but probably depending on the on/off rate of the propeptide. The efficiency by which FLEEL, bEELoMe, d-OC⁷⁻²⁵, d-OC¹⁰⁻²⁵ and d-OC¹³⁻²⁵ are carboxylated is strongly reduced because of a low affinity of these substrates. The low affinity explains why not more than one Glu/mol is formed before enzyme/substrate dissociation and thus why efficiency of carboxylation is low for short peptide substrates^{20,21}. Obviously, this is undesirable for protein precursors in which formation of a complete multi-carboxylated product is required during one cycle of enzyme-substrate interaction. ProlX-59 Q/S is processively carboxylated, and the dissociation of the propeptide from the enzyme is rate limiting in this process¹⁸. All 12 glutamate residues are carboxylated by the enzyme in one binding event, thereby ensuring maximal carboxylated ProlX-59 Q/S to be formed. Since *in vivo* the substrate concentration is well below the K_m value, replacement of bound substrate by an excess of free substrate will be an unlikely event, except in cases of unusual accumulation of precursor proteins for instance during vitamin K-deficiency or coumarin treatment. Indeed partially carboxylated coagulation factors have been reported to circulate in coumarin-treated humans²².

It remains unclear however why d-OC¹⁻⁴⁹ is carboxylated with a 2 to 3-fold higher efficiency as propeptide-containing substrates are. The question remains whether this is the reason why in humans undercarboxylated osteocalcin can be found in both bone²³, and serum²⁴⁻²⁸. Alternatively, a local or tissue-specific vitamin K-deficiency in human bone might explain the occurrence of undercarboxylated osteocalcin.

Although carboxylase displayed a low K_m value for d-OC¹⁻²⁵ (table2), the efficiency by which it is carboxylated still is ~12-fold lower than for d-OC¹⁻⁴⁹ (table 3). It is clear that the C-terminal domain from residues 26 to 39 contributes to the carboxylation efficiency of d-OC¹⁻⁴⁹ to a major extent.

However, the $V_{\max}$ values of the N-terminal deleted d-OC peptides show a slight decrease when compared to d-OC¹⁻⁴⁹, GSTrhOC¹⁻⁴⁹ and GSTrhOC¹⁻³⁹. Possibly, substrates which bind with high affinity at either the propeptide binding site or the osteocalcin binding site on carboxylase induce a conformational change in the catalytic domain of the enzyme, thereby directing the active site of the enzyme towards the substrate glutamate residues²⁹. This subsequently results in the carboxylation of the glutamate residues on the substrate. If the affinity of these substrates is reduced by deletion of specific amino acids, the conformational change in the catalytic domain can be less pronounced and thereby lower the $V_{\max}$.

From the results presented here we can furthermore draw the conclusion that the EXXXEXC consensus sequence, as was postulated by Price et al.⁸, does not contribute to the high affinity recognition of osteocalcin by carboxylase. The d-OC¹³⁻²⁵ peptide, which consists of residues 13 to 25 of descarboxy-osteocalcin (P D P L E P R R E V C E L; the EXXXEXC-part is underlined), has a K_m that is 3 orders of magnitude higher than that of d-OC¹⁻⁴⁹ and d-OC¹⁻²⁵. This is in agreement with results found by Ratcliffe et al., who used prothrombin mutants in which glutamic acid residues were modified to aspartic acid, generating fully carboxylated proteins³⁰. Furthermore it was shown by several authors using *in vitro* substrates consisting of a propeptide followed by a random Glu-containing protein sequence, that the EXXXEXC motif is redundant in substrate recognition^{1,2,31}.

In conclusion, we have demonstrated that the d-OC molecule binds to carboxylase in a different way when compared to the other known vitamin K-dependent proteins. The results of the experiments described in this paper indicate that carboxylase recognizes a d-OC by two specific regions flanking the Gla-domain, which is distinct from the propeptide domain. The carboxylase binding domain described here seems specific only for osteocalcin, but might also be available for currently undiscovered vitamin K-dependent proteins lacking a propeptide motif. These proteins of yet unknown structure and function could be capable of binding carboxylase with high affinity in a propeptide-independent way.

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Chapter 7

SUMMARY, CONCLUSIONS AND DISCUSSION

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Summary, conclusions and discussion

Chapter 1

During synthesis *in vivo*, proteins undergo extensive post-translational processing before being able to function appropriately. Some of these modified proteins are secretory proteins such as the Gla-proteins involved in blood coagulation or tissue-mineralization processes. Carboxylation of specific glutamate (Glu) residues into γ -carboxy glutamate (Gla) residues is one of the postranslational processes and is accomplished by the vitamin K-dependent enzyme γ -glutamyl carboxylase. Vitamin K-hydroquinone (KH_2) is the cofactor and an absolute requirement in the carboxylation process. During the conversion of Glu into Gla, KH_2 is oxidized to vitamin K 2,3 epoxide (KO) in a 1:1 stoichiometry via an intermediate vitamin K alkoxide. Subsequently, KO is reduced in two steps; first to K by the enzyme KO-reductase and then to KH_2 by the enzyme K-reductase. Due to this "recycling" mechanism, the daily requirement in humans for vitamin K is relatively low. The conversion of Glu to Gla is necessary for the calcium-binding properties of the Gla-proteins. Only a small number of all the known proteins in man are Gla-proteins. Therefore the Gla-proteins have to be distinguishable from other proteins so they can be recognized by the vitamin K-dependent carboxylase. For that aim, the precursor forms of the Gla-proteins possess a domain, a so-called propeptide, which makes them capable of binding to carboxylase with high affinity. The goal of this thesis was to gain insight in the determinants responsible for the recognition of the Gla-precursor molecule and carboxylase. For that purpose we have chosen to use substrates derived from the bone Gla-protein osteocalcin, from the precursor protein of human coagulation factor IX, and from the precursor protein of human prothrombin. In this chapter a review on the literature is presented concerning the history and current status of our knowledge regarding carboxylase, its cofactors and substrates. Moreover the function of the various Gla-proteins is discussed.

Chapter 2

The bone Gla-protein osteocalcin is unique in its ability to bind carboxylase with high

affinity. To obtain sufficient amounts of this bone protein, several strategies can be applied. The first method is to purify the native protein from (bovine/human) bone. However, this technique has several disadvantages; (i) it is laborious with relatively low yields and in case of isolation from human bones (ii) the source is limited and there is a potential risk of infection. Besides these drawbacks, the protein obtained from bone is carboxylated and thus not useful as an *in vitro* substrate for carboxylase. Thermal decarboxylation of native osteocalcin can be applied but may induce unwanted and unpredictable damage to the protein due to the reaction conditions of the procedure. As a second possibility, recombinant DNA techniques can be utilized to produce unlimited amounts of osteocalcin in bacteria (*Escherichia coli*). The osteocalcin produced in *E.coli* is not carboxylated and can be used as a substrate for carboxylase. Besides the synthesis in *E.coli*, chemical synthesis is a third possibility to obtain both carboxylated- (OC) and descarboxy-osteocalcin (d-OC) of high purity. Nevertheless, this technique has its limitations, and the length of the OC molecule (49 amino acids) is approximately the maximum which can be routinely synthesized. In this thesis we have chosen to study the substrate properties of osteocalcin using both organically synthesized OC and d-OC. Once the proper protocol has been selected to produce sufficient amounts of d-OC, carboxylase from various tissue types can be used to study the properties of d-OC and other substrates. Furthermore the isolation of carboxylase in the form of washed microsomes from various types of tissue, and its use in enzyme substrate interactions is described here.

Chapter 3

One of the high affinity substrates used in carboxylase research is ProIX-59 Q/S. ProIX-59 Q/S consists of the propeptide covalently attached to the descarboxy Gla-domain of factor IX, and functions as a good substrate for carboxylase. The interactions of the propeptide and the Gla-domain is studied using purified bovine carboxylase. The importance of the propeptide for the carboxylation of the glutamate residues in the factor IX Gla-domain, the effect of the propeptide on the carboxylation of a non-Gla-domain sequence containing 5 Glu residues, and the substrate properties of synthetic

d-OC for purified bovine carboxylase is described in this chapter. From the results reported in this chapter we can draw several conclusions: (i) the affinity of the carboxylase substrate and processivity is induced by the propeptide without requiring the conserved Gla-domain sequences and (ii) factor IX and osteocalcin may have distinct mechanisms of interaction with the carboxylase.

Chapter 4

Since the results described in chapter 3 were obtained using purified carboxylase from bovine liver containing at least 2 μ M propeptide a new protocol for the production of carboxylase was developed. For this aim we have collaborated with the group of Prof. Dr. Stafford (Department of Biology, The University of North Carolina at Chapel Hill, U.S.A), who cloned the cDNA sequence of human γ -glutamyl carboxylase and expressed it in an insect cell line, normally free of carboxylase. The carboxylase purified from these cells was completely free of any interfering propeptide or propeptide containing substrates (endogenous substrate). Therefore the purified recombinant human carboxylase is the system of choice to study enzyme / substrate interactions where the absence of a propeptide is required (e.g. d-OC).

Although the unique properties of d-OC already have been discussed in chapters 2 and 3, its characteristics are studied more extensively in this chapter. Chapter 4 describes the absence of inhibition by free propeptide of factor IX (Pro^{FIX}) on d-OC, whereas Pro^{FIX} strongly inhibits ProIX-59 Q/S carboxylation. Furthermore d-OC carboxylation is not stimulated by Pro^{FIX} in contrast to bEELoMe. Pro^{FIX} lowers the K_m of short pentapeptide substrates 10 fold. The propeptide of osteocalcin (Pro^{OC}) was incapable of either inhibiting ProIX-59 Q/S and d-OC carboxylation. Moreover Pro^{OC} could not stimulate bEELoMe carboxylation, nor could it lower the K_m of bEELoMe. Synthetic carboxylated osteocalcin (OC) inhibited both d-OC and bEELoMe, whereas it was incapable of inhibiting ProIX-59 Q/S carboxylation. These results implicate that Pro^{OC} does not contribute to the high affinity carboxylase binding of the osteocalcin precursor molecule and that d-OC binds carboxylase at a site distinct from the propeptide binding site. However, it remains unclear whether d-OC binds to the active site, or on a distinct

osteocalcin binding site on carboxylase.

Chapter 5

In this chapter definite proof is given that d-OC binds carboxylase with high affinity on a specific d-OC binding domain. By using a stereospecific active site inhibitor (S -MeTPT) for carboxylase we have shown that bEELOMe is competitively inhibited by S -MeTPT. ProlX-59 Q/S is competitively inhibited by Pro^{FIX}, since ProlX-59 Q/S initially binds the propeptide binding site on carboxylase prior to its carboxylation. Addition of S -MeTPT to ProlX-59 Q/S carboxylation resulted in a non-competitive inhibition of ProlX-59 Q/S. This is in agreement with the proposed mechanism by which ProlX-59 Q/S interacts with carboxylase. Since d-OC is inhibited by neither Pro^{FIX} nor Pro^{OC} it was expected that d-OC would bind the active site on carboxylase and therefore competitively inhibited by S -MeTPT. However, addition of S -MeTPT to d-OC carboxylation resulted in a non-competitive inhibition of d-OC. Since d-OC lacks a propeptide these results give rise to postulate that carboxylase possesses a specific d-OC binding domain, where d-OC can bind with high affinity in a propeptide independent way.

Chapter 6

Here we describe the domain by which d-OC is capable of binding with high affinity to carboxylase. From other Gla-proteins it is known that the precursor proteins contain a high affinity propeptide at the N-terminus, immediately adjacent to the Gla domain. Matrix Gla protein (MGP) is the only known Gla-protein to contain an internal propeptide which is homologous to the propeptide, but which is not removed from the protein after carboxylation. In the N-terminus of the d-OC molecule no homology can be found with the propeptide of either the other known Gla-proteins, or MGP. Nevertheless, we expected the high affinity carboxylase recognition sequence (CRS) of d-OC to be located N-terminally adjacent to the Gla-domain, between residues 1 and 16. The Gla-domain consists of residues 17 to 24. We therefore generated various d-OC peptides, d-OC¹⁻²⁵, d-OC³⁻²⁵, d-OC⁵⁻²⁵, d-OC⁷⁻²⁵, d-OC¹⁰⁻²⁵, and d-OC¹³⁻²⁵, all containing the

non carboxylated Gla-domain, which may serve as a substrate in the *in vitro* carboxylase assay. The kinetic parameters of these peptides were compared with the intact d-OC molecule. Due to the different N-terminal deletions it became evident that indeed substantial CRS activity is located between residues 1 and 13. Some of the residues proved to be particularly important, since deletion resulted in dramatic increases in the K_m of the peptides. However, the K_m of the d-OC¹⁻²⁵ still differed 10-fold when compared to the intact d-OC molecule. The efficiency of carboxylation was the highest for d-OC¹⁻⁴⁹, GST-rhOC¹⁻⁴⁹ and GST-rhOC¹⁻³⁹ when compared to the short d-OC peptides, the propeptide-containing substrates or the short tri- and pentapeptides. Furthermore, a secondary structural component, located between residues 26 and 39 of the d-OC molecule is also involved in the high affinity binding of osteocalcin to carboxylase, since this domain is capable of lowering the K_m of d-OC 10-fold and increasing the efficiency of carboxylation ~12-fold.

Summarizing, the major findings presented in this thesis are (I) that the vitamin K-dependent carboxylase, besides the active site, possesses at least two substrate recognition domains. One recognition site is available for the "coagulation factor-type" Gla-proteins, the other site is essential for "OC-type" substrates. The first site recognizes propeptides, the latter recognizes a sequence present on OC which has not been defined previously.

(II) Closer analysis of the sequence composition revealed that the carboxylase recognition sequence on d-OC is composed of two units. The first unit consists of residues 1-13 (N-terminal of the Gla-domain), and the second unit is composed of residues 26-39 (C-terminal of the Gla-domain). The fact that a carboxylase recognition site exists which is composed of two segments, divided by a Gla-domain, is new.

(III) The efficiency of d-OC carboxylation is the highest when compared to propeptide-containing substrates and short pentapeptides.

(IV) *In vivo*, the precursor molecule of osteocalcin is synthesized containing a propeptide. Nonetheless, this propeptide has no measurable affinity for carboxylase. Therefore, the function of the osteocalcin propeptide remains unclear. It is possible that

the osteocalcin propeptide is required for a more efficient utilization of KH_2 , by lowering its K_m .

Discussion

Recombinant synthesis of descarboxy osteocalcin in *E. coli*, either with or without an attached propeptide has turned out to be more difficult than was expected. A single explanation for the low expression levels we obtained cannot be given. What remains is that only very few groups in the world have succeeded in producing low amounts of bacterially synthesized osteocalcin. The fact that bovine decarboxylated osteocalcin (bd-OC) already displayed unique substrate properties was one of the main reasons to start our line of investigations. Our experimental data from organically synthesized human descarboxy osteocalcin (d-OC) have confirmed and founded the previous results; osteocalcin possesses unique properties as a substrate for carboxylase. Our experimental data have substantiated the previous results obtained with bd-OC in washed microsomes, the interpretation of which was difficult due to the fact both the substrate and the enzyme were from bovine origin and the properties could therefore be distinct from the human proteins. Besides that, the possible damaging of bd-OC by thermal decarboxylation could not be assessed. In the experiments described in this thesis we have used purified human recombinant carboxylase (chapters 4, 5 and 6) as a source for carboxylase, free of contaminating and interfering propeptides or endogenous substrates, which makes our experimental results more convincing. The results presented in this thesis have added new insights to the mechanism by which Gla proteins are able to bind carboxylase. What previously was unknown was that carboxylase has at least two high affinity binding sites for the Gla precursor proteins. Whether these two binding sites are the only ones present on carboxylase remains to be awaited, but might have considerable consequences for optimal synthesis of, for instance, recombinant Gla proteins in human eukaryotic cell lines, necessary for the treatment of hemophilia. However, a proper understanding of the structure and interactions between the Gla precursor proteins and carboxylase is required to obtain maximal efficient production and carboxylation.

The recent discovery of new Gla-proteins like the carboxylase itself, or proteins that are not involved in blood coagulation but in cellular growth (Gas-6), and other yet unknown processes (the putative PRGP 1 and PRGP 2) indicates that the discovery of other new Gla-proteins is likely ^{1,2}. The type of interaction between the yet undiscovered Gla-proteins interact with carboxylase might be, like osteocalcin, in a propeptide independent way. This can be illustrated using a hypothetical calculation; It is expected that the human genome contains 100.000 genes. From these genes, 30% is known, which results in 30.000 proteins that have been currently discovered. If the known 13 Gla-proteins represent 30% of the total amount of Gla-proteins, this means that there are still 30 Gla-proteins to be discovered. Of these 30 Gla-proteins, at least 2 proteins will bind carboxylase in a propeptide independent way. Maybe, this could indicate that carboxylase is a much more versatile enzyme as was expected, capable of binding various types of proteins.

The role of Gla as a Ca^{2+} (or other divalent metal-ions) binding residue remains to be discussed. A large number of other proteins are very well capable of binding Ca^{2+} without the need for Gla. Osteonectin, a non-Gla basement membrane protein found in bone and platelets contains a glutamic acid-rich Ca^{2+} binding domain. Gas-6 for instance, functions as a ligand for different protein kinases which are involved in cellular growth regulation. A deletion mutant of Gas-6, lacking the Gla domain, is very well capable of binding to the extracellular domains of two cell adhesion molecule-related tyrosine kinase receptors, Rse and Axl, and thereby activating phosphorylation³. Therefore the question remains what is the the function of the Gla residues within Gas-6 or other (yet unknown) Gla-proteins. The vitamin K-dependent carboxylase itself is also a Gla-protein, which possesses 3 Gla residues ⁴. The function and the location of these Gla residues is still unknown. Furthermore it is unclear whether carboxylase is capable of functioning without being carboxylated itself. Gla residues also have been discovered already in 1982 in hermatypic corals, but the origin of the Gla remained unclear ⁵. Gla-proteins have also been discovered in the venom duct of the predatory marine snail, *Conus geographus* ⁶. This was the first invertebrate organism reported to possess the posttranslational carboxylation mechanism, which was thought to be

restricted to rather specialized mammalian systems. The invertebrate Gla-protein, called conantokin-G, is a 17 amino acid peptide that inhibits the *N*-methyl-D-aspartate receptor, thereby paralyzing the fish and thus become prey for the fish-hunting snail. Besides conantokin-G there are at least two other conantokins that contain Gla-residues, namely conantokin-T and conantokin-R^{7,8}. The conantokin precursor proteins contain a propeptide which binds *Conus* carboxylase with high affinity, but not the mammalian enzyme. This implies a difference in either the enzyme or the propeptide of the conantokin precursor proteins versus the mammalian propeptides. Whether d-OC is able to bind *Conus* carboxylase remains to be investigated, but this is very well possible, and might thus form additional evidence for human descent and evolution.

The importance of the newly discovered carboxylase recognition sequence is that it offers a possibility for substrates to be carboxylated at a significant distance from the N-terminus. Normally, in propeptide-containing substrates, carboxylation takes place within the first 40 N-terminal amino acids. The chimeric osteocalcin molecules synthesized by Käkönen et al. (chapter 6, this thesis) showed that OC-type recognition can occur at any position within a larger protein. Using this knowledge it may be possible to search for new members of this class of Gla-proteins.

The results presented in this thesis will add to the understanding of the characteristics required in the mechanism of the high affinity association between the various Gla-precursor proteins, osteocalcin in particular, and the vitamin K-dependent γ -glutamyl carboxylase.

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Samenvatting, conclusies en discussie

Hoofdstuk 1

Tijdens de *in vivo* synthese van eiwitten ondergaan deze een groot aantal post-translationele modificaties alvorens ze hun functie goed kunnen uitoefenen. Deze bewerkte eiwitten zijn secretair, zoals de Gla-eiwitten welke betrokken zijn bij bloedstollings- of weefselmineralisatie processen. De carboxylering van specifieke glutaminezuur (Glu) residuen tot γ -carboxy glutaminezuur (Gla) is een van de post-translationele modificaties die wordt uitgevoerd door het vitamine K-afhankelijke enzym γ -glutamyl carboxylase. Dit carboxyleringsproces is alleen mogelijk in de aanwezigheid van de cofactor vitamine K hydroquinon (KH_2). Tijdens de omzetting van Glu naar Gla wordt KH_2 in een 1:1 verhouding geoxideerd tot vitamine K 2,3 epoxide (KO) via een intermediair vitamine K alkoxide. KO wordt vervolgens in twee opeenvolgende stappen gereduceerd; eerst tot K door het enzym KO-reductase en vervolgens tot KH_2 door het enzym K-reductase. Als gevolg van dit "recycling" proces is de humane dagelijkse behoefte aan vitamine K relatief gezien erg laag. De omzetting van Glu naar Gla is nodig voor de calcium-bindende eigenschappen van de Gla-eiwitten. Slechts een beperkt aantal van alle bekende eiwitten in de mens zijn Gla-eiwitten. Daarom is het noodzakelijk dat deze Gla-eiwitten zich onderscheiden van andere eiwitten zodat ze herkenbaar zijn voor het vitamine K-afhankelijke carboxylase. Derhalve bezitten de voorloper vormen van de Gla-eiwitten een domein, het zogenaamde propeptide, dat hen in staat stelt met een hoge affiniteit te binden aan het carboxylase. Het doel van dit proefschrift was het verschaffen van inzicht in de determinanten die noodzakelijk zijn voor de herkenning van de Gla-voorloper eiwitten door het carboxylase. Daarvoor is er besloten gebruik te maken van substraten afgeleid van het bot Gla-eiwit osteocalcine, van het voorlopereiwit van de humane bloedstollingsfactor IX, en van het voorlopereiwit van humaan prothrombine. In dit hoofdstuk wordt een literatuuroverzicht gegeven van de ontdekking van vitamine K in 1935 tot het huidige kennisniveau en inzichten over het carboxylase, de cofactoren en substraten. Tevens worden de functies van de verschillende humane Gla-eiwitten besproken.

Hoofdstuk 2

Het bot Gla-eiwit osteocalcine bezit een aantal unieke eigenschappen waardoor het in staat is om met een hoge affiniteit aan carboxylase te binden. Om voldoende van het osteocalcine te verkrijgen kunnen er verschillende methodes worden toegepast. De eerste mogelijkheid is om het natieve eiwit op te zuiveren uit (bovine/humane) botten. Aan deze techniek kleven echter een aantal nadelen; (i) het is een arbeidsintensieve methode met een lage opbrengst en in het geval van isolatie uit humane botten (ii) is de bron gelimiteerd en bestaat er een aanzienlijk infectierisico. Bovendien is het osteocalcine dat op deze wijze wordt gezuiverd reeds gecarboxyleerd, waardoor het niet bruikbaar is als *in vitro* substraat voor carboxylase. Thermische decarboxylering van natief osteocalcine kan worden toegepast maar zou ongewenste en onvoorspelbare schade kunnen induceren in het eiwit als gevolg van de reactieomstandigheden. Recombinant DNA technieken kunnen worden toegepast om ongelimiteerde hoeveelheden osteocalcine te produceren in bacteriën (*Escherichia coli*). Het door *E. coli* geproduceerde osteocalcine is niet gecarboxyleerd en kan daardoor worden gebruikt als *in vitro* substraat voor carboxylase. Naast de synthese van osteocalcine door *E. coli* is het ook mogelijk om zowel gecarboxyleerd osteocalcine (OC) als descaboxy-osteocalcine (d-OC) in zuivere vorm te verkrijgen met behulp van chemische synthese. Niettemin heeft ook deze techniek zijn beperkingen en de lengte van het OC molecuul (49 aminozuren) is ongeveer de maximum lengte die routinematig kan worden gesynthetiseerd. In dit proefschrift is ervoor gekozen om de substraateigenschappen van osteocalcine te bestuderen aan de hand van zowel organisch gesynthetiseerd OC als d-OC. Indien het juiste protocol is gekozen om voldoende hoeveelheden d-OC te genereren, kan er carboxylase worden gebruikt dat afkomstig is uit verschillende weefseltypen, om zo de eigenschappen van d-OC en andere substraten te bestuderen. Bovendien wordt de isolatie van het carboxylase uit verschillende weefseltypen in de vorm van gewassen microsomen en het gebruik hiervan bij enzym/substraat interacties in dit hoofdstuk beschreven.

Hoofdstuk 3

Een van de substraten die wordt gebruikt in het carboxylase onderzoek is ProlX-59 Q/S. Dit is een recombinant eiwit dat is afgeleid van de voorloper van het humane bloedstollingseiwit factor IX dat bestaat uit het propeptide wat covalent gebonden zit aan het descarboxy Gla-domein en hierdoor een hoge affiniteit heeft voor carboxylase. De interacties van het propeptide met carboxylase zijn bestudeerd in gezuiverd bovine carboxylase. Het ontbreken van het propeptide en het effect hiervan op de substraateigenschappen van het descarboxy Gla-domein van factor IX, het effect van het propeptide op de carboxylering van een non-Gla-domein sequentie met 5 Glu residuen en de substraateigenschappen van synthetisch d-OC voor het gezuiverde bovine carboxylase worden beschreven in dit hoofdstuk. Uit de resultaten die hierin zijn besproken kunnen een aantal conclusies worden getrokken: (i) de bindingsaffiniteit van het substraat voor het carboxylase en de processiviteit van deze reactie wordt geïnduceerd door het propeptide zonder de noodzaak van geconserveerde Gla-domein sequenties en (ii) factor IX en osteocalcine hebben mogelijk verschillende interactiemechanismen met betrekking tot de carboxylasebinding.

Hoofdstuk 4

Aangezien de resultaten in hoofdstuk 3 zijn verkregen met behulp van gezuiverd carboxylase afkomstig uit bovine lever dat minimaal 2 μM propeptide bevatte, werd er een nieuw protocol voor de zuivering van carboxylase ontwikkeld. Hiervoor hebben we samengewerkt met de groep van Prof. Dr. Stafford (Department of Biology, The University of North Carolina at Chapel Hill, U.S.A.) die de cDNA sequentie van humaan γ -glutamyl carboxylase gecloneerd en tot expressie gebracht hebben in een insecten cellijn, die normaliter geen carboxylase bevat. Het carboxylase dat uit deze cellen werd opgezuiverd was volledig vrij van storend propeptide en/of propeptide bevattende substraten (endogeen substraat). Hierdoor vormt het het gezuiverde recombinante humane carboxylase het systeem bij uitstek om enzym/substraat interacties te meten waarbij de afwezigheid van propeptides gewenst is (bijv. d-OC).

Hoewel de unieke substraateigenschappen van d-OC reeds werden aangehaald in de

hoofdstukken 2 en 3, worden de karakteristieken uitvoeriger besproken in dit hoofdstuk. In hoofdstuk 4 wordt de afwezigheid van remming door het vrije propeptide van factor IX (Pro^{FIX}) op d-OC beschreven terwijl Pro^{FIX} de carboxylering van ProlX-59 Q/S zeer sterk remt. Tevens wordt aangetoond dat d-OC carboxylering niet gestimuleerd wordt door Pro^{FIX} , dit in tegenstelling tot bEELoMe, dat wel wordt gestimuleerd door Pro^{FIX} . Pro^{FIX} verlaagt de K_m van korte penta-peptiden met een factor 10. Het propeptide van osteocalcine (Pro^{OC}) was niet in staat om de carboxylering van zowel ProlX-59 Q/S als d-OC te remmen. Bovendien was Pro^{OC} niet in staat om de carboxylering van bEELoMe te stimuleren of de K_m van bEELoMe te verlagen. Synthetisch gecarboxyleerd osteocalcine (OC) inhibeerde zowel de d-OC als de bEELoMe carboxylering, terwijl het niet in staat bleek de ProlX-59 Q/S carboxylering te remmen. Deze resultaten houden in dat Pro^{OC} niets bijdraagt aan de hoge affiniteit dat het osteocalcine voorlopereiwit heeft voor carboxylase en dat d-OC aan het carboxylase bindt op een plaats die afwijkt van de propeptide bindingsplaats. Uit deze resultaten blijkt echter nog niet of d-OC op het katalytisch domein bindt of op een specifieke osteocalcine bindingsplaats op het carboxylase.

Hoofdstuk 5

In dit hoofdstuk wordt het definitieve bewijs geleverd dat d-OC het carboxylase bindt met een hoge affiniteit op een specifiek osteocalcine bindend domein. Door gebruik te maken van een specifieke katalytisch domein remmer (S-MeTPT) voor carboxylase hebben we aan kunnen tonen dat bEELoMe competitief wordt geremd door S-MeTPT. ProlX-59 Q/S wordt competitief geremd door Pro^{FIX} , aangezien ProlX-59 Q/S allereerst bindt op de propeptide bindingsplaats op carboxylase om vervolgens te worden gecarboxyleerd. Toevoeging van S-MeTPT aan ProlX-59 Q/S carboxylering resulteerde in een non-competitieve inhibitie van ProlX-59 Q/S. Dit is in overeenstemming met het voorgestelde mechanisme volgens welk ProlX-59 Q/S interageert met carboxylase. Aangezien d-OC carboxylering niet werd geremd door zowel Pro^{FIX} als Pro^{OC} werd verondersteld dat d-OC op het katalytisch domein van carboxylase zou binden en dus competitief geremd zou worden door S-MeTPT. Dit bleek echter niet het geval te zijn.

Toevoeging van S-MeTPT aan d-OC carboxylering resulteerde in een non-competitieve remming van d-OC. Omdat d-OC géén propeptide bezit mag de stelling geponeerd worden dat carboxylase naast een propeptide bindingsplaats ook over een specifieke osteocalcine bindingsplaats beschikt waar osteocalcine op een propeptide-onafhankelijke manier kan binden.

Hoofdstuk 6

In dit hoofdstuk beschrijven wij het domein waarmee d-OC met hoge affiniteit bindt aan carboxylase. Dankzij andere Gla-eiwitten is het duidelijk geworden dat voorlopereiwitten over een propeptide beschikken dat zich N-terminaal naast het Gla-domein bevindt. Matrix Gla-proteïne (MGP) beschikt als enige Gla-eiwit over een intern propeptide dat homoloog is aan de bekende propeptides, maar wat niet wordt afgesplitst van het eiwit nadat dit is gecarboxyleerd. In de N-terminus van het d-OC molecuul kan géén homologie worden gevonden met de propeptides van de andere Gla-eiwitten noch met het interne propeptide van MGP. Niettemin veronderstelden we dat de herkenningsequentie voor het carboxylase (CRS) zich bevindt in de N-terminus van d-OC, vóór het Gla-domein tussen residuen 1 en 16. Het Gla-domein van osteocalcine bestaat uit residuen 17-24. Daarom zijn er verschillende d-OC peptiden gesynthetiseerd, te weten d-OC¹⁻²⁵, d-OC³⁻²⁵, d-OC⁵⁻²⁵, d-OC⁷⁻²⁵, d-OC¹⁰⁻²⁵ en d-OC¹³⁻²⁵, die allemaal het niet gecarboxyleerde Gla-domein bevatten zodat ze als substraat zouden kunnen dienen voor de *in vitro* carboxylase test. De kinetische parameters van deze peptiden werden vergeleken met het intacte d-OC molecuul. Als gevolg van de verschillende N-terminale deleties van de peptiden werd duidelijk dat de CRS zich voor het grootste gedeelte bevindt tussen residuen 1 en 13. Sommige aminozuren in dit domein bleken bijzonder belangrijk, aangezien deletie van een aantal residuen resulteerde in een verhoging van de K_m van de peptides. Het verschil in K_m tussen d-OC¹⁻²⁵ en d-OC bedroeg echter nog altijd een factor 10. De carboxyleringsefficiency was het hoogst bij d-OC¹⁻⁴⁹, GST-rhOC¹⁻⁴⁹ en GST-rhOC¹⁻³⁹ in vergelijking tot de korte d-OC peptiden, de propeptid- bevattende substraten of de korte tri- en penta-peptiden. Bovendien bleek een secundaire structurele component

die gelegen is tussen residuen 26 en 39 van het intacte d-OC molecuul ook betrokken te zijn bij de hoge affiniteit van het osteocalcine molecuul voor carboxylase. Dit 26-39 domein bleek in staat om de K_m van d-OC met een factor 10 te verlagen en de carboxylerinsefficiency met een factor 12 te verhogen.

Concluderend zijn de belangrijkste bevindingen in dit proefschrift (I) dat het vitamine K-afhankelijke carboxylase, naast het catalytisch domein, tenminste twee substraat herkenning domeinen bezit. Een herkenning domein is beschikbaar voor "stofactor-type" Gla-eiwitten, het andere domein is essentieel voor "OC-type" substraten. Het eerste domein herkent propeptides, het laatste domein herkent een sequentie aanwezig op d-OC, die nog niet eerder beschreven was.

(II) Nadere analyse van de sequentiesamenstelling toonde aan dat de carboxylase herkenningsequentie op d-OC uit twee gedeelten bestaat. Het eerste gedeelte bestaat uit de residuen 1-13 (N-terminaal van het Gla-domein) en het tweede gedeelte bevindt zich tussen de residuen 26-39 (C-terminaal van het Gla-domein). Het feit dat er een carboxylase herkenningsequentie uit twee delen bestaat die worden gescheiden door het Gla-domein is nieuw.

(III) De efficiency waarmee d-OC werd gecarboxyleerd was het hoogst wanneer vergeleken met propeptide-bevattende substraten en korte penta-peptiden.

(IV) *In vivo* wordt het voorloper molecuul van osteocalcine gesynthetiseerd met een propeptide. Niettemin heeft dit propeptide geen meetbare affiniteit voor carboxylase. Hierdoor blijft de functie van het osteocalcine propeptide onduidelijk. Het is mogelijk dat dit propeptide noodzakelijk is voor een efficiënter gebruik van KH_2 , via een verlaging van de K_m van deze cofactor.

Discussie

Recombinante synthese in *E. coli* van descarboxy osteocalcine al dan niet met een propeptide is moeilijker gebleken dan werd verondersteld. Een enkele verklaring voor het lage expressienivo van osteocalcine dat wij vonden is niet te geven. Een feit is echter dat slechts zéér weinig onderzoeksgroepen in de wereld er in geslaagd zijn

kleine hoeveelheden bacterieel gesynthetiseerd osteocalcine te produceren. Aangezien gedecarboxyleerd bovine osteocalcine (bd-OC) al unieke substraateigenschappen vertoonde in het verleden was één van de redenen om onze onderzoekslijn te starten. Onze experimentele data, afkomstig van organisch gesynthetiseerd decarboxy osteocalcine (d-OC) hebben de al bekende resultaten bevestigd en verder gefundeerd: osteocalcine bezit een aantal unieke substraateigenschappen. Verder gefundeerd aangezien interpretatie van resultaten uit het verleden, verkregen met bd-OC in gewassen microsomen moeilijk was aangezien zowel het substraat als het enzym van bovine oorsprong was, waardoor de eigenschappen zouden kunnen afwijken van de humane eiwitten. Bovendien was het niet mogelijk om beschadigingen aan het bd-OC als gevolg van de thermische decarboxylering vast te stellen. In de experimenten beschreven in dit proefschrift hebben we gebruik gemaakt van gezuiverd humaan recombinant carboxylase (hoofdstukken 4, 5 en 6). Dit carboxylase is vrij van contaminerende en belemmerende propeptides of endogene substraten wat onze experimentele resultaten verder versterkt. De resultaten in dit proefschrift hebben nieuwe inzichten verschaft in de wijze waarop Gla-eiwitten in staat zijn aan carboxylase te binden. Wat voorheen onbekend was, is dat carboxylase over tenminste twee hoog-affiniteits bindingsplaatsen voor Gla-voorlopereiwitten beschikt. Of deze twee bindingsplaatsen de enigen zijn op carboxylase is nog onbekend, maar dit kan aanzienlijke consequenties hebben voor de optimale synthese van bijvoorbeeld recombinante Gla-eiwitten in eukaryote cellen, noodzakelijk voor de behandeling van hemofiliepatiënten. Echter, een gedegen begrip van de structuur en de interacties van de Gla-eiwitten met het carboxylase zijn noodzakelijk om maximale en efficiënte synthese en carboxyleringsgraad te verkrijgen.

De recente ontdekking van nieuwe Gla-eiwitten waaronder het carboxylase zelf, of Gla-eiwitten die zijn betrokken celgroei (Gas-6) of bij nog onbekende processen (PRGP-1 en PRGP-2) geeft aan dat de ontdekking van nieuwe Gla-eiwitten niet uit te sluiten is ^{1,2}. De wijze van interactie tussen deze nog te ontdekken Gla-eiwitten en het carboxylase zou, net als osteocalcine, kunnen plaatsvinden op een propeptide onafhankelijke wijze. Dit kan worden geïllustreerd met het volgende hypothetische

rekenvoorbeeld; Men vermoedt dat het menselijke genoom uit circa 100.000 genen bestaat. Hiervan is 30 % inmiddels bekend, wat overeenkomt met 30.000 verschillende eiwitten. Indien de nu 13 bekende Gla-eiwitten 30 % vertegenwoordigen van het totale aantal Gla-eiwitten houdt dit in dat er nog 26 Gla-eiwitten kunnen worden ontdekt. Van deze 26 Gla-eiwitten zullen ten minste 2 eiwitten het carboxylase binden zonder de noodzaak van een propeptide. Dit houdt in dat carboxylase een veelzijdig enzym is dat in staat is om verschillende typen eiwitten te binden.

De rol van Gla als Ca^{2+} (of andere divalente metaal-ionen) bindend aminozuur blijft discutabel. Er bestaan veel eiwitten die zeer goed in staat zijn om Ca^{2+} te binden zonder de noodzaak van Gla-residuen. Osteonectine, een non-Gla basaal membraaneiwit afkomstig uit bot en bloedplaatjes bevat een glutaminezuurrijk Ca^{2+} bindend domein. Gas-6 bijvoorbeeld, fungeert als een ligand voor verscheidene proteïne kinasen die betrokken zijn bij cellulaire groeiregulatie. Een deletiemutant van Gas-6, waarvan het volledige Gla-domein ontbreekt, is zeer goed in staat om aan de extracellulaire domeinen van twee celadhesie-molecuul gerelateerde tyrosine kinase receptoren Rse en Axl te binden en zo de fosforylering te activeren ³ Hierdoor blijft onduidelijk wat de functie van de Gla-residuen in Gas-6 of andere (nog onbekende) Gla-eiwitten is. Het vitamine K-afhankelijke carboxylase zelf is eveneens een Gla-eiwit, met 3 Gla-residuen ⁴. De functie en de positie van deze Gla-residuen in het carboxylase molecuul zijn nog niet bekend. Bovendien is het nog niet bekend of carboxylase wel in staat is te functioneren zonder Gla-residuen. Gla residuen zijn ook al in 1982 ontdekt in hermatypische koralen, maar de herkomst van dit Gla bleef onbekend ⁵. Er zijn tevens Gla-eiwitten ontdekt in de gifklieren van de roof-zeeslak *Conus geographus* ⁶. Dit was het eerste en voorlopig enige ongewervelde organisme waarvan bekend werd dat het posttranslationeel carboxyleerde. Tot dan toe werd aangenomen dat carboxylering uitsluitend was voorbehouden aan gespecialiseerde zoogdiersystemen. Het *Conus* Gla-eiwit, conantokine-G genaamd, is een peptide met een lengte van 17 aminozuren met daarin 5 Gla-residuen. Het conantokine-G remt de *N*-methyl-D-aspartaat receptor, waardoor vissen worden verlamd en zo een gemakkelijke prooi vormen voor de op vis jagende zeeslak. Naast conantokine-G zijn er tenminste 2

andere Gla-residu bevattende conantokines bekend, namelijk conantokine-T en conantokine-R^{7,8}. De voorlopereiwitten van de conantokines bezitten eveneens een propeptide dat met grote affiniteit bindt aan het *Conus*carboxylase, maar niet aan zoogdiercarboxylase. Dit houdt in dat er verschillen bestaan in het enzym of het *Conus* propeptide versus de zoogdierpropeptiden. Of het d-OC in staat is om met hoge affiniteit te binden aan het *Conus*carboxylase dient nog onderzocht te worden, maar zou zeer wel mogelijk kunnen zijn. Dit zou additioneel bewijs kunnen vormen voor de evolutionaire overgang van ongewervelde naar gewervelde dieren en zo een aanvulling kunnen zijn voor de theorieën omtrent de evolutie van de mens.

Het belang van de nieuw ontdekte carboxylase herkenningssequentie is dat het voor substraten de mogelijkheid biedt om carboxylering plaats te laten vinden op een significante afstand van de N-terminus. Normaliter vindt de carboxylering van propeptide-bevattende substraten plaats binnen de eerste 40 N-terminale aminozuren. Dankzij de door Käkönen et al. gesynthetiseerde chimere osteocalcine moleculen is aangetoond dat carboxylering op iedere plaats in een groot eiwit mogelijk is (hoofdstuk 6). Met deze kennis is het wellicht mogelijk om nieuwe leden van deze klasse van Gla-eiwitten op te sporen.

De resultaten welke zijn beschreven in dit proefschrift zullen ertoe bijdragen dat de eigenschappen welke noodzakelijk zijn in het mechanisme van de hoge-affiniteits associatie tussen de verschillende Gla-eiwitten, met name osteocalcine, en het vitamine K-afhankelijke γ -glutamyl carboxylase beter begrepen worden.

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Curriculum Vitae

De auteur van dit proefschrift werd op 3 augustus 1968 geboren te Goirle. Na een kortstondig verblijf in Tilburg verhuisde hij naar Vught. Hier werd basisschool "De Baarzen" doorlopen, waarbij het eindrapport van de 6^e klas aanleiding gaf om in 1980 zijn middelbare schoolcarrière te starten aan het "Maurick College", eveneens gevestigd te Vught. De brugklas bleek toch lastiger dan verwacht, zodat na de nodige taken gedurende de zomervakantie, werd gepoogd het HAVO met goed gevolg af te leggen. In 1986 werd dit diploma behaald, waarna het VWO de volgende uitdaging vormde.

In 1988 werd ook dit met goed gevolg afgelegd, zodat Roger eindelijk in Maastricht kon gaan studeren aan wat toen nog de Rijksuniversiteit Limburg was. Gezondheidswetenschappen bleek bij nader inzien een zeer goed alternatief te vormen voor Geneeskunde, en met name de afstudeerrichting "Biologische Gezondheidkunde" waarbinnen het biomedische wetenschappelijk onderzoek een prominente rol speelde, had zijn warme belangstelling. In december 1992 werd begonnen met de afstudeerstage bij de vakgroep Moleculaire Celbiologie en Genetica, aan de faculteit der Geneeskunde, eveneens in Maastricht. Hier werd door Dr. Guillaume van Eys en Dr. Rob Slobbe getracht Roger te bekwamen in de recombinant DNA-technologie.

In oktober 1993 werd Roger benaderd door Dr. Cees Vermeer en Dr. Berry Soute (vakgroep Biochemie, faculteit der Geneeskunde te Maastricht) om als student-assistent de recombinant DNA-technologie te implementeren binnen de divisie vitamine K. In juli 1994 werd de doctoraalbul Gezondheidswetenschappen behaald, waarna hij in september 1994, gevrijwaard van militaire dienst, assistent in opleiding werd bij de divisie vitamine K (Dr. C. Vermeer, vakgroep Biochemie, faculteit der Geneeskunde te Maastricht). Gedurende deze periode heeft hij in november 1995 twee weken fermentatieonderzoek gedaan bij Dr. Robert Azerad (Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques Université R. Descartes, Paris, France) en heeft hij gedurende de maanden mei en juni 1998 onderzoek gedaan in het laboratorium van Prof. Dr. Darrel W. Stafford, (Department of Biology, The University of North Carolina at Chapel Hill, USA). De resultaten van het vierjarig promotieonderzoek staan beschreven in dit proefschrift.

Roger is getrouwd met Jacqui, woonachtig te Amersfoort en thans werkzaam als medical advisor bij Excerpta Medica Medical Communications te Almere-Stad.

Bedankt / Thanks / Merci

Alhoewel de kaft en titelpagina doen vermoeden dat ik al het onderzoek, waarvan de resultaten in dit boekje staan beschreven, helemaal in mijn eentje heb uitgevoerd, is het tegendeel waar. Een proefschrift schrijf je nooit alleen en zeker niet dit exemplaar.

Allereerst wil ik mijn beide promotores Prof. Dr. H.C. Hemker en Prof. Dr. F.C.S. Ramaekers bedanken; beste Coen en Frans, bedankt voor het vertrouwen door jullie in mij gesteld en het verlenen van de nodige vrijheid bij het tot stand komen van dit proefschrift.

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De beide periodes die wij samen in het buitenland hebben doorgebracht heb ik als enorm prettig ervaren, en waren daarom ook nooit vervelend. We hebben vaak discussies gevoerd en dan bleek maar al te vaak dat je niet te snel mensen moet beoordelen op grond van wat anderen over die persoon vertellen. Je eigen indruk is nog altijd de beste. Berry, het ga je goed.

Leon, wij zijn ongeveer tegelijkertijd begonnen en wilden allebei dolgraag onder militaire dienst uit. Jij koos de wel een erg drastische oplossing, je besloot namelijk te trouwen met Romy. Ikzelf probeerde het op onmisbaarheid voor het onderzoek te gooien en ook dat lukte. Jij en Romy hebben inmiddels een dochtertje, dus heb je uiteindelijk de juiste keuze gemaakt. Leon, jij was een hele prettige collega met een aanstekelijke vette lach en een geweldig gevoel voor humor, vooral als je de avond van tevoren voetbaltraining had gehad. Jouw spontane manier van mensen benaderen gecombineerd met inzicht en motivatie zal ervoor zorgen dat we in de toekomst nog veel van je zullen horen.

Paul, het oermens. Gij mee oew brabantse humor, ook al ben je van origine geen brabander. Jij bent door een hele donkere periode gegaan na het overlijden van Yvette. De wijze waarop jij je hebt teruggevochten uit dit dal boezemde bij mij enorm veel ontzag in. Je reist iedere dag vanuit Eindhoven met de trein op en neer, in je vrije tijd mountainbike je, klim je wat en trek je zo af en door de wildernis van Canada. Vooral om dat laatste benijd ik je zeer. Je hebt de zaken goed voor elkaar en bent een keiharde werker al ben je af en toe wel erg eigenwijs. Niet voor niets is er nu een MGP-testkit. Respect Paul, respect.

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Mocht ik, ondanks dit uitvoerige dankwoord, dan toch nog iemand vergeten zijn;

BEDANKT HÉ !

A handwritten signature in black ink. It features a large, stylized capital 'R' that loops around the word 'Roger', which is written in a cursive script.