Enzyme/substrate interactions of the vitamin K-dependent carboxylase

ENZYME/SUBSTRATE INTERACTIONS OF THE VITAMIN K-DEPENDENT CARBOXYLASE

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ENZYME/SUBSTRATE INTERACTIONS OF THE VITAMIN K-DEPENDENT CARBOXYLASE

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Het leek me beter alles nog es over te doen

Herman van Veen

CONTENTS

List of abbreviations

BGP	Bone Gla protein (osteocalcin)
BiP	Binding protein
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
d-	Refers to a decarboxylated protein
DP	Docking protein
DT	DPNH (NADH) and TPNH (NADPH)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FLEEL	The pentapeptide phenylalanine-leucine-glutamic acid-glutamic acid-leucine
FLEEV	The pentapeptide phenylalanine-leucine-glutamic acid-glutamic acid-valine
FPLC	Fast protein liquid chromatography
FIX	Factor IX
γ-CRS	Gamma-glutamyl carboxylase recognition site
GCI	Glycoprotein crystallization protein
Gla	Gamma-carboxyglutamic acid
Glu	Glutamic acid
HPLC	High performence liquid chromatography
K	Vitamin K quinone
KH_2	Vitamin K hydroquinone
KO	Vitamin K 2,3 epoxide
MGP	Matrix Gla protein
M_r	Relative molecular mass
NAD(P)H	Nicotinamide adenine dinucleotide(phosphate) reduced form
PBS	Phosphate buffered saline
PGP	Plaque Gla protein
Pro	Propeptide
PT	Prothrombin
RER	Rough endoplasmic reticulum
RIA	Radioimmuno assay
SDS	Sodium dodecyl sulfate
SGP	Sperm Gla protein
SRP	Signal recognition particle
TCA	Trichloroacetic acid

CHAPTER 1

INTRODUCTION

INTRODUCTION

Historical background of vitamin K and vitamin K antagonists

Vitamin K was discovered by Henrik Dam in 1935 when he was studying the effects of a cholesterol-free diet on chickens (1). He observed that after some weeks the animals developed a bleedings tendency and that their blood exhibited a prolonged coagulation time in in vitro coagulation assays. This effect could not be counteracted by the addition of purified cholesterol to the diet. During the extraction of the cholesterol from the food another compound apparently had been extracted simultaneously. This fat-soluble vitamin was called Koagulations-vitamin (coagulation vitamin) or vitamin K. Nowadays several compounds are known to contain vitamin K activity: Phyloquinone (vitamin K_1) which is present in green vegetables and the menaquinones (vitamin K_2 or MK) a group of related products from microbial origin (fig.1).

The bleeding symptoms, provoked by the vitamin K deficient diet, were similar to those described in the 1920's when a hemorrhagic disease prevailed among cattle in Canada and the United States of America. This disease was found to be caused by feeding the animals with improperly cured sweet clover hay (2). The active compound, 3,3'-methylene-bis-(4-hydroxycoumarin) (dicoumarol) (3), was shown to act as an antagonist of vitamin K (fig.1). On the basis of its structure a number of derivatives have been synthesized all

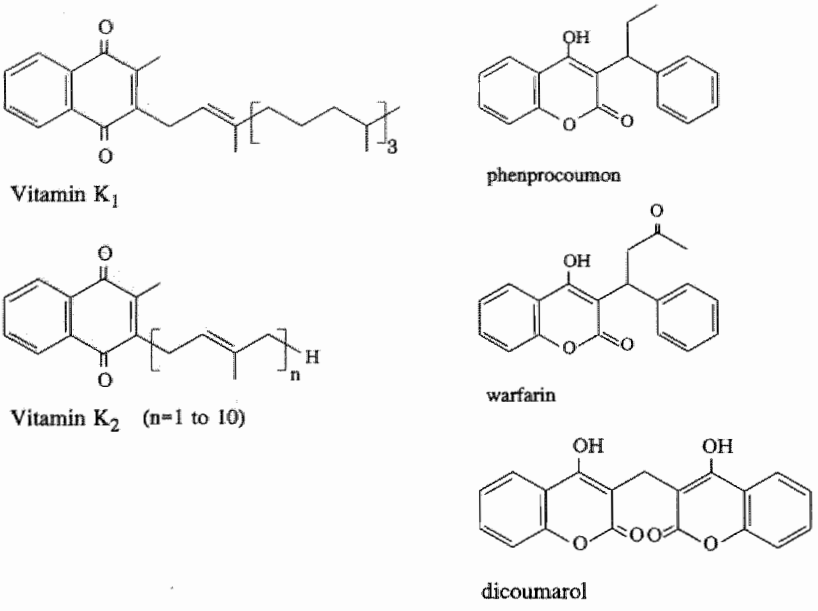

Fig. 1. Structure of vitamin K_1 and K_2 and some vitamin K antagonists.

exhibiting anticoagulant activity. These components are used in the production of rodenticides (warfarin, brodifacoum, flocoumafen) as well as for treatment and profylaxis of thrombotic episodes in men (acenocoumarol, phenprocoumon, warfarin).

Gamma-carboxyglutamic acid

Hemker et al. (4) postulated on kinetic grounds that plasma from dicoumarol treated patients contained an abnormal form of prothrombin (factor II) which they called PIVKA II (protein induced by vitamin K antagonists). This abnormal protein was considered to be the precursor of prothrombin. Ganrot and Nilehn (5) and Josso (6) supported this hypothesis by demonstrating the presence of a protein in the plasma of patients receiving dicoumarol which was immunochemically similar to prothrombin, but inactive in the in vitro coagulation assay. The abnormal form of prothrombin was not able to bind calcium ions (7,8). The presence of other abnormal coagulation factors (factors VII, IX and X) was reported by several authors (9-16) shortly afterwards. It was not until 1974, that the nature of this abnormality was identified. Several groups reported independently (18-20) that the calcium binding sites in

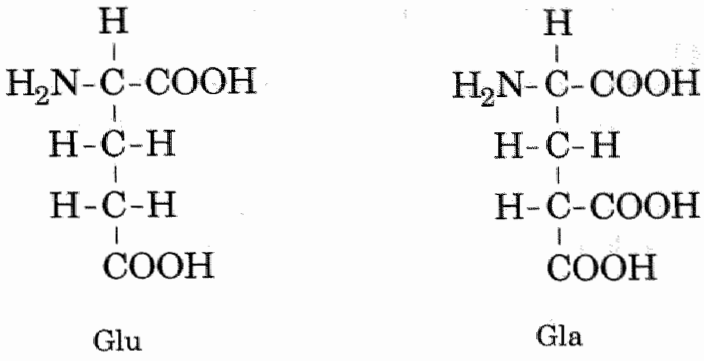

Fig. 2. Structures of glutamic acid (Glu) and γ-carboxyglutamic acid (Gla).

prothrombin were gamma-carboxyglutamic acid residues (Gla, see fig. 2). In abnormal prothrombin these Gla-residues were not present. From these data it was concluded that vitamin K was involved in the conversion of a number of glutamic acid residues (Glu) into Gla (19,20). Later it was found that this conversion is accomplished in the post-translational phase of protein biosynthesis.

For a long time it has been thought that the four Gla-containing coagulation factors (prothrombin and the factors VII, IX and X) were the only proteins requiring vitamin K for their synthesis. During the last decade, however, several other Gla-containing proteins have been isolated. A common feature of these proteins is that they all bind calcium ions and that they rely on their Gla residues for this binding. In those cases in which the function of Gla-proteins is known, the presence of Gla is essential for their biological activity. Other examples of Gla-containing proteins are the coagulation inhibiting proteins protein C and S and several proteins not related to blood clotting like the bone proteins osteocalcin and matrix Gla protein. These proteins will be discussed later (page 20).

Vitamin K-dependent carboxylation in vitro

Suttie (29) demonstrated an accumulation of the vitamin K-dependent coagulation factor precursors in the liver of rats which had been treated with warfarin prior to sacrifice. An in vitro system for the detection of vitamin K-dependent carboxylase activity was developed, and the enzyme was found to be present in the post-mitochondrial fraction of rat liver homogenates (28).

Radiolabeled $^{14}CO_2$ could be incorporated into the endogenous precursor proteins in the presence of vitamin K. The label was incorporated into Gla residues at the N-terminus of the mature protein.

The enzyme vitamin K-dependent carboxylase is located at the luminal side of the rough endoplasmic reticulum (RER). The enzyme activity can be extracted from the membrane of the microsomal fraction with the aid of detergents. Additional requirements for the in vitro carboxylation reaction are O_2, CO_2, a carboxylatable substrate, and vitamin K hydroquinone.

To be able to study the mechanism of the vitamin K-dependent carboxylation reaction it was necessary to develop exogenous substrates. Comparison of the N-termini of the Gla-containing proteins show homologous regions (fig. 3).

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

Fig. 3. Conserved amino acid in the Gla-domains of various vitamin K-dependent proteins. The predicted amino acid sequences are based upon published cDNA sequences for human prothrombin (25), factor VII (38), factor IX (26), factor X (37), protein C (39), osteocalcin (46) and bovine protein S (40), protein Z (143) and MGP (amino acid residues 32-61)(17). Sequences are given in standard one-letter symbols. X represents Gla. Possition number 1 corresponds with the first amino acid residue of osteocalcin. The sequences have been aligned to give maximum homology.

Peptides, based upon these amino acid sequences in which Gla was replaced by Glu, have been synthesized (30-34), and were active as substrates for the in vitro carboxylase system. Another set of exogenous substrates used to study this enzyme reaction is formed by the so called "natural substrates". These substrates were derived from non-carboxylated or decarboxylated Gla-proteins (35,36).

Non-hepatic vitamin K-dependent carboxylase

For many years the only Gla-containing proteins known were the four coagulation factors and it was supposed that the vitamin K-dependent carboxylase was exclusively present in the liver, despite the fact that vitamin K could be extracted from other tissues as well (122,123). The only function

known for vitamin K in vertebrates is to mediate in the vitamin K-dependent carboxylase reaction. In 1976 Hauschka et al. (103) demonstrated vitamin K-dependent carboxylase activity to be present in kidney microsomes. Later on several authors reported the occurrence of this enzyme system in a variety of other tissues, like spleen, testis, lung, pancreas, placenta, vessel wall and several isolated tumors (113-117). Also cultured cells, like osteoblasts, endothelial cells, hepatocytes and various cultured tumor cells were reported to contain the vitamin K-dependent carboxylase (118-121). Vermeer et al. (113) reported that, like in the liver, also in extrahepatic tissues vitamin K antagonists induce the accumulation of precursor proteins capable of acting as an "endogenous" substrate for carboxylases in vitro. A few extrahepatic Gla-containing proteins have been characterized, but their physiological importance is not clear at this time.

Vitamin K reductases

The role of vitamin K in blood coagulation has been reviewed on several occasions (49-52). It is widely accepted now that the reduced form of vitamin K, vitamin K hydroquinone (KH_2), is the cofactor for the vitamin K-dependent carboxylase. In the liver two other forms of vitamin K have been identified, vitamin K 2,3-epoxide (KO) and vitamin K quinone (K). It has been postulated that these three forms of vitamin K are interconverted into each other in a cyclic way. Several arguments support this assumption: 1. The dietary supply of vitamin K from food occurs primarily in its quinone form so that a reduction step has to precede the carboxylation reaction; 2. It may be calculated that on a molar base the urinary Gla excretion is several thousand fold higher than the vitamin K ingestion. An efficient recycling of the vitamin would explain the discrepancy; 3. Unless the reduction of KO is blocked (with coumarin derivatives) the epoxide is not set free in circulation. This is consistent with the hypothesis that the majority of the KO formed is metabolized by the reductases of the vitamin K cycle; 4. In vitro the carboxylation reaction can be started with either form of the vitamin, provided that a reducing agent is added and no reductase inhibitor is present.

Figure 4 shows the cyclic pathway of vitamin K. In step 1 vitamin KH_2 is converted to vitamin KO by the action of vitamin K epoxidase (133). The activity of the epoxidase is closely associated with the vitamin K-dependent carboxylase activity, but not strictly coupled. Addition of CN^- has been demonstrated to uncouple both enzyme activities in vitro (61,63). Also in the

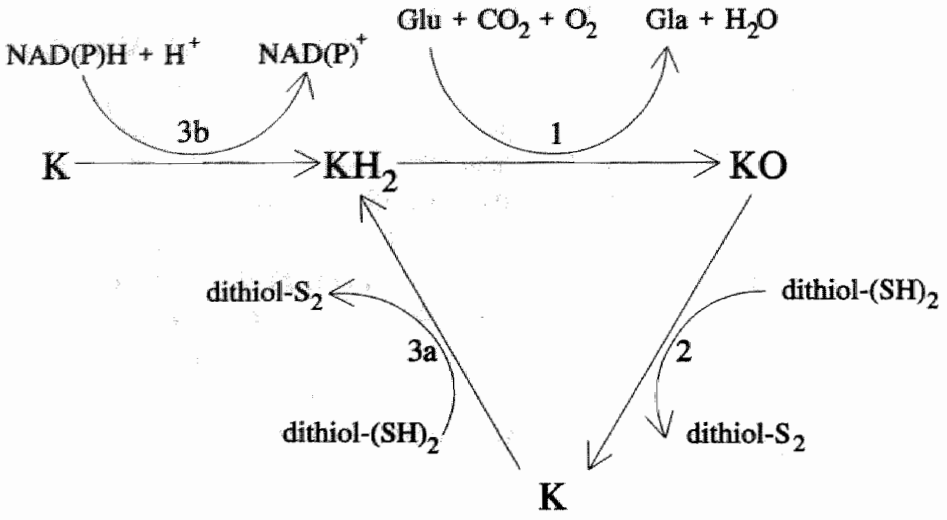

Fig. 4. Schematic representation of the vitamin K cycle.

absence of CO_2 or a carboxylatable substrate the formation of KO continues, while carboxylation stops (61,62). It is generally accepted now that the energy required for the carboxylation of glutamic acid residues is generated by the oxidation of KH_2 by O_2 (58-60). In step 2 vitamin KO is reduced to vitamin K by the action of vitamin KO reductase. In vitro the reducing equivalents required for the reduction of KO may be provided by dithiothreitol (DTT), but its physiological counterpart has not been found yet. Recent studies (53) have postulated a model in which thioredoxin plays an important role in this reaction. In step 3a and 3b vitamin K is reduced to KH_2. In vitro this reaction can be catalyzed by at least two enzymes, one driven by DTT and the other(s) by NAD(P)H.

Whether the DTT-dependent reduction of KO and K is accomplished by the same enzyme is not clear, but it is at least striking that both enzymatic activities share a number of characteristics, e.g. their extreme sensitity to coumarin derivatives (64,124). Wallin et al. (65) has purified the NAD(P)H-dependent DT-diaphorase from rat liver. The enzyme was found to be present in both, cytosol and microsomes. The NAD(P)H-dependent enzyme is relatively insensitive to coumarin derivatives (66,67). It is assumed that under normal physiologic conditions the DTT-dependent reductases play a dominant role in the generation of vitamin KH_2. However, if these enzymes are inhibited by coumarin derivatives, high doses of vitamin K can activate the NAD(P)H-dependent reductase, and thus help to overcome the action of the drugs.

Synthesis of Gla-containing proteins

All Gla-containing proteins known so far are secretory proteins. These proteins are secreted into the extracellular fluid or to the outer cellular membrane after maturation. The membrane translocation and synthesis of secretory proteins in general has been studied extensively by Walter and Blobel (21) and Meyer et al. (22,23). The biosynthesis of the Gla-containing blood coagulation factors has been studied by several authors (24-26,38,48,125-128), whereas the group of Price investigated the molecular biology of the Gla-containing bone proteins (47,88,129).

Secretory proteins are synthesized in a precursor form which contains a hydrophobic leader sequence at the N-terminus of the growing polypeptide. Once this signal peptide has been synthesized it is recognized by a signal recognition particle (SRP), which occurs in the cytoplasm. Further translation of the mRNA comes to a halt upon binding of the SRP to the signal peptide. At the surface of the endoplasmic reticulum an SRP receptor or docking protein (DP) is present. The formation of the ribosome-SRP-DP complex results in the continuation of polypeptide chain elongation. The hydrophobic signal sequence penetrates into the RER facilitating the transport of the growing protein to the luminal side of the ER. Here the enzymes are located which mediate posttranslational modifications such as glycosylation, gamma-glutamyl carboxylation, hydroxylation and also the cleavage of the leader sequence. Presently over a hundred of those post-translational modifications are known. Obviously not all proteins undergo all possible modifications, which means that the enzymes involved in the post-translational processing of proteins must be able to discriminate between the various secretory proteins.

Substrate specificity

The first synthetic peptides used in the in vitro vitamin K-dependent carboxylation reaction were based upon the homologous regions in the N-termini of the mature proteins. The ones used most frequently are Phe-Leu-Glu-Glu-Leu and Phe-Leu-Glu-Glu-Val, homologous with amino acids 5 to 9 of several Gla-containing blood coagulation factors (fig. 3). Although these peptides were carboxylated readily, the fact that the Km^{app} values were in the millimolar range demonstrate that their affinity for the enzyme is very low. Apparently a recognition site for carboxylase is missing in these small peptides. Attempts to create good substrates by decarboxylation of mature clotting factors

failed. The size of the protein and its tertiary structure might prohibit the right configuration of the enzyme-substrate complex, or even prevent the formation of the complex at all. Smaller Gla-containing proteins or proteolytic digests of clotting factors were prepared and decarboxylated. Substrates like descarboxy osteocalcin or descarboxy fragment Su (amino acid residues 13-29 of prothrombin), have Km^{app} values which are three orders of magnitude lower than those of the synthetic pentapeptides. These 'natural' substrates probably do possess a recognition site for carboxylase. A real breakthrough in finding the recognition site for carboxylase came when the vitamin K-dependent proteins were cloned and the amino acid sequences were determined.

Searching for the human factor IX cDNA in a human liver c-DNA library, Kurachi and Davie (26) identified a plasmid containing the factor IX cDNA insert. The DNA of the selected clone was longer than might be expected from the molecular weight of the mature protein. Besides the coding region for the mature plasma protein it contained a leader sequence of 137 base pairs at the 5' end and a 48 base pairs fragment at the 3' end of the DNA. The leader sequence consisted of the signal peptide which is present in all secretory proteins and a pro-sequence which was unique in this protein. At about the same time Degen et al. (25) cloned the cDNA for human prothrombin. The cDNA did not contain the complete signal peptide but it did have a pro-sequence between the signal peptide and the mature protein. The pro-sequence was also found in the cDNA's of other Gla-containing (37-40), and if properly aligned the various prosequences show considerable homology (fig. 5). Therefore the pro-sequence was thought to play an important role in the recognition of precursor proteins by the vitamin K-dependent carboxylase (40,42,43,45-47). Several naturally occurring mutant forms of factor IX have been isolated from the plasma of

Fig. 5. The amino acid sequences of the propeptide regions of various Gla-containing proteins. The predicted amino acid sequences are based upon published cDNA sequences for human factor II (25), factor VII (38), factor IX (26,142), factor X (37), protein C (39), osteocalcin (46) and bovine protein S (40) and MGP (amino acid sequence 7-31 of the mature protein) (17).

patients suffering from hemophilia B (42-44,130,131). The factor IX mutants are inactive in the clotting cascade, and have molecular weights which are slightly higher than those of the normal plasma protein. The mutant proteins contained an 18 amino acid sequence at the N-terminal of the mature protein, that normally was cleaved before secretion. Further characterization of the abnormal proteins showed that a mutation had occurred in the propeptide region. Factor IX San Dimas, factor IX Oxford-3, factor IX Kawachinagano and factor IX Troed-y-Rhiw were found to exhibit the same mutation: the substitution of arginine -4 by glutamine (43,44,130,131). Factor IX Cambridge had a point mutation at -1 where arginine had been replaced by serine (42). Contradicting results about the carboxylation of these proteins have been presented in the literature. Only factor IX Kawachinagano was reported to be fully carboxylated (131).

Jorgensen et al. (48) demonstrated the importance of the propeptide in experiments with recombinant factor IX. Modifications were made in the propeptide region of the cDNA of factor IX. These "mutant" forms of factor IX were expressed in Chinese hamster ovary cells. The amount of Gla-residues per molecule of factor IX was determined. Deletion of the complete propeptide region prevented the protein from being carboxylated. Mutations made at the highly conserved amino acid residues, phenylalanine -16 and alanine -10 almost completely abolished carboxylation, but did not interfere with the propeptide cleavage. It has been widely accepted now that the amino acid residues at position -1 to -4 are the propeptidase recognition site. The proposal of Furie and Furie (68) that the carboxylation recognition site is located exclusively in the propeptide cannot explain the low Km of fragment Su and descarboxy-osteocalcin in the in vitro carboxylation system. Wether the consensus sequence Glu-X-X-X-Glu-X-Cys might add to the vitamin K-dependent recognition site, as was suggested by Price et al. (69), remains to be seen, however.

The vitamin K-dependent proteins

Injury of a blood vessel initiates various complex events resulting in the formation of the clot. One of the components of a clot is fibrin which is formed after a series of sequential activations of pro-enzymes to enzymes. Three of these activation steps occur at phospholipid surfaces, and in all three steps Gla-containing blood coagulation factors are involved. The latters only bind to the negatively charged phospholipid surfaces in the presence of calcium ions. The calcium binding causes conformational changes in the Gla-domain of the

protein, resulting in the exposure of the phospholipid-binding site at the surface of the protein (55-57). The actual binding site, however, has not yet been determined.

Three other Gla-containing proteins in blood plasma are the proteins C, S and Z. The first two have a regulatory function in the blood coagulation. Protein C is an anticoagulant which, upon activation by thrombin (70,71), degrades the factors Va and VIIIa (71). In its unbound form protein S stimulates the activation of protein C. About 60% of the circulating protein S, forms a 1:1 complex with C_4-binding protein (73). The function of protein Z is unknown.

Several Gla-containing proteins have been found in calcified tissues. The one studied most extensively is osteocalcin. This protein, also called bone Gla protein (BGP), is among the six most abundant proteins in man and is present in bone, dentin and cementum (74-77,82). It contains 47-50 amino acids, depending on the species, of which the residues at positions 17, 21, and 24 are Gla. A model of the protein in its three dimensional structure based upon Laser Raman Spectroscopy (78) and nuclear magnetic resonance (NMR) (79-81) showed that the three Gla residues are located at one side of the molecule, and that the spacing between the Gla residues is the same as the spacing of the calcium ions in the hydroxyapatite crystal. The binding of osteocalcin to the hydroxyapatite matrix of the bone is very tight, and is highly dependent on the degree of carboxylation of the protein. In man the glutamic acid residue at position 17 is not carboxylated (97). This may explain why in man the concentration of osteocalcin in bone is only 10-20% of that in other species. Comparison of the amino acid sequence of osteocalcin from several species shows that its primary structure is highly conserved during evolution (fig. 6). After it has been synthesized by the osteoblasts most of the secreted osteocalcin is bound to the hydroxyapatite crystal matrix of the bone and only a

```
Cow          YLDHWLGAOAPYPDPLXPKRXVCXLNPDCDELADHIGFQEAYRRFYGPV
Monkey       --YQ-------------------------------------------
Man          --YQ----PV------E-R-----------------------------
Cat          --APG---------------I----------------D-------T-
Rat          --NNG-------------H--------N-----------D--K-I--TTV
Pig          ----G-------------R-------------------------IA
Goat         ----G-------------------------------------------
Sheep        ---PG-------------R-----------------------------
Wallaby      --YQT--F-------QEN--------------------S--------TA
Chicken    H-AQDSGV-G-- -N---AQ------S--------Q-------------
```

Fig. 6 The amino acid sequences of osteocalcin in various species. The sequences are in the one-letter code. O stands for hydroxyproline and X for Gla. Amino acid sequences for monkey (99), human (46,97), cow (98), cat (145), rat (88), goat, sheep, pig and wallaby (146) and chicken (144) osteocalcins have been aligned to give maximum homology.

minor fraction is released into the blood stream. Synthesis of the protein in the absence of vitamin K or in the presence of vitamin K antagonists results in a decreased Gla content and in a reduced affinity for the hydroxyapatite matrix (83-95). Price and Kaneda (96) showed that in vivo vitamin K can reverse the effect of warfarin on the blood coagulation factors but not that on the binding of serum osteocalcin to hydroxyapatite.

Another Gla-containing protein in bone is matrix Gla protein (MGP). MGP is synthesized in osteoblasts, cartilage and in a variety of soft tissues. The protein consists of a single polypeptide chain of 79 amino acids and contains 5 Gla residues. MGP is the only known Gla-containing protein so far lacking the propeptide in the precursor protein. However, a region in the mature protein was shown to be homologous with the propeptides of other Gla-containing proteins (fig.3). Although the precise function of both bone Gla proteins is still unknown, several reports have been published illustrating their importance for bone metabolism. In 1975 Pettifor and Benson (134) described fetal warfarin syndrome, a congenital malformation of bones in infants, caused by oral anticoagulants taken by the mother during the first trimester of pregnancy. The fetal warfarin syndrome (chondrodysplasia punctata) is characterized by excessive mineralization of the epiphyses and malformations of the axial skeleton, the proximal and facial bones (139-141). The same defects were seen in a boy with a congenital deficiency of KO-reductase (132). Complete growth plate closure due to excessive calcification and subsequent inhibition of longitudinal growth could be provoked in experimental animals (rats and chickens) by treating them with either warfarin or a vitamin K-deficient diet. Although osteocalcin has been shown to inhibit precipitation of calcium salts in vitro (72,74,97), it is not clear whether this protein also prevents calcification of the epiphysis in vivo. It has been suggested by Price (101) that MGP might be involved in this process because it is synthesized in cartilage. As early as in 1971 a Japanese group (135) reported a study in which three postmenopausal patients with severe osteoporosis were treated with menaquinone. The treatment with this form of vitamin K resulted in a decrease of urinary calcium excretion of 18 to 50%. In our laboratory studies were performed to investigate the influence of vitamin K. The results indicate that in postmenopausal women the daily intake of vitamin K may significantly reduce the calcium excretion among the group with a high urinary calcium excretion. The Gla-content of serum osteocalcin in the majority of postmenopausal women was 60-70% of normal, administration of vitamin K lead to a normalization of the Gla content as well as to an increase in circulating osteocalcin antigen. Vitamin K-antagonists probably have the opposite effect on the urinary calcium excretion. The distribution of

urinary calcium excretion in a group of young men receiving oral anticoagulant therapy was different than that in an age and sex matched control group (147). Recently Fiore et al. (138) demonstrated that in a group of women receiving long-term anticoagulant therapy the mineral content as well as the bone mass were significantly reduced as compared to a matched control group. Although the data described above suggest that Gla-containing proteins play an important role in bone metabolism, the actual mechanism of their regulatory role remains unclear.

Several other vitamin K-dependent proteins have been found over the years. Possible products of renal vitamin K-dependent carboxylase are the Gla proteins found in kidney, renal stones and urine (103-105). From human urine we have isolated a mixture of proteins containing one or more Gla-proteins (136). These proteins had a remarkable potency of inhibiting the precipitation of various calcium salts from supersaturated solutions. Nakagawa et al. (105) also reported a urinary Gla-containing protein, Glycoprotein Crystallization Inhibitor (GCI), which was purified to homogeneity. The authors claimed that GCI represented 90% of the total crystallization inhibitory capacity of urine, and that in a number of idiopathic renal stone formers GCI was Gla-deficient (100). Gla-containing proteins have also been found in calcified tissues like coral and calcified lesions of the skin (106-108). The function of these proteins has not been determined yet, but they possibly play a role in the regulation of the precipitation of calcium salts. Recently Van Haarlem (102) and Gijsbers et al. (137) have reported the isolation of a Gla-protein from human atherosclerotic plaques, which they called PGP. Neither its origin nor its function are known, however.

Sperm Gla-protein (SGP) was isolated from human spermatozoa (138). Because the hepatic Gla-proteins are mainly serine proteases, and because also spermatozoa contain several serine proteases, it seemed plausible that one of these proteins was identical to SGP. On the basis of its preliminary characteristics (M_r, amino acid composition) we have concluded, however, that SGP is dissimilar from any of the known sperm proteins characterized thus far.

Another group of Gla-containing proteins is that found in the venoms from various snakes (109,110). These proteins are able to activate the blood coagulation by binding to phospholipid micelles. The Gla-containing peptide from the venom of the fish hunting snail Conus geographus (111) are neurotoxins which induces a sleeplike state in mice younger than two weeks. Older mice become hyperactive when they are injected with these "sleeper" peptides.

Introduction to this thesis

In 1982, when the work presented in this thesis was initiated, the only tissues known to contain the vitamin K-dependent carboxylase were the liver and kidney. Since that time we have actively participated in demonstrating carboxylase activity in a wide variety of tissues and cultured eukaryotic cells. The first part of this thesis describes the discovery of a number of extrahepatic carboxylases, and summarizes our attempts to find tissue specific differences among these enzymes notably with respect to their substrate specificity and sensitivity towards coumarin derivatives. A major breakthrough in carboxylase research was the discovery of the role of the propeptide in the enzyme/substrate recognition. In the second part of this thesis we describe some structural requirements for this pro-sequence to be recognized by the carboxylase, and the use of propeptide as an affinity-ligand during the purification of carboxylase. In the last chapter we report on the unusual stability of the bone Gla protein osteocalcin in fossil bone, and the implications of this finding for phylogenesis.

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CHAPTER 2

COMPARISON OF HEPATIC AND NONHEPATIC CARBOXYLASE/REDUCTASE SYSTEMS

Summary in retrospect of the following papers:

- Vermeer, C. and Ulrich, M. Thrombos. Res. 28: 171-177, 1982
- Ulrich, M.M.W., Soute, B.A.M., de Boer-van den Berg, M.A.G. and Vermeer, C. Biochim. Biophys. Acta 830: 105-108, 1985
- Roncaglioni, M.C., Ulrich, M.M.W., Muller, A.D., Soute, B.A.M., De Boer-van den Berg, M.A.G. and Vermeer, C. Trombos. Res. 42:727-736, 1986
- Ulrich, M.M.W., Soute, B.A.M., van Haarlem, L.J.M. and Vermeer, C. Thromb. Haemost. 57: 17-19, 1987
- Van Haarlem, L.J.M., Ulrich, M.M.W., Hemker, H.C., Soute, B.A.M. and Vermeer, C. Biochem. J. 245: 251-255, 1987
- Ulrich, M.M.W., Knapen, M.H.J., Herrmann-Erlee, M.P.M and Vermeer, C. Thrombos. Res. 46: 27-32, 1988

COMPARISON OF HEPATIC AND NONHEPATIC CARBOXYLASE/REDUCTASE SYSTEMS

Introduction

During the last decade the presence of vitamin K-dependent carboxylase has been demonstrated in almost all tissues investigated. Examples are: liver, lung, kidney, bone and testis (1-5). Obviously the physiological functions of these various tissues are widely different, and hence also the nature and function of the respective Gla-proteins produced will differ substantially. This is illustrated, for instance, by the differences between the Gla-containing blood coagulation factors (which are all of hepatic origin) and the bone Gla-proteins. The question arose, therefore, whether the hepatic and extrahepatic carboxylase/reductase systems are similar, or whether small differences exist, for instance in their affinity for peptide or protein substrates, for vitamin K or for vitamin K-antagonists. In this chapter we will summarize our early attempts to answer these questions.

The recognition of carboxylatable proteins and peptides

After the structure of Gla had been identified (6-8), it was clear that the vitamin K-dependent step in protein biosynthesis is a carboxylation reaction. The

requirements for a peptide or protein to be recognized by carboxylase were unknown, however. In the initial attempts to study the carboxylation reaction in vitro, use was made of liver homogenates prepared from vitamin K-deficient animals. During the period of vitamin K deprivation non-carboxylated precursor proteins had accumulated in the liver, and it was demonstrated by Esmon et al. (9) that on addition of vitamin K and radiolabeled CO_2 to a postmitochondrial supernatant, these precursor proteins were carboxylated in vitro. Later on short synthetic peptides were prepared, which were analogous to amino acid sequences in the Gla-domain of the blood coagulation factors, but in which the Gla-residues were replaced by Glu. Several of these peptides turned out to function as substrates for vitamin K-dependent carboxylase in vitro (10-12). The development of these substrates was a milestone in carboxylase research, enabling the investigation of the enzyme in the absence of unknown and limiting amounts of endogenous precursor proteins. Instead the exogenous substrates could be added to the reaction mixtures in well known or even in saturating concentrations. A drawback of the short peptide substrates (generally not longer than 3-5 amino acid residues) is that their Km^{app} values are high (in the millimolar range), suggesting that they lack a recognition site for carboxylase. Increasing the length of the synthetic peptides did not improve this situation (13).

Substrate	origin	range of Km^{app} value	ref
FLEEL	synthetic based upon amino acid 5-9 human coagulation factor VII	2000-8000 μM	(33,13)
FLEEV	synthetic based upon amino acid 5-9 bovine prothrombin	2000-8000 μM	(13)
d-prothrombin	plasma prothrombin decarboxylated	400 μM	(33)
d-fragment 1	plasma prothrombin fragment 1 decarboxylated	400 μM	(13)
d-fragment Su	plasma prothrombin proteolytic (subtilisin) fragment of decarboxylated prothrombin, amino acid 13-29	1-3 μM	(14,33)
d-osteocalcin	bone protein decarboxylated osteocalcin	1-30 μM	(33,34)
d-SGP	spermatozoa decarboxylated sperm Gla protein	85-96 μM	(33,34)

Table I. Various substrates for the in vitro vitamin K-dependent carboxylation reaction and their Km^{app} values for the liver enzyme system.

We have followed another strategy and have tried to prepare carboxylatable substrates from naturally occurring Gla-proteins. After thermal decarboxylation the smaller proteins (osteocalcin, sperm Gla-protein) turned out to function as relatively good substrates for carboxylase, with Km^{app} values in the micromolar range. Larger proteins like decarboxylated prothrombin had first to be degraded by limited proteolysis before a comparably active substrate could be isolated (14). In table I we have summarized the various exogenous substrates and their Km^{app} values for bovine liver carboxylase, and it is clear that the affinity for hepatic carboxylase varies substantially from one substrate to the other. To answer the question whether nonhepatic carboxylases exhibit different substrate specificities, we have selected four substrates from table I and tested them in bovine carboxylating systems obtained from four different tissues. The results of this experiment are shown in table II. The four tissues chosen were: liver, kidney, lung and testis; the four substrates used were: the non-selective pentapeptide FLEEL, d-osteocalcin (which may be regarded as heterologous in all four systems), d-fragment Su (prepared from prothrombin and thus homologous in the liver system), and d-SGP (prepared from spermatozoa and probably homologous in testis carboxylase). The Km^{app} for FLEEL and d-osteocalcin varied 2-3 fold in the four carboxylases, but the differences obtained with d-fragment Su and d-SGP were much larger. In this respect it is striking that the lowest Km^{app} values for both substrates were obtained in the systems which were defined earlier as 'homologous'. The ratio between the Km^{app} for d-fragment Su and d-SGP was 0.035 in liver carboxylase and 2.3 in thetesticular enzyme system. This is a more than 65-fold difference, which is suggestive for some form of substrate specificity of carboxylases from different tissues.

	Km^{app} (μM)			
substrate	liver	kidney	lung	testis
FLEEL	2600	7100	5300	6500
d-osteocalcin	28	27	8	11
d-fragment Su	3	6	3	28
d-sperm Gla protein	85	158	204	12

Table II. Apparent Km values of different substrates in various enzyme systems. The apparent Km values were determined from initial carboxylation rates at various substrate concentrations under standard conditions.

Additional evidence for a putative role of the Gla-domain structure in its recognition by carboxylase was obtained from experiments in which we compared the ability of d-osteocalcin from different sources to function as a substrate for bovine liver carboxylase (32). It turned out that d-osteocalcin from the cow was a far better substrate than that from chicken, monkey or man. The

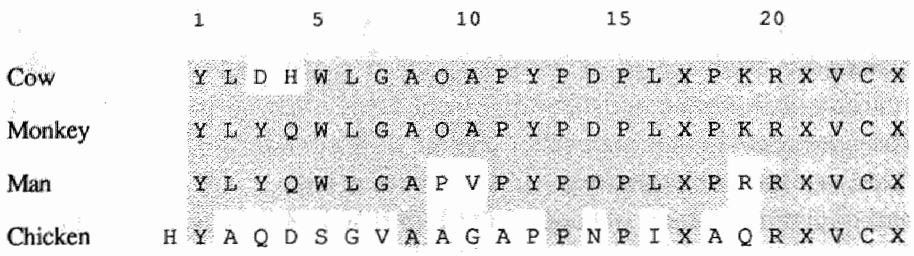

Fig. 1. N-terminal sequences of osteocalcin from various species. The sequences are in the one letter code. O stands for hydroxyproline and X for Gla. Amino acid sequences for monkey (37), human (35,38), cow (36) and chicken (39) osteocalcins have been aligned to give maximum homology.

substrate activity of the latter two proteins was only 5 and 10% of that of bovine d-osteocalcin. As can be seen from figure 1 osteocalcin is highly conserved during evolution, and there is not more than a two amino acid residues difference at position 3 and 4 between the primary structure of the bovine and the monkey protein. The fact that in spite of a more than 94% identity between the two molecules such large differences were found in their ability to function as a substrate for bovine liver carboxylase is consistent with the idea that besides the pro-sequence (see chapter 3) also the Gla-domain of the mature protein contributes to its recognition by vitamin K-dependent carboxylase.

Inhibitors of the vitamin K cycle

Many different compounds are known to interfere with the recycling of vitamin K, examples are: indandiones, chloro-K, coumarin derivatives and salicylates. Only the latter two groups are of present clinical relevance, which is the reason why we have restricted ourselves to these two drugs. As early as in 1922 it was known that cows fed with improperly cured clover hay developed a bleeding tendency with frequent haemorrhages (15). Almost 20 years later 4-hydroxycoumarin was identified as the active component, and since that time many derivatives have been synthesized which are used as rodenticides as well as for oral anticoagulant therapy in man. Their general mode of action is that they

block the dithiothreitol(DTT)-dependent reductase(s) in the vitamin K cycle via a mechanism not yet completely understood (16,17). By this blockade the recycling of vitamin KO comes to a halt, and the hepatic store of KH_2 is rapidly exhausted. This results in the appearance of undercarboxylated blood coagulation factors in the blood stream, and in increased liver and serum levels of KO.

Acetylsalicylate, inhibit the blood coagulation system in two ways: at low concentrations they effectively prevent blood platelet aggregation, but at higher concentrations they also induce a decrease of the plasma concentration of the Gla-containing coagulation factors (18-20). Here we will restrict ourselves to the latter property, which was shown to be caused by salicylate. Although salicylate-induced hypoprothrombinaemia was reported as early as in 1943 (21,22), the molecular basis of this interaction is not completely understood. Using perfused rat livers, Owens and Cimino demonstrated that the synthesis of prothrombin and factor VII were inhibited by salicylate, but that only for prothrombin this inhibition could be reversed by vitamin K (30,31). Also the presence of salicylate in the perfusion buffer resulted in the accumulation of vitamin KO in the liver. Except for the remarkable inability of vitamin K to counteract the effect of salicylate on the factor VII synthesis these results are very similar to those expected for inhibition by coumarin drugs. As long as no data are available for the other Gla-containing coagulation factors, we are inclined to assume that the confusing data on factor VII were due to its artificial activation, which is a well known problem in the determination of this coagulation factor.

In the early 1980's almost nothing was known about the effect of coumarin derivatives and salicylate on nonhepatic carboxylase/reductase systems. In a first attempt to study the effects of these drugs we looked at the accumulation of noncarboxylated precursor proteins in horse liver, kidney and spleen after a daily treatment with 1 g of 3-(acetonylbenzyl)-4-hydroxycoumarin (warfarin) during one week (23). Our data clearly demonstrated the presence of vitamin K-dependent carboxylase in all three tissues mentioned above. Moreover, it turned out that in the treated animals the microsomal levels of noncarboxylated precursor proteins were 12, 6 and 2 times those in a series of non-treated control animals. On the basis of these results we have concluded that both hepatic and nonhepatic carboxylase/reductase systems are inhibited by coumarin derivatives. These preliminary data were the starting point for more elaborate investigations, which showed that vitamin K-dependent carboxylase belongs to the standard machinery of all types of cells, and that it may be identified in almost all mammalian tissues (1-5).

In a subsequent project we have investigated whether - like warfarin - also salicylate inhibits the nonhepatic recycling of vitamin K. For these studies rats were chosen as our experimental animals. Our first aim was to find out the dose/response effects of both drugs on the plasma levels of the various coagulation factors. As is shown in table III both drugs inhibit the production of the Gla-containing coagulation factors, and not that of factor V. Far higher doses of salicylate are required, however, to obtain an effect comparable to that

drug	amount mg/kg	plasma level of coagulation factor F II	F VII	F X	F V	endogenous substrate % of control animals liver	lung
none		100	100	100	100	100	100
salicylate	4x 50	98	67	83	100	611	250
salicylate	4x100	79	37	51	104	665	41
salicylate	4x200	52	29	39	94	852	591
salicylate + vitamin K	4x200 1x 20	109	98	96	97	67	6
warfarin	1x0.2	78	40	78	94	422	257
warfarin	1x0.5	30	12	32	98	551	475
warfarin	1x1.0	22	5	5	96	800	857
warfarin + vitamin K	1x1.0 1x 20	85	97	85	100	56	60

Table III. The effect of warfarin and salicylate on blood coagulation factors and on the synthesis of Gla-containing proteins in liver and lung in rats. The in vivo effects of various amounts of salicylate and warfarin on plasma levels of coagulation factors and endogenous substrate levels in liver and lung. The drugs were administrated by peritoneal injection, because of the short half-life time of salicylate in blood the injections with this drug was repeated every 6 hours. Vitamin K (if added) was mixed with the drugs shortly before injection. After 20 hours blood was taken by heart puncture and the livers and lungs were removed. The coagulation factors were measured in one-stage coagulation assay. The incorporation of $^{14}CO_2$ in endogenous substrate of liver and lung microsomes was measured under standard conditions in the absence of exogenous substrate.

of warfarin. The inhibitory effect of both drugs could be counteracted by an additional dose of vitamin K. We also demonstrated a parallel and dose-dependent accumulation of non-carboxylated precursor proteins in hepatic and nonhepatic microsomes after the administration of either warfarin or salicylate. Our data therefore strongly suggest a similar physiological effect of both drugs on the vitamin K-dependent enzyme system.

Finally we have tried to identify the target enzymes for both drugs. Varying concentrations of either warfarin or salicylate were added to the in vitro carboxylase/reductase system, and as a coenzyme we used vitamin K either in its hydroquinone, its quinone or its epoxide form. No significant inhibition was found in the presence of KH_2, demonstrating that the KH_2-dependent carboxylase was not the target enzyme for warfarin nor for salicylate (see fig. 2). In the vitamin quinone-directed reaction K has first to be reduced to KH_2, and this step may be accomplished by two reductases: an NADH-dependent one and a DTT-dependent one. We have tested the inhibitory effect of warfarin and

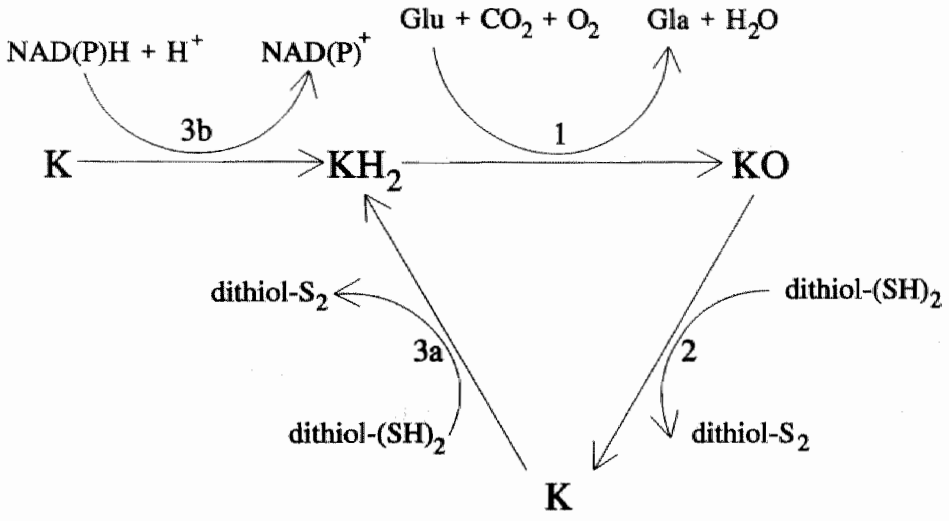

Fig. 2. The vitamin K cycle. Step 1: the conversion of KH_2 in KO by vitamin K epoxidase, Glu is converted to Gla simultaneously. Step 2: reduction of KO to K by KO reductase. The reaction is stimulated by dithiols. Step 3: Reduction of K to KH_2. This reaction can be accomplished by either a DTT-dependent reductase (3a) or a NAD(P)H-dependent reductase (3b).

salicylate on both reactions, and it is clear that these drugs interfere almost exclusively with the DTT-dependent reductase. The inhibition was even more pronounced in the KO-directed carboxylation reaction, in which two DTT-dependent reduction steps are required for the production of KH_2 (see fig. 2). Hence we concluded that warfarin and salicylate have the same target enzyme(s): the DTT-dependent reductase(s) of the vitamin K cycle. This conclusion is in contrast with that of Hildebrandt and Suttie (25,26), who claimed that salicylate inhibits the NADH-dependent reductase. Because this enzyme is able to catalyze only the reduction of vitamin K and not that of KO it can not readily be seen how these authors explain the observed inhibition of blood coagulation factor synthesis.

Involvement of NADH-dependent reductase in vitamin K metabolism

The reduction of vitamin KO to K is the crucial event in the vitamin K cycle. Only one enzyme is known to catalyze this step: the DTT-dependent KO-reductase. In vitro generally synthetic dithiols like DTT are used as a reducing cofactor, but also natural compounds like thioredoxin may fulfil this function (27). The physiological cofactor for KO-reductase is still unknown at this time. Also the conversion of K to KH_2 may be accomplished in a DTT-dependent way, and many arguments favour the assumption that both reduction steps are catalyzed by the same enzyme. In liver a second pathway for the reduction of vitamin K quinone is known. The enzyme involved may use NADH and NADPH as reducing cofactors. Because only K quinone and not KO are reduced by this NAD(P)H-dependent reductase, the enzyme is unable to maintain the vitamin K cycle, which is the reason why in vivo this pathway plays a minor role. Another difference with the DTT-dependent reductases is that the NAD(P)H-dependent enzyme is relatively insensitive for coumarin drugs. Therefore the enzyme becomes of vital importances in those cases in which patients have ingested an overdose of oral anticoagulants. Under those circumstances the DTT-dependent pathway is totally blocked, and the NAD(P)H-dependent reductase is the only enzyme capable of generating KH_2. Because each molecule is used only once, high doses of vitamin K should be given to counteract the effect of the coumarins on the hepatic Gla-containing proteins. Recently, Price and Kaneda demonstrated that in rats also the Gla-content of the bone Gla-protein osteocalcin is strongly affected by warfarin administration (28). In this case, however, the effect of warfarin could not be counteracted by vitamin K. On the basis of these data we have put forward the hypothesis that the hepatic carboxylase/reductase system differs from the osteoblasts in its NAD(P)H-dependent reductase content. We have tested this hypothesis using the osteoblast-like rat osteosarcoma cell line UMR-106, which was used to produce sufficient amounts of cultured osteosarcoma cells as well as tumor tissue in hairless rats (29).

In a first experiment we have compared the DTT- and the NADH-dependent pathway in an in vitro rat liver carboxylase/reductase enzyme system, using vitamin K quinone as a coenzyme for carboxylase and an excess of either DTT or NADH as a reducing cofactor. The Km^{app} for vitamin K was 18 μM in the DTT-dependent pathway and 275 μM in the NADH-dependent one. It cannot be concluded from our data whether this difference is the result of a low affinity of vitamin K for the NADH-dependent reductase or whether it is due to the absence of recycling in this pathway. The data do show, however, that in order to compare both pathways properly vitamin K has to be added to the reaction

mixtures in rather high concentrations. In the experiments reported in this paragraph the vitamin K concentration was 0.4 mM. Washed microsomes were prepared from rat liver and tumor tissue, as well as from cultured osteosarcoma cells, and in these systems we compared the DTT- and the NADH-dependent pathway. As is shown in table IV in liver the NADH-dependent route amounted 22% of the DTT-dependent one, which is quite normal under the conditions employed. In the systems derived from tumor tissue and from cultured cells no activity was found in the NADH-containing systems, suggesting that the NADH-

	Carboxylase activity (%)		
Cofactor	liver	tumor	cell lysate
KH_2	100	100	100
K+DTT	69	68	26
K+NADH	15	0.4	0

Table IV. Carboxylase activity using different cofactors. Vitamin K-dependent carboxylase activity was measured in tumor and liver microsomes or cell lysate under standard conditions, using vitamin K and either 2 mM NADH or 4 mM DTT. Values are expressed as percentages of the vitamin K hydroquinone driven reaction.

dependent K-reductase is absent in osteosarcoma UMR-106. If a similar situation would occur in normal osteoblasts, this would provide a good explanation for the data reported by Price and Kaneda. More elaborate investigations are required to learn whether the absence of NADH-dependent K-reductase is a general characteristic of the nonhepatic carboxylases. We would like to stress, however, that the combined carboxylase/reductase assay - though quick and reproducible - is an indirect reductase test. In order to be able to draw definite conclusions a direct and quantitative measurement of K-reductase should be developed. Preliminary attempts in our lab to solve this problem were hampered by the rapid reoxidation by molecular oxygen of the KH_2 formed.

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CHAPTER 3

SUBSTRATE RECOGNITION OF PROPEPTIDE-CONTAINING SYNTHETIC PEPTIDES BY THE VITAMIN K-DEPENDENT CARBOXYLASE

Based on:

- Ulrich, M.M.W., Furie, B., Jacobs, M., Vermeer, C. and Furie, B.C. J. Biol. Chem. 263: 9697-9702, 1988
- Hubbard, B.R., Jacobs, M., Ulrich, M.M.W., Walsh, C., Furie, B. and Furie, B.C. J. Biol. Chem. 264: 14145-14159, 1989

A SYNTHETIC PEPTIDE BASED UPON THE γ-GLUTAMYL CARBOXYLATION RECOGNITION SITE SEQUENCE OF THE PROTHROMBIN PROPEPTIDE IS AN ACTIVE SUBSTRATE FOR THE CARBOXYLASE IN VITRO

Summary

The vitamin K-dependent blood-clotting proteins contain a γ-glutamyl carboxylation recognition site in the propeptide, between the signal peptide and the mature protein, that directs γ-glutamyl carboxylation of specific glutamic acid residues. To develop a better substrate for the in vitro assay of the vitamin K-dependent γ-glutamyl carboxylase and to understand the substrate recognition requirements of the carboxylase, we prepared synthetic peptides based upon the structure of human proprothrombin. These peptides were employed as substrates for in vitro carboxylation using a partially purified form of the bovine liver carboxylase. A 28-residue peptide (HVFLAP QQARSLLQRVRRANTFLEEVRK), based on residues -18 to +10 in proprothrombin, includes the complete propeptide and the first 10 residues of acarboxyprothrombin. Carboxylation of this peptide is characterized by Km of 3.6 μM. In contrast, FLEEL is carboxylated with a Km of about 2200 μM. A 10-residue peptide (ANTFLEEVRK), based on residues +1 to +10 in prothrombin, and a 20-residue peptide (ARSLLQRVRRANTFLEEVRK), based on residues -10 to +10 in proprothrombin, are also poor substrates for the carboxylase. Replacement of phenylalanine with alanine at residue 3 (equivalent to position -16 in proprothrombin) in the 28-residue peptide

significantly alters the Km to 200 μM. A synthetic propeptide (HVFLAPQQAR SLLQRVRRY), homologous to residues -18 to -1 in proprothrombin, inhibited carboxylation of the 28-residue peptide substrate with a Ki of 3.5 μM, but modestly stimulated the carboxylation of the 5- and 10-residue peptide substrates. These results indicate that an intact carboxylation recognition site is required for efficient in vitro carboxylation and that this site includes critical residues in region -18 to -11 of proprothrombin. The carboxylation recognition site in the propeptide binds directly to the carboxylase or to a closely associated protein.

Introduction

The vitamin K-dependent blood coagulation proteins are components of a family of calcium-binding proteins that function in an essential role during the initiation and regulation of blood coagulation. These proteins contain 10-12 γ–carboxyglutamic acid residues near the NH_2 terminus (1,2) and have, as a special property, the ability to interact with membrane surfaces in the presence of metal ions (3-5). The vitamin K-dependent proteins, including prothrombin, factors VII, IX, X, and the proteins C, Z and S are synthesized in a precursor form that includes a typical signal peptide for translocation of the nascent polypeptide chain, a propeptide, and a mature zymogen of a serine protease (6-12). Based upon the marked sequence homology of the propeptides predicted from the cDNA clones of proteins that contain γ–carboxyglutamic acid (13) and the observation that factor IX Cambridge, a naturally occurring mutant with a defect in the propeptide region, has a defect in γ-glutamyl carboxylation (14), we examined the role of the propeptide of factor IX in directing vitamin K-dependent γ-glutamyl carboxylation. The expression of mutant factor IX species, prepared by site-directed mutagenesis and expressed in heterologous mammalian cells, demonstrated that forms lacking the propeptide (residues -18 to -1) or containing point mutations at the highly conserved phenylalanine -16 or alanine -10 were not carboxylated (15,16). Similar studies have shown the importance of the propeptide in the carboxylation of protein C (17).

The post-translational carboxylation of specific glutamic acid residues in the precursor forms of the vitamin K-dependent proteins is catalyzed in the rough endoplasmic reticulum (18) by the vitamin K-dependent carboxylase, a membrane-bound enzyme (19,20). In the presence of reduced vitamin K, oxygen, and CO_2, a γ–proton on specific glutamic acids is removed, and the additional carboxyl group is added (21,22). Knobloch and Suttie (23) have

recently demonstrated that synthetic peptides based upon factor IX and X propeptides stimulate the in vitro carboxylase assay, perhaps indicating a regulatory role for the propeptide.

The details of the carboxylation reaction remain to be elucidated. Despite considerable efforts of a number of laboratories (24-27), the purification of the vitamin K-dependent carboxylase has not been complete. The preparation of synthetic pentapeptides FLEEV (Phe-Leu-Glu-Glu-Val) (28) and FLEEL (Phe-Leu-Glu-Glu-Leu) (29) that could be carboxylated in an in vitro system facilitated efforts to partially purify the enzyme and to characterize its properties. However, their high Km (2-8 mM), the minimal conversion of substrate to product, and the inability to carboxylate adjacent glutamic acid residues have raised questions as to the relationship of these synthetic substrates to the natural substrate (27,30).

Based upon our identification of a γ-glutamyl carboxylation recognition site in the propeptides of the vitamin K-dependent proteins (15), we have designed a new synthetic peptide substrate based upon the primary structure of human proprothrombin. This peptide contains the 18-residue propeptide domain and the first 10 residues of the γ–caboxyglutamic acid-rich domain of prothrombin, including glutamic acid residues 6 and 7. In this work, we demonstrate that this 28-residue peptide is efficiently carboxylated with a Km 3 orders of magnitude lower than that of FLEEL (23). These results confirm the importance of the γ-glutamyl carboxylation recognition site in directing carboxylation and suggest that this recognition element is in direct contact with the carboxylase. The availability of an efficient substrate may facilitate the purification of the enzyme and the study of its mechanism of action.

Materials and Methods

Synthesis of Peptides. Peptides were synthesized by the solid-phase method using an Applied Biosystems Model 430A peptide synthesizer (31). The t-butoxycarbonyl-amino acids were sequentially coupled as symmetric anhydrides onto a butoxycarbonylaminoacyl-OCH_2-pyridine-2-aldoxine methiodide resin. Double-coupling cycles were used for all asparagine, arginine, and glutamine residues. The coupling efficiency at each step was determined by ninhydrin analysis. The cleavage of the benzyl-protected peptide from the resin and simultaneous removal of the side chain-protecting groups were performed using anhydrous hydrogen fluoride The cleavage reactions were performed in HF:anisole:resin (10:1:1) at -10°C for 30 min and for an additional 45 min at

0°C. Extraction of cleaved peptide from resin was performed using 50 ml of 30% acetic acid in water after a rinse with 50 of anhydrous ethyl ether. The cleaved, deprotected peptide was purified by high performance liquid chromatography using a reverse-phase column and a Beckman HPLC system. Each peptide was purified using a 10 mm x 25-cm Ultrasphere ODS column (Beckman Instruments) with a flow rate of 4 ml/min (solvent A = 0.1% aqueous trifluoroacetic acid; solvent B = 0.1% trifluoroacetic acid in acetonitrile). A linear gradient of 15-50% solvent B for 70 min was employed. The purity of each peptide was evaluated using a 4.6 mm x 25-cm Ultrasphere ODS column with a flow rate of 1 ml/min. A linear gradient of 0-50% solvent B for 50 min was used. The sequence of each peptide was verified by automated Edman degradation using an Applied Biosystems Model 470 Protein Sequencer (32) and an on-line Model 120A PTH analyzer.

Preparation of Partially Purified Carboxylase. Crude microsomes were prepared from bovine liver and washed three times with buffer A (0.1 M NaCl, 50 mM Tris-HCl, pH 7.4), followed by buffer A containing 1 M NaCl. These microsomes were used to prepare partially purified carboxylase. The microsomes were resuspended in 0.5% (w/v) CHAPS (Sigma) in buffer B (0.5 M NaCl, 20 mM Tris-HCl, pH 7.4) and sedimented by centrifugation at 150,000 x g for 1 h. The carboxylase activity was solubilized by resuspending the pellet in 1% (w/v) CHAPS in buffer B (27). Solid ammonium sulfate was added to 25% saturation. After 30 min at 4°C, the nonsolubilized material was removed by centrifugation at 150,000 x g for 1h. The carboxylase was precipitated by adding ammonium sulfate to 50% saturation to the supernatant. The precipitate was recovered by centrifugation at 10,000 x g for 20 min and dissolved in buffer B (27). The partially purified carboxylase was stored at -80°C until use.

Preparation of Vitamin K Hydroquinone. Vitamin K quinon (Merck) was reduced as described earlier (44).

Carboxylase Assay. The amount of $^{14}CO_2$ incorporated into exogenous peptide substrates was measured in reaction mixtures of 125 µl containing 1 mg of partially purified carboxylase, 0.4 mM vitamin K hydroquinone (Merck), 1.4 mM $NaH^{14}CO_3$ (10 µCi; Du Pont-New England Nuclear), and 4 mM dithiothreitol (33). In some instances, 1 M ammonium sulfate was added (34). Exogenous substrates were added as indicated. The reaction mixtures were incubated in sealed tubes at 25°C; and, at the indicated times, the reaction was stopped by adding 2 ml of 5% (w/v) trichloroacetic acid. Traces of nonbound $^{14}CO_2$ remaining dissolved after acidification were removed by boiling the mixture for 1 min before 10 ml of Atomlight (Du Pont-New England Nuclear)

was added. The samples were assayed in a Beckman LS1801 liquid scintillation counter. A blank value, obtained by carrying out the reactions in the absence of vitamin K, was subtracted from all reported data.

Results

Synthesis of Peptide Substrates and Inhibitors. Six peptides were prepared by solid-phase peptide synthesis (Fig. 1). These peptides include Peptide I (designated proPT28), a 28-residue peptide based on residues -18 to +10 in human proprothrombin. This peptide incorporates the complete propeptide and 10 residues of the mature NH_2 terminus of acarboxyprothrombin. Peptide II (designated proPT20, based on residues -10 to +10, incorporates a truncated propeptide and 10 residues of the mature NH_2 terminus of acarboxyprothrombin. Peptide III (designated PT/1-10), is based on residues +1 to 10 of acarboxyprothrombin. Peptide IV (FLEEL) is homologous to residues 4-9 in acarboxyprothrombin and has been one of the best synthetic substrates available for the carboxylase (29). Peptide V (designated proPT28[FA-16]) is identical to proPT28 except for the modification of residue 3 from phenylalanine to alanine. This peptide is analogous to the form of recombinant factor IX (FIX/FA-16), prepared by site-specific mutagenesis (15, 16). Peptide VI, the propeptide from residues -18 to -1, includes an extra COOH-terminal tyrosine for purposes of radioiodination. After cleavage of the peptides from the resin, each peptide was purified by reverse-phase chromatography to yield homogeneous material. The purified peptides were reanalyzed by HPLC under

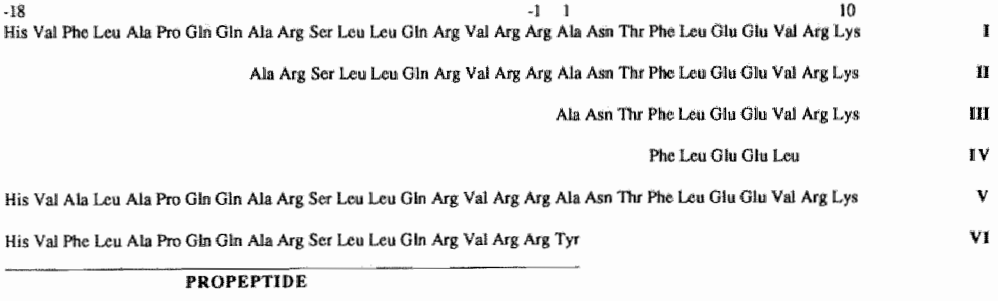

Fig. 1. Primary structure of the synthetic peptides and their relationship to the structure of the propeptide and the γ-carboxyglutamic acid-rich domain of prothrombin. The propeptide length, known to be 18 residues for factor IX (14), is assumed to be equivalent for prothrombin. The propeptide extends from residue -18 to -1. The glutamic acid substrate for the carboxylase is located at residues 6 and 7. I, proPT28; II, proPT20; III, PT/1-10; IV, FLEEL; V, proPT28[FA-16]; VI, propeptide.

different solvent conditions to evaluate purity. ProPT28 and the other peptides demonstrated a single, symmetrical peak. These peptides were subjected to automated Edman degradation to confirm the sequence and homogeneity of the sample. These analyses verified the structure and purity of each of the peptides.

Comparison of Carboxylation of Synthetic Peptides. Using the in vitro carboxylase assay and a partially purified form of the bovine liver carboxylase, we analyzed the incorporation of $^{14}CO_2$ into the synthetic peptides. In these experiments, the peptide concentrations were fixed at 40 μM. As shown in Fig. 2, proPT28 readily incorporated $^{14}CO_2$. This reaction was almost linear for 60 min, but did not approach maximal incorporation until 6 h. Although the actual concentration of CO_2 in the system is uncertain (since the system includes endogenous unlabeled CO_2 as well as $^{14}CO_2$), calculation of the amount of $^{14}CO_2$ incorporated into proPT28 can only provide an estimated lower limit. Experiments performed at low proPT28 concentration suggest that at least 40% of the glutamic acid residues in proPT28 are carboxylated. In contrast, insignificant CO_2 is incorporated into FLEEL, proPT20, and PT/1-10 at these substrate concentrations.

The carboxylations of proPT20, PT/1-10, and FLEEL were compared at higher substrate concentrations. At 1 mM, these substrates demonstrated the incorporation of $^{14}CO_2$ (Table I). However, using conditions for carboxylation optimized for FLEEL, FLEEL appeared to be a better substrate than either proPT20 or PT/1-10.

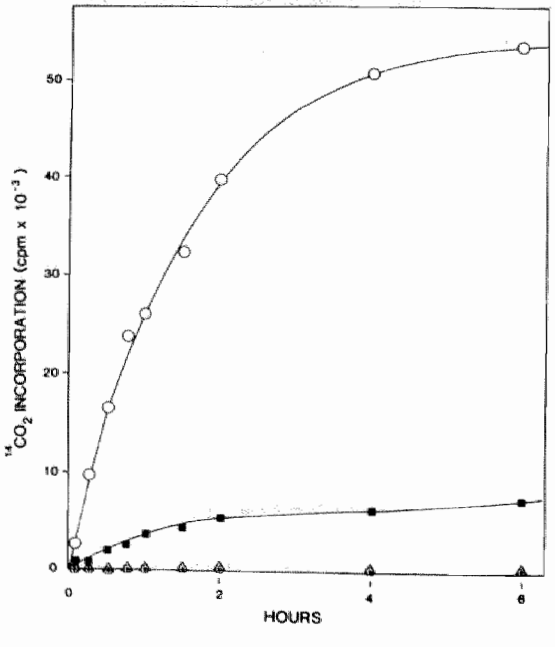

Fig. 2. Comparison of the in vitro carboxylaton of synthetic peptides. The incorporation of $^{14}CO_2$ was measured as a function of time. The substrates used included proPT28 (O), proPT28[FA-16] (■), proPT20 (❑), PT/1-10 (Δ) and FLEEL (●). The peptide substrate concentration was 40 μM.

Substrate	No addition	+propeptide	+Ammonium sulfate
		(cpm)	
FLEEL	27,228	85,880	69,083
PT/1-10	6,222	19,968	10,585
proPT20	13,242	17,808	9,668
proPT28[FA-16]	32,641	27,577	34,098

Table I. Comparison of the effect of the propeptide and ammonium sulfate on carboxylation. Numbers (the average of duplicate reactions) represent ^{14}C incorporated into the substrates. The concentrations of propeptide and ammonium sulfate were 10 μM and 1 M, respectively. The concentration of peptide substrates used was 1 mM.

We have previously demonstrated that a point mutation, from phenylalanine to alanine at residue -16, in the propeptide of human factor IX almost obliterates γ-glutamyl carboxylation (15). Therefore, we prepared a synthetic peptide, proPT28 [FA-16], in which the phenylalanine is replaced by alanine. At 40 μM, the carboxylation of this peptide was significantly impaired (Fig. 2). These results emphasize the importance of phenylalanine -16 in the carboxylation recognition site in in vitro carboxylation, extending the observation made originally in an in vivo recombinant expression system (15).

Kinetic Analyses of Vitamin K-dependent Carboxylation of Synthetic Peptides. The carboxylation of the peptide substrates, as monitored by the incorporation of $^{14}CO_2$ for over 30 min, was determined in the in vitro carboxylation assay.

As demonstrated in Fig. 3A, the initial velocity of the reaction was determined using a proPT28 concentration range of 0-10 μM. A double-reciprocal plot of the results indicates a linear relationship between 1/V versus 1/S. Under the conditions employed, the proPT28 peptide has a Km of 3.6 μM and a Vmax of 667 cpm/min. For comparison, a similar experiment was performed using the pentapeptide FLEEL concentration range of 0-4 mM. A double-reciprocal plot of the results indicates a linear relationship between 1/V versus 1/S. Under these conditions, a Km of 2200 μM and a Vmax of 2000 cpm/min were measured. Kinetic analysis performed on the peptide substrate proPT20 indicated a Km of 850 μM and a Vmax of 333 (Fig. 3C). A similar kinetic analysis performed on the peptide substrate proPT28[FA-16] revealed aKm of 200 μM and a Vmax of 770 cpm/min (Fig. 3D). These results indicatethat the velocity of each of these reactions is similar at saturating levels of substrate. However, the binding of these substrates to the carboxylase varies

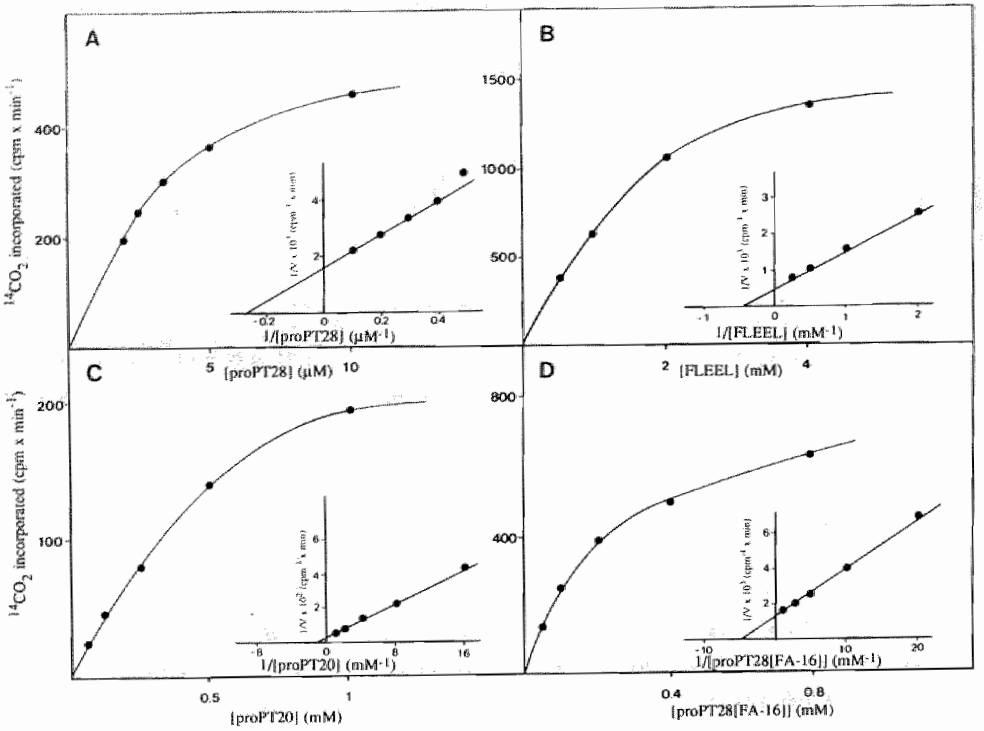

Fig. 3. Kinetic analyses of carboxylase action on the synthetic peptides. The reaction mixture included partially purified carboxylase (1 mg), buffer A, peptide substrate at indicated concentrations, and $Na^{14}CO_3$ in a volume of 125 μl. After incubation at 25°C for 30 min, the amount of ^{14}C incorporated into the substrate was measured. A, proPT28; B, FLEEL; C, proPT20; D, proPT28[FA-16].

almost 3 orders of magnitude. The proPT28 peptide binds to the carboxylase about 600-fold more tightly than does FLEEL and 250-fold more tightly than does proPT20. The substitution of a phenylalanine by alanine in proPT28[FA-16] significantly impairs binding of the peptide to the carboxylase by over 50-fold, indicating the importance of phenylalanine -16 in the recognition site.

Effect of Propeptide on Carboxylation Recently, Knobloch and Suttie (23) have demonstrated that the propeptides of factors IX and X stimulate the carboxylation of Boc-Glu-Glu-Leu-OMe in an in vitro carboxylation system similar to that described here. For these reasons, we evaluated the effect of the synthetic propeptide of prothrombin (Peptide VI) on the carboxylation of the synthetic peptide substrates. In a carboxylase assay that included 1 M ammonium sulfate, which stimulates carboxylase activity (34), no propeptide stimulation of the carboxylation of FLEEL, proPT20, and PT/1-10 was observed. However, if ammonium sulfate was deleted from the carboxylation system, stimulation of the carboxylase by propeptide was noted (Table I). The

incorporation of CO_2 into FLEEL was increased about 3-fold in the presence of the propeptide as compared to the assay system lacking the propeptide. This stimulation is higher than the stimulation of carboxylase activity by 1 M ammonium sulfate. Whereas the Km for FLEEL is unaltered by ammonium sulfate, a Km for FLEEL of 700 μM was measured in the presence of 10 μM propeptide (data not shown). Similarly, the propeptide increased the carboxylation of PT1/10 about 3-fold. ProPT20 and proPT28[FA-16] carboxylations were not stimulated by either the propeptide or ammonium sulfate. Only a small fraction (<0.1%) of the peptide substrates were carboxylated regardless of the presence of the propeptide. Importantly, the carboxylation of the PT/1-10 (residues 1-10) substrate in the presence of the propeptide (residues -18 to -1) does not yield carboxylation parallel to proPT28 (residues -18 to 10). Thus, the covalent attachment of the propeptide and the substrate glutamic acids is a critical requirement for efficient carboxylation of substrate. The suggestion that the propeptide is an allosteric regulator of the carboxylase has been based on the observed stimulation of the carboxylase action on Boc-Glu-Glu-Leu-OMe (23). For these reasons, we determined the effect of the propeptide on the carboxylation of the proPT28 substrate. As shown in Fig. 4, the synthetic propeptide is an inhibitor of the carboxylation of proPT28 by the carboxylase, and the kinetics of inhibition fit a model forcompetitive inhibition (Ki = 3.5 μM). These results suggest that the propeptide binds to the enzyme with an association constant similar to that of the substrate proPT28 and that the propeptide and proPT28 bind to the same site on the enzyme.

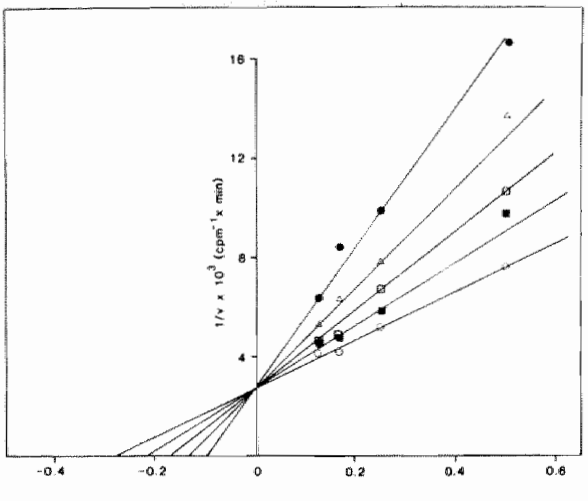

Fig. 4. Inhibition of the proPT28 substrate by the synthetic propeptide. The reaction mixture included partially purified carboxylase (1 mg), buffer A, peptide substrate at indicated concentrations, and $Na^{14}CO_3$ in a volume of 125 μl. The concentration of propeptide was 0 (O), 1 (■), 2 (❑), 4 (Δ), and 6 (●) μM. After incubation at 25°C for 30 min, the amount of ^{14}C incorporated into the substrate was measured. A Ki of 3.5 μM was determined. In contrast, the propeptide stimulated the carboxylation of PT/1-10 and FLEEL.

Discussion

Since the discovery of γ–carboxyglutamic acid (1,2), progress has been made by a number of laboratories toward a better understanding of the enzymes involved in the synthesis of this amino acid. Carboxylation of protein or peptide substrates requires vitamin K, oxygen, CO_2, and a membrane bound enzyme, the vitamin K-dependent carboxylase. This enzyme has been partially purified from microsomal preparations in a detergent-solubilized form. Because of its relative instability, this enzyme has not been purified to homogeneity. For these reasons, experiments defining its mechanism of enzyme action, the structure of the protein, and the relationship of this enzyme to other microsomal enzyme activities have met with limited success. The discovery of the synthetic peptide substrate Phe-Leu-Glu-Glu-Val and subsequently, a related peptide, Phe-Leu-Glu-Glu-Leu, gave considerable impetus to the study of the enzymology of this system (28,29). However, these synthetic substrates have had limitations. The Km values of these substrates have been determined to be in the range of 2-7 mM, depending upon the preparation and source of the carboxylase and the conditions of assay. Furthermore, minimal amounts of the substrate are converted to product, raising questions about the relationship of these synthetic substrates to the endogenous substrates (27). Finally, only the first of the two glutamic acids is converted to γ–carboxyglutamic acid (30). Hexapeptide analogs, based on residues 18-23 in bovine prothrombin, were unusually poor substrates (37,38). A large number of peptide analogs, based on residues 5-9 in bovine prothrombin, also proved no better then FLEEL or FLEEV (39). Furthermore, acarboxyprothrombin (35) is only minimally carboxylated. Descarboxy bone Gla protein is a better substrate for the carboxylase (34), as are some proteolytic products of acarboxyprothrombin (39,35). The reason why these substrates, lacking a prosequence, are carboxylated remains unclear.

Our discovery of the γ-glutamyl carboxylation recognition site on factor IX (15) and the discovery of a similar site on protein C (17) suggested to us that the physiologic substrate for the carboxylase was not the uncarboxylated protein with the mature NH_2 terminus, but rather the uncarboxylated protein precursor containing a propeptide extension on the NH_2 terminus. For these reasons, we synthesized proPT28 as a synthetic analog of proprothrombin to test as a synthetic substrate. This peptide is readily carboxylated in the in vitro carboxylation assay using the detergent-solubilized partially purified carboxylase. The Km is about 3 μM, almost 3 orders of magnitude lower than

that of FLEEL; and much of the substrate is converted to product. At present, we are uncertain whether both glutamic acid residues become carboxylated.

The specificity of the carboxylase for this peptide was determined using a number of related peptides. The observation that proPT28[FA-16] was only minimally carboxylated emphasizes the requirement for an intact carboxylation recognition site. The expression of recombinant factor IX (15) and recombinant protein C (17) with defects in the propeptide region has begun to delineate the size of this carboxylation recognition site. Point mutations at residue -16 or -10 nearly eliminate carboxylation of factor IX (15,16). Deletion mutations in the propeptide of protein C in region -18 to -11 also impair carboxylation (17). These results are internally consistent with the concept of a carboxylation recognition site that stretches from residue -18 to at least residue -10. However, Suttie et al. (40) noted preferential carboxylation of recombinant protein C in an in vitro carboxylation system if the recombinant protein included a truncated propeptide, from residues -10 to -1, on the NH_2 terminus of protein C compared to no propeptide. The in vivo carboxylation results would not have predicted this carboxylation preference since protein C has a propeptide of 24 residues (17). For this reason, we synthesized proPT20, based on residues -10 to 10 of proprothrombin, to compare as a substrate to PT/1-10, based on residues 1-10. Since the Km for proPT20 is 850 μM, we conclude that neither substrate is carboxylated significantly better than FLEEL. This result is consistent with a requirement for an intact carboxylation recognition site including residues -18 to -11. Since the incorporation of CO_2 in the recombinant protein C containing the truncated propeptide is only slightly above that of protein C (40), we believe that these in vitro experiments, combined with our current results, do not support a conclusion that the propeptide residues -10 to -1 are sufficient to direct carboxylation.

The propeptide has now been shown to direct carboxylation of the vitamin K-dependent proteins during post-translational processing (15,17). The observation that von Willebrand factor multimerization is eliminated in heterologous cells transfected with von Willebrand factor cDNA constructs lacking the propolypeptide indicates a requirement of the propolypeptide in the multimerization process (41). However, multimerization can be rescued in these cells synthesizing von Willebrand factor dimer by cotransfection with cDNA coding for the propolypeptide (42). Thus, the propolypeptide can participate in multimerization whether or not it is covalently attached to the NH_2 terminus of the mature von Willebrand factor. In contrast, addition of the prothrombin propeptide to the substrate PT/1-10 stimulated substrate carboxylation modestly, but was several orders of magnitude less effective a

substrate than proPT28, a peptide equivalent to a single-chain form of these two peptides. These results suggest that the propeptide plays a dominant role as a recognition element for carboxylation by binding to the carboxylase or to a closely associated protein. Price et al. (43) have recently suggested that, in addition to the carboxylation recognition site, a consensus sequence in the γ–carboxyglutamic acid domain of the vitamin K-dependent proteins may play a role in carboxylase recognition. ProPT28 lacks this consensus sequence and yet is well carboxylase for the natural substrate is significantly lower than 3 μM. If it is , the apparent Km of proPT28 may be artifactually elevated due to the intrinsic conformational flexibility of the peptide that may not be characteristic of this region in the intact protein. Alternatively, residues beyond position +10 may contribute to the binding energy. It remains uncertain whether the carboxylation recognition site on the propeptide is not only required but is also sufficient for γ–glutamyl carboxylation. The expression of γ–glutamyl carboxylated hybrid proteins containing the prothrombin leader sequences and a mature protein that is otherwise not γ–glutamyl carboxylated (experiments in progress) should allow critical evaluation of this hypothesis. Finally, the availability of the synthetic substrate proPT28 that is readily carboxylated and binds tightly to the enzyme should allow the design of experiments to purify the enzyme, to characterize its structure, and to understand the mechanism by which vitamin K facilitates enzyme catalysis.

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IN VITRO MODIFICATION OF SYNTHETIC PEPTIDES CONTAINING THE γ-GLUTAMYL CARBOXYLATION RECOGNITION SITE

Summary

Synthetic peptides including the γ-glutamyl carboxylation recognition site and acidic amino acids were compared as substrates for vitamin K-dependent γ-glutamyl carboxylation by bovine liver carboxylase. The 28-residue proPT28 (proprothrombin -18 to +10) and proFIX28 (profactor IX -18 to +10) were carboxylated with a Km of 3 μM. The Vmax of proPT28 was 2-3 times greater than that of proFIX28. An analog of proFIX28 that contained the prothrombin propeptide had a Vmax 2-3 fold greater than an analog of proPT28 that contained the factor IX propeptide. ProFIX28/RS-1, based upon factor IX Cambridge, proFIX28/RQ-4, based upon factor IX Oxford 3, and proFIX28 had equivalent Km and Vmax values, Analogs of proPT28 containing Ala^6-Glu^7 or Glu^6-Ala^7 were carboxylated at equivalent rates. A peptide containing Asp^6-Asp^7 was carboxylated at a rate of about 1% of that of Glu carboxylation. Carboxylation of peptides containing Asp^6-Glu^7 and Glu^6-Asp^7 yielded results identical with peptides containing Ala^6-Glu^7 and Glu^6-Ala^7. Carboxymethylcysteine was not carboxylated when substituted for Glu^6 in a peptide containing Asp^7. These results indicate that the prothrombin propeptide is more efficient in the carboxylation process than is the factor IX propeptide, but that both propeptides direct carboxylation; the γ-glutamyl

carboxylation recognition site does not include residues -4 and -1; aspartic acid and carboxymethylcysteine are poor substrates for the carboxylase, but aspartic acid does not inhibit the carboxylation of adjacent glutamic acids.

Introduction

The vitamin K-dependent proteins represent a unique class of calcium binding proteins that contain γ-carboxyglutamic acid (1,2). These proteins, which include the blood clotting and regulatory proteins prothrombin, factor IX, factor X, factor VII, and protein C, are synthesized in the liver in a precursor form (for review, see Ref. 3). They are synthesized containing a signal peptide, a propeptide, and the mature zymogen that circulates in the blood. After translocation to the rough endoplasmic reticulum, these proteins undergo post-translational modifications that include the conversion of a specific set of glutamic acids near the NH_2 terminus to γ-carboxyglutamic acid (4). This reaction is catalyzed by a vitamin K-dependent carboxylase that is thought to be an integral membrane protein (5). Reduced vitamin K, molecular oxygen, and carbon dioxide are required to generate γ-carboxyglutamic acid.

The propeptides of the vitamin K-dependent proteins contain a recognition site that designates the precursor proteins (e.g. profactor IX) for carboxylation (6-8). The propeptide regions of all of the vitamin K-dependent proteins demonstrate marked sequence homology (9), suggesting a role for this region in carboxylation. Structural analysis of a mutant factor IX, factor IX Cambridge, from a patient with hemophilia B revealed that the propeptide of factor IX stretched from residue -18 to residue -1, that mutation of residue -1 from arginine to serine precluded propeptide cleavage, and that this mutation within the propeptide impaired γ-glutamyl carboxylation (10). These results provided direct evidence for a relationship between the propeptide and carboxylation. Similar results have since been found in the natural mutant factor IX San Dimas ($Arg^{-4} \rightarrow Gln$) (11) and the same mutant ($Arg^{-4} \rightarrow Gln$) prepared by site-specific mutagenesis and expressed in heterologous cells (12).

Based upon these results, we have shown the presence of a γ-glutamyl carboxylation recognition site in the propeptide of factor IX (6,8). Using site-specific mutagenesis, we demonstrated that factor IX lacking the 18-residue propeptide was not carboxylated, and that point mutations at either highly conserved phenylalanine -16 or alanine -10 eliminated carboxylation. Using deletion mutants of protein C, Foster et al. (7) also demonstrated the requirement of the propeptide in the carboxylation of protein C. These results

indicated the presence of a recognition element, termed the γ-glutamyl carboxylation recognition site or γ-CRS (6), within the propeptide of the vitamin K-dependent proteins. During post-translational processing of the prozymogen, this site in the propeptide allows recognition of the protein as a substrate for the carboxylase. Glutamic acids in the region adjacent to the propeptide are thought to undergo γ-glutamyl carboxylation prior to cleavage of the propeptide from the mature zymogen.

Based upon this analysis, we anticipated that synthetic peptides that contained the γ-glutamyl carboxylation recognition site and glutamic acid residues adjacent to this site would serve as substrates for in vitro carboxylation (13). The vitamin K-dependent carboxylase binds tightly to the γ-CRS on a 28-residue γ–CRS-containing peptide substrate, proPT28, based on the structure of proprothrombin. Peptides lacking the intact γ-CRS, including FLEEL and FLEEV (13-15), bind poorly to the carboxylase and are thus poor substrates. Propeptide analogs stimulate the carboxylation of small substrates, suggesting that the carboxylation recognition site may be an allosteric modifier of the carboxylase when small substrates are employed (16). However, the synthetic prothrombin propeptide inhibits the carboxylation of peptides that include the carboxylation recognition site (13). Taken as a whole, these results indicate by in vivo and in vitro studies that the γ–CRS is critical to carboxylation. In the current study, we have used analogs of proPT28 to explore the size of the γ-CRS, the effect of adjacent amino acids on carboxylation of glutamic acid residues, and the relationship between the γ-CRS of factor IX and prothrombin.

Materials and Methods

Synthesis of Peptides. Peptides were synthesized by the solid phase method using an Applied Biosystems Model 430A Peptide Synthesizer (17), proPT28/EA7 was synthesized using t-butoxycarbonyl/N-methylpyrrolidone chemistry (18). Amino acids were coupled as 1-hydroxybenzotriazole esters and sequentially coupled onto an OCH_2-phenylacetamidomethyl resin. Following each coupling step, all uncoupled α-amino termini were acetylated. ProPT28/EA6 and proFIX/PT were synthesized using a small scale rapid t-Boc chemistry. Sequential deprotection of the α-amino termini prior to coupling was achieved with neat trifluoroacetic acid. Amino acids were coupled as either symmetric anhydrides or 1-hydroxybenzotriazole esters onto 0.1 mmol of OCH_2-phenylacetamidomethyl resin. Prior to HF cleavage, the 2,4-dinitro

phenyl protecting group on histidine was removed from the resin-bound peptide using 20% β-mercaptoethanol, 10% diisopropylethylamine/dimethylformamide. A reaction mixture of 10 ml of β-mercaptoethanol/diisopropylethylamine/dimethylformamide per 500 mg of resin was thrice mixed for 30 min at room temperature. The resin was then washed extensively with dimethylformamide followed by dichloromethane. All other peptides were synthesized using standard t-Boc chemistry. The t-Boc amino acids were sequentially coupled as symmetric anhydrides onto an OCH_2-phenylacetamidomethyl resin. Double coupling cycles were used for all asparagine, arginine, and glutamine residues. The coupling efficiency at each step was determined by ninhydrin analysis. The cleavage of the benzyl-protected peptide from the resin and simultaneous removal of the side chain-protecting groups was performed using anhydrous hydrogen fluoride. The cleavage reactions were performed in HF/anisole/resin (10:2:1) or in HF/anisole/dimethyl sulfide resin (10:2:2:1) for cysteine-containing peptides. Reactions proceeded at -10°C for 30 min and for an additional 45 min at 0°C. Extraction of cleaved, deprotected peptide from resin was performed using 50 ml of 30% acetic acid in water after a rinse with 50 ml of anhydrous ethyl ether. The cleaved, deprotected peptide was purified by high performance liquid chromatography using a reverse phase column and a Waters high performance liquid chromatography system. Each peptide was purified using a Hi-Pore 318 column (21.5 mm x 25 cm) (Bio-Rad) with a flow rate of 10 ml/min (solvent A = 0.1% aqueous trifluoroacetic acid; solvent B = 0.1% trifluoroacetic acid in acetonitrile). A linear gradient of 15 to 45% solvent B in 75 min (0.4%B/min) was employed. The sequence of each peptide was verified by automated Edman degradation using an Applied Biosystems Model 470 Protein Sequencer (19) and Model 120A PTH analyzer.

The synthetic peptide proPT28/EC6/ED7 that contained cysteine was modified by carboxymethylation using iodoacetic acid (20). A 2 mg/ml solution of peptide was prepared in 6 M guanidine HCl, 5 mM EDTA, 0.5 M Tris-HCl, pH 8.6, β-Mercaptoethanol was added to a final concentration of 25 mM, and the solution was incubated under nitrogen overnight at 25°C in the dark. Iodoacetic acid was added to a final concentration of 50 mM, and the solution was incubated for 15 min. The reaction was stopped with the addition of 1% (v/v) β-mercaptoethanol. The modified peptide was repurified by high performance liquid chromatography, as above, and its structure was analyzed by automated Edman degradation using a phenylthiohydantoin-carboxymethylcysteine standard.

Preparation of Partially Purified Carboxylase. Crude microsomes were prepared from bovine liver and washed three times with buffer A (0.1 M NaCl, 50 mM Tris-HCl, pH 7.4) followed by buffer A containing 1 M NaCl. The microsomes were resuspended in buffer B (0.5 M NaCl, 25 mM Tris-HCl, pH 7.4) to a protein concentration of 40 mg/ml and adjusted to 0.5% (w/v) CHAPS (Sigma). The carboxylase activity was sedimented by centrifugation at 150,000 x g for 1 h. The pellet was resuspended in buffer B and solubilized by adjusting the CHAPS to 1% (w/v) (21). Solid ammonium sulfate was added to 25% saturation. After 30 min at 4°C, the nonsolubilized material was removed by centrifugation at 150,000 x g for 1 h. The carboxylase was precipitated by adding ammonium sulfate to 50% saturation to the supernatant. The precipitate was recoverd by centrifugation at 10,000 x g for 20 min and dissolved in buffer B containing 0.1% (w/v) CHAPS (21). The partially purified carboxylase was stored at -80°C until use.

Preparation of Vitamin K Hydroquinone. Vitamin K quinon (Merck) was reduced as described earlier (44).

Carboxylase Assay. The amount of $^{14}CO_2$ incorporated into exogenous peptide substrates was measured in reaction mixtures of 125 µl containing 1 mg of partially purified carboxylase, 0.4 mM vitamin K hydroquinone, 0.74 mM $NaH^{14}CO_2$ (5 µCi; Amersham), and 8 mM dithiothreitol (22). Exogenous peptide substrates were added as indicated. The reaction mixtures were incubated sealed tubes at 25°C and at indicated times, the reaction was stopped by adding 2 ml of 10% (w/v) trichloroacetic acid. Traces of nonbound $^{14}CO_2$ remaining dissolved after acidification were removed by boiling the mixture for 10 min, then 5 ml of Atomlight (Du Pont-New England Nuclear) was added. The samples were assayed in a Beckman LS1801 liquid scintillation counter. A blank value, typically about 300 to 500 cpm, was obtained by carrying out the reactions in the absence of peptide substrate; this value was subtracted from all reported data.

Determination of Kinetic Parameters. The initial rate of incorporation $^{14}CO_2$ into each peptide substrate was determined by preincubating a stock solution of partially purified carboxylase at 25°C for 4 min in the presence of dithiothreitol and vitamin KH_2. To this mixture $NaH^{14}CO_3$ was added, and the reaction was initiated by the addition of peptide substrate. The stock solution was divided into 125 µl aliquots and incubated in sealed tubes at 25°C. Initial rate measurements were taken in duplicate for incubation times of 5, 7.5, and 10 min at substrate concentrations of 2.5, 5.0, 10.0 and 20.0 µM. The kinetic parameters were calculated using the kinetics program HYPER (23).

Results

Synthesis of Potential Peptide Substrates. Peptides were prepared by solid phase peptide synthesis. Extending the observation that proPT28, a 28-residue peptide based upon the sequence of human proprothrombin from residue -18 to residue +10 (13), is efficiently carboxylated, we have synthesized a series of 28-residue peptides that contain the γ-CRS and the NH_2 terminus of the acarboxy form of prothrombin or factor IX, or analogs thereof. After cleavage of the peptides from the resin, each peptide was purified by reverse phase liquid chromatography to yield homogeneous material. The sequence of each peptide was verified by automated Edman degradation.

Carboxylation of Synthetic Peptides Based upon Profactor IX. We have previously demonstrated the efficient carboxylation of proPT28, a 28-residue peptide based upon the sequence of human proprothrombin from residue -18 to residue +10 [HVFLAPQQARSLLQRVRRANTFLEEVRK] (fig. 1) (13). In

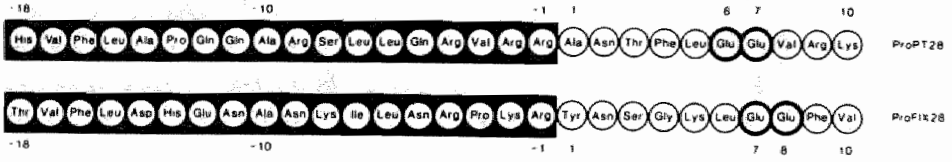

Fig. 1. Amino acid sequence of proPT28 and proFIX28. The propeptide, from residues -18 to -1, is indicated by the solid bar. Glutamic acid 6 and glutamic acid 7 in the prothrombin-based peptide are substrates for the carboxylase; the homologous glutamic acids in factor IX are located at residues 7 and 8. The numbering system is based upon that of human prothombin and human factor IX.

the current work, carboxylation of this peptide was characterized by a Km of 2.2 μM, a Vmax of 2173 cpm/min, and a Vmax/Km of 1003 (cpm/min/μM). To permit generalization that a synthetic peptide based upon the amino acid sequence of the propeptide and NH_2 terminus of other vitamin K-dependent proteins would be carboxylated, we synthesized proFIX28, [TVFLDHENAN KILNRPKRYNSGKLEEFV]. This 28-residue peptide is based upon the sequence of human profactor IX from residue -18 to residue +10 (24,25). As shown in Fig. 2 the kinetics of carboxylation of proFIX28 are similar to those of proPT28. Carboxylation of this peptide was characterized by a Km of 3.1 μM, a Vmax of 1000 cpm/min, and Vmax/Km of 328. These results indicate that the γ–CRS of both prothrombin and factor IX bind similarly to the carboxylase, but the Vmax for the substrate containing the prothrombin

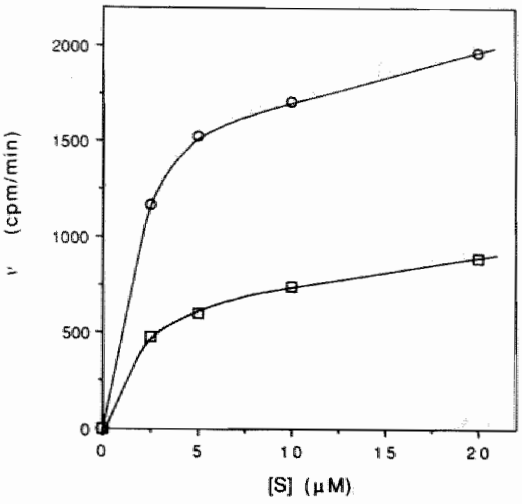

Fig. 2. Comparison of the rates of carboxylation of proFIX28 (□), based upon the structure of profactor IX, and proPT28 (O), based upon the structure of proprothrombin. The reaction mixture included partially purified carboxylase (1 mg), buffer A, peptide substrate at the indicated concentration S, and $NaH^{14}CO_3$ in a volume of 125 μl. After incubation of samples in duplicate at 25°C for 5, 7.5 and 10 min, the amount of ^{14}C incorporated into the substrate was measured at each substrate concentration.

propeptide is carboxylated 2- to 3-fold more rapidly than the substrate containing the factor IX propeptide.

Interchange of the γ-Glutamyl Carboxylation Recognition Site of factor IX and Prothrombin. To determine whether the rates of carboxylation of the proPT28 and proFIX28 substrates were intrinsically related to the differences in their γ-glutamyl carboxylation recognition sites, we prepared peptides in which the propeptide of factor IX was substituted for that of prothrombin (proFIX/PT28) and the propeptide of prothrombin was substituted for that of factor IX (proPT/FIX28). The carboxylation of these peptides were compared with proPT28 and proFIX28. ProPT/FIX28 [HVFLAPQQARSLLQRVRRYNS GKLEEFV], based upon residues -18 to -1 in prothrombin and residues +1 to +10 in factor IX, was carboxylated at a rate similar to proPT28. The kinetic constants are given in Table I. ProFIX/PT28 [TVFLDHENANKILNRPKRA NTFLEEVRK], based upon residues -18 to -1 in factor IX and residues +1 to +10 in prothrombin, was carboxylated at a rate similar to proFIX28. These results indicate that althought the factor IX and prothrombin γ-glutamyl carboxylation recognition sites bind to the carboxylase with similar affinity, the substrate containing the prothrombin γ-CRS is more efficiently carboxylated. The context of the glutamic acids does not appear to influence the rate of carboxylation significantly.

Size of the γ-Glutamyl Carboxylation Recognition Site. Factor IX Cambridge ($Arg^{-1} \rightarrow Ser$), factor IX San Dimas ($Arg^{-4} \rightarrow Gln$) and possibly factor IX Oxford 3 ($Arg^{-4} \rightarrow Gln$) are hemophiliac factor IX mutants that contain the propeptide and are characterized by incomplete carboxylation (10,11,26). Similar findings have been observed for a factor IX ($Arg^{-4} \rightarrow Gln$) prepared

		Km μM	Vmax cpm/min	Vmax/Km	Relative Rate
proPT28	HVFLAPQQARSLLQRVRRANTFLEEVRK	2.2	2173	1003	100
proFIX28	TVFLDHENANKILNRPKRYNSGKLEEFV	3.1	1000	328	46
proPT/FIX28	HVFLAPQQARSLLQRVRRYNSGKLEEFV	3.9	2569	658	118
proFIX/PT28	TVFLDHENANKILNRPKRANTFLEEVRK	1.6	1192	712	55

Table I. Carboxylation of peptides containing either the prothrombin propeptide or the factor IX propeptide.

by site-specific mutagenesis (12). The molecular basis for this incomplete carboxylation is unclear, but the possibility that the γ-glutamyl carboxylation recognition site includes the COOH terminus of the propeptide, including residues -4 and -1, has been suggested by us and others (10,12). To test this hypothesis, we prepared proFIX28/RS-1 [TVFLDHENANKILNRPKSYNS GKLEEFV], based upon factor IX Cambridge ($Arg^{-1} \rightarrow Ser$), and proFIX28/RQ-4 [TVFLDHENAN KILNQPKRYNSGKLEEFV], based upon factor IX Oxford 3 and factor IX San Simas ($Arg^{-4} \rightarrow Gln$), and evaluated their potential as carboxylase substrates. These synthetic peptides are substrates for the carboxylase in vitro. The kinetics of carboxylation are similar to those of proFIX28 (Table II). Therefore, although factor IX Cambridge and factor IX San Dimas are partially carboxylated, the similarity of the Km for the synthetic substrates based upon these structures and the Km of proFIX28 argues for the presence of an intact γ-CRS, despite the presence of a mutation in the propeptide.

		Km μM	Vmax cpm/min	Vmax/Km	Relative Rate
proFIX28	TVFLDHENANKILNRPKRYNSGKLEEFV	3.1	1000	328	100
proFIX28/RX-1	TVFLDHENANKILNRPKSYNSGKLEEFV	3.4	998	290	100
proFIX28/RQ-4	TVFLDHENANKILNQPKRYNSGKLEEFV	3.4	866	253	87

Table II. Effect of amino acid substitution in the propeptide of factor IX on the interaction of the γ-glutamyl carboxylation recognition site with the carboxylase.

Comparison of Carboxylation of Glu^6 and Glu^7. The relative rates of carboxylation of Glu^6 and Glu^7 in proPT28 are not known nor is it certain that glutamic acid residues at both positions get carboxylated in the in vitro

assay using proPT28 (13). To evaluate this question, we prepared analogs of proPT28 in which an alanine was substituted for either Glu^6 or Glu^7. These peptides, proPT28/EA6 [HVFLAPQQARSLLQRVRRANTFLAEVRK] and proPT28/EA7 [HVFLAPQQARSLLQRVRRANTFLEAVRK], were evaluated as substrates for the carboxylase. $^{14}CO_2$ was incorporated into both substrates, indicating carboxylation of glutamic acid at both position 6 and position 7. The rate of incorporation of CO_2 into these peptides was equivalent and about 30% lower than that of proPT28 (Fig. 3.). The Vmax/Km of Glu at position 7 was within experimental error of that of Glu at position 6. Thus glutamic acid at position 6 or at position 7 is carboxylated in a simplified substrate containing a single site for carboxylation.

SUBSTRATE	K_m (μM)	V_{max} (CPM/min)	$\frac{V_{max}}{K_m}$	Rate (Normalized)
~~ Glu_6-Glu_7 ~~~	2.2	2173	1003	1.00
~~ Glu_6-Ala_7 ~~~	5.8	1780	308	0.82
~~ Ala_6-Glu_7 ~~~	5.6	1522	273	0.70
~~ Glu_6-Asp_7 ~~~	6.5	1199	183	0.55
~~ Asp_6-Glu_7 ~~~	6.7	1469	220	0.68
~~ Asp_6-Asp_7 ~~~	3.7	14	4	0.01

Fig. 3. Comparison of the carboxylation of glutamic acid and aspartic acid in synthetic peptides based upon prothrombin containing the γ–CRS. Km is presented in μM, Vmax in cpm/min, and Vmax/Km in cpm/min/μM. The Vmax of the reactions are normalized, with that of the Glu^6 - Glu^7 peptide defined as 1.00.

Carboxylation of Aspartic Acid. An analog of proPT28 was prepared in which aspartic acid residues were substituted for glutamic acid residues at positions 6 and 7. The carboxylation of the peptide [HVFLAPQQARS LLQRVRRANTFLDDVRK] was minimal, with incorporation of CO_2 within the background of the assay. If the ^{14}C measured were completely associated with aspartic acid in the form of β-carboxyaspartic acid, the upper limit of the rate of carboxylation of aspartic acid is less than 1% that of the carboxylation of glutamic acid (Fig.3.). These results indicate that aspartic acid is a poor substrate for the carboxylase.

The effect of aspartic acid on the carboxylation of an adjacent glutamic acid was measured in two peptides containing Asp-Glu or Glu-Asp at positions 6 and 7. ProPT28/ED6 [HVFLAPQQARSLLQRVRRANTFLEDVRK], was carboxylated with a Km of 6.5 μM, a Vmax of 1199 cpm/min, and a Vmax/Km of 183. These Km, Vmax and Vmax/Km values for proPT28/ED7 and

proPT28/ED6 are within experimental error. These results suggest that the presence of an aspartic acid next to a glutamic acid did not alter the rate or minimally alters the rate of carboxylation of the adjacent glutamic acid. The similarity of the carboxylation rates or proPT28/ED6, proPT28/ED7, proPT28/EA6, and proPT28/EA7 emphasize that the negatively charged aspartic acid does not inhibit or only minimally alters carboxylation of the adjacent glutamic acid.

Carboxylation of Carboxymethylcysteine. Carboxymethylcysteine, a homoglutamic acid analog, was introduced into a proPT28 analog by chemical modification. ProPT28/EC6/ED7, [HVFLAPQQARSLLQRVRRANTFLCD VRK], was prepared. No significant carboxylation of this peptide was observed in the in vitro carboxylation assay. This peptide was carboxymethylated using iodoacetic acid, yielding carboxymethylcysteine at position 6. The structure of this peptide was confirmed by automated Edman degradation. When this peptide was evaluated as a substrate for carboxylation, no incorporation of CO_2 was observed. The significant decrease in the fixation of CO_2 in the reaction mixture when this peptide was present compared to reactions in which a peptide substrate was deleted from the reaction suggests that this peptide may be a potent competitor of the endogenous substrate that contaminates this carboxylase preparation.

Discussion

The vitamin K-dependent carboxylase is an enzyme found in the endoplasmic reticulum that is responsible for the post-translational modificaton of specific glutamic acid residues of a select group of proteins to form γ-carboxyglutamic acid. This amino acid confers metal binding properties on the vitamin K-dependent proteins and is required for the interaction of these proteins with phospholipid vesicle surfaces. From in vivo and in vitro experiments (6-8, 13), it appears that the carboxylation of the precursor forms of the the vitamin K-dependent proteins involves a recognition element adjacent to the glutamic acid-rich region that serves as substrate for the carboxylase. In the current work, we have evaluated a series of synthetic peptides to obtain a better understanding of the substrate requirements for carboxylation. We have previously demonstrated that an essential element for efficient carboxylation is the presence of a propeptide containing an intact γ-glutamyl carboxylation recognition site (6,13). Truncation of the propeptide or the replacement of the highly conserved phenylalanine-16 disrupted carboxylation by markedly reducing the binding affinities of these peptides for

the enzyme or enzyme complex (13). Although Phe^{-16} and Ala^{-10} have been implicated in this recognition site from in vivo experiments (6), the role of Arg^{-4} and Arg^{-1} in this site has been uncertain. Inspection of the propeptide cleavage sites of the vitamin K-dependent proteins clearly indicates that residues -4 to -1 define the propeptidase cleavage site (10,26), but the role of this region in carboxylation is more problematic. The impaired carboxylation of factor IX mutants containing mutations at -4 and -1 has implicated these residues in carboxylation. These mutations may disrupt the carboxylation recognition site or may alter the carboxyolation of adjacent glutamic acids. To resolve this issue, we prepared 28-residue peptide substrates based upon residues -18 to +10 of factor IX, factor IX Cambridge, and factor IX San Dimas. These peptides are substrates for the carboxylase and are characterized by K_m values of about 3 μM. These results indicate that these peptides contain an intact carboxylation recognition site with the ability to interact with the carboxylase with normal affinity, and thus Arg^{-4} and Arg^{-1} are not part of this site. It would appear that the impaired carboxylation of the factor IX mutants is not a result of the decreased affinity of the precursor protein for the carboxylase due to a disrupted carboxylation recognition site, but instead this defect interferes with the carboxylation of glutamic acids within the mature zymogen. Although our results would suggest that Glu^7 and Glu^8 are efficiently carboxylated in the peptide, thus indicating that the carboxylation of other more distal glutamic acid residues in factor IX is impaired, it also remains possible that the inherent flexibility of the short synthetic peptide imposes less contraints on the conformation of the region of Glu^7 and Glu^8 in the peptide than in the natural protein substrate. Localization of glutamic acid and γ–carboxyglutamic acid residues in the NH_2 terminus of factor IX Cambridge and factor IX San Dimas would likely identify specific glutamic acid residues whose carboxylation is impaired and place constraints on carboxylase mechanistic proposals.

Suttie and colleagues (27) have previously analyzed small peptide substrates containing aspartic acid and demonstrated the formation of β–carboxyaspartic acid from aspartic acid by the rat liver vitamin K-dependent carboxylase (27). Boc-Asp-Asp-Leu-methyl ester was carboxylated at 0.5% of the relative activity of Boc-Glu-Glu-Leu-methyl ester. Boc-Asp-Glu-Leu-methyl ester and Boc-Glu-Asp-Leu-methyl ester were intermediate substrates. The rates of carboxylation of FLEDL and FLDEL were 10% and 19%, repsectively, of the rate of carboxylation of the prototypic substrate FLEEL. These results, although indicating the carboxylation of aspartic acid, demonstrate a wide variation in the efficiency of carboxylation depending upon the sequence

context of the aspartic acid. Furthermore, some of these results could be interpreted as suggesting that the aspartic acid, although carboxylated, inhibits the carboxylation of the adjacent glutamic acid. These substrates have a Km in the millimolar range; a Km^{app}, of 7.3 mM and Vmax of 940 is reported for Boc-Asp-OBzl and a Km^{app} of 6.8 mM and Vmax 0f 11,550 is reported for Boc-Glu-OBzl (27). In our study, the aspartyl peptide substrates evaluated contained a γ-glutamyl carboxylation recognition site, and the Km values measured were about 3 μM, about 1,000-fold lower than the small substrates noted above. Using higher affinity substrates, we observe poor incorporation of CO_2 into peptides that contain aspartic acid. However, although aspartic acid is not a good substrate for the carboxylase, it does not interfere with the carboxylation of adjacent glutamic acids. We conclude that the bovine liver vitamin K-dependent carboxylase is not likely to significantly carboxylate aspartic acid residues under physiologic conditions. Furthermore, carboxymethylcysteine, an analog of homoglutamic acid that has been used elsewhere to evaluate the function of acidic amino acid side chains (28), is not a substrate for the carboxylase. These results suggest an enzyme mechanism in which there is a fastidious requirement for the precise localization for the methylene proton that is extracted. Carboxymethylcysteine and aspartic acid, in which this proton is displaced in space about 1 Å further or closer, respectively, from the Cα compared to glutamic acid, are not suitable substrates (Fig. 4).

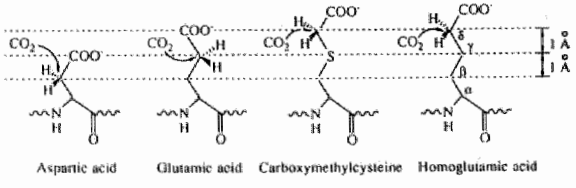

Fig. 4. Spatial relationships of the methylene proton extracted in the vitamin K-dependent reaction to form the carbanion

Our results furthermore indicate the common functional features of the carboxylation recognition sites of factor IX and prothrombin. These sites do not appear to be integrally joined or annealed to the glutamic acid-rich regions in the NH_2 termini of these zymogens. Interchange of the recognition sites of factor IX and prothrombin lead to efficient carboxylation. These results emphasize that, despite the sequence differences in these two proteins, both recognition sites serve to effect tight binding of the substrate to the carboxylase. Furthermore, the similarities of the affinities of these substrates for the carboxylase, regardless of the alterations in the substrate region of the peptide, suggest that the affinity of interaction between the carboxylase and the

synthetic peptides is dominated by the interaction of the carboxylation recognition site with the carboxylase or carboxylase complex. We have previously observed increased efficiency of the in vivo carboxylation of prothrombin compared to factor IX in a mammalian heterologous expression system (29,30). Prothrombin was fully carboxylated, even when prothrombin synthesis was increased by gene amplification. In contrast, factor IX was only about 70% carboxylated at low expression levels. The current in vitro studies reflects a similar phenomenon, suggesting that the prothrombin propeptide promotes more rapid carboxylation than does the factor IX propeptide. The molecular basis for this observation awaits further study. The recent purification of the vitamin K-dependent carboxylase will now allow analysis of its mechanism of action with special attention to the specific role of vitamin K (31,32).

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CHAPTER 4

PARTIAL PURIFICATION OF THE VITAMIN K-DEPENDENT CARBOXYLASE, BY AFFINITY CHROMATOGRAPHY USING A SYNTHETIC PEPTIDE BASED UPON THE PROPEPTIDE OF HUMAN PROTHROMBIN

Based on:

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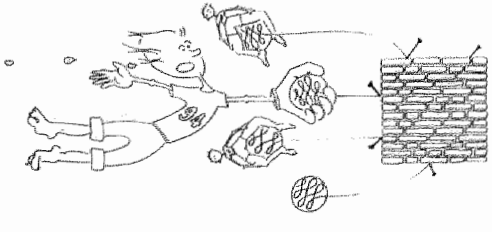

PARTIAL PURIFICATION OF THE VITAMIN K-DEPENDENT CARBOXYLASE, BY AFFINITY CHROMATOGRAPHY USING A SYNTHETIC PEPTIDE BASED UPON THE PROPEPTIDE OF HUMAN PROTHROMBIN

Abstract

The vitamin K-dependent carboxylase catalyzes the post-translational modification of specific glutamic acid residues to form γ-carboxyglutamic acid residues within the vitamin K-dependent proteins. This enzyme recognizes the γ-glutamyl carboxylation recognition site on the propeptide of the precursor forms of the vitamin K-dependent blood coagulation proteins. We attempted to purify the enzyme by affinity chromatography using a synthetic peptide based upon the amino-acid sequence of the propeptide of prothrombin. Elution with 10 mM propeptide with detergent and phospholipids gave us a preparation in which only minimal amount of carboxylase activity could be detected. High concentrations of propeptide, however, also inhibit the vitamin K-dependent carboxylation reaction in crude microsomes. On SDS gel electroforesis this preparation showed one major band with a molecular weight of 78,000. A biologically active carboxylase preparation was obtained by coupling a similar synthetic peptide via a cysteine to a activated thiol-Sepharose column. The carboxylase-propeptide complex was eluted at room temperature by reduction of the disulfide bond with dithiotreitol (DTT) in the presence of detergent and phospholipids. This preparation also showed a major band of 78,000 Dalton on SDS gel electroforesis, which was characterized as binding protein (BiP).

Carboxylase had been purified more than 10,000 fold by the affinity chromatography step, which may prove to be a valuable tool in our attempts to purify the enzyme to homogeneity.

Introduction

γ-carboxyglutamic acid is a component of an unique class of calcium-binding proteins that require vitamin K for their complete synthesis (1,2). Of the vitamin K-dependent proteins, prothrombin, factor IX, factor X, factor VII, protein C, and protein S are plasma proteins involved in blood coagulation or the regulation of coagulation (3,4). These proteins bind to calcium ions through γ-carboxyglutamic acid and undergo a calcium-induced conformational transition that is associated with the expression of a membrane binding site (5-10). The role of the γ–carboxyglutamic acid-rich domain involves calcium-dependent binding of the vitamin K-dependent proteins to membrane surfaces, allowing the formation of protein complexes on membranes for the efficient activation of these proenzymes to their active form (11).

The vitamin K-dependent blood coagulation proteins are synthesized in the liver in a precursor form (12,13). Specific glutamic acid residues are converted to γ-carboxyglutamic acid by a vitamin K-dependent carboxylase located in the rough endoplasmic reticulum. This reaction requires reduced vitamin K, molecular oxygen, and carbon dioxide (14). The prozymogens, including proprothrombin and profactor IX, contain a propeptide between the signal peptide and the mature zymogen (15-17). The propeptides of all of the vitamin K- dependent proteins have sequence homology (18). On the basis of this homology and the observation that a defect in the propeptide of factor IX Cambridge and factor IX San Dimas impaired carboxylation (19,20), we proved that an intact propeptide is required for carboxylation (21). Mutation of phenylalanine -16 or alanine -10, two highly conserved residues in the propeptide, or deletion of the propeptide (-18 to -1) in factor IX obliterated in vivo carboxylation of mutant proteins expressed in heterologous mammalian cells (21,22). Similar results with the propeptide of protein C confirmed these findings (23).

A recognition element, known as the γ-glutamyl carboxylation recognition site, is contained within the propeptide (21). Synthetic 28-residue peptides containing this γ-glutamyl carboxylation recognition site and the NH_2-terminal residues of the mature zymogen undergo efficient vitamin K-dependent carboxylation in vitro when a partially purified form of bovine liver

carboxylase is used (24,25). Synthetic peptides homologous to the propeptide also stimulate the carboxylation of a small peptide substrate, Phe-Leu-Glu-Glu-Leu (FLEEL) (26). These studies have suggested that the γ-glutamyl carboxylation recognition site in the propeptide binds directly to the carboxylase.

The vitamin K-dependent carboxylase is an integral membrane protein. Although it has attracted considerable interest since its discovery (27), the protein has been refractory to complete purification because of problems with its solubilization and stability (27-36). For all mechanistic and kinetic studies performed up till now, the enzyme was used as crude microsomes or as the insolubilized form Solid Phase carboxylase (31). For more detailed mechanistic studies of the action of the enzyme, the carboxylase should be purified to homogeneity. In this paper we describe a method, based upon affinity chromatography, in which in one step a substantial purification is obtained. The product thus obtained can be used as the starting material for further purification steps of the carboxylase.

Materials and Methods

Synthesis of Peptides. Peptides were synthesized on an Applied Biosystems model 430A peptide synthesizer (37) and purified as previously described (24,25). The cleavage reactions were performed in HF/anisole/dimethyl sulfide/resin (10:2:2:1) for cysteine-containing peptides. The sequence of each peptide was verified by automated Edman degradation, using an Applied Biosystems model 470 protein sequencer.

Vitamin K-dependent Carboxylase Assay. The amount of $^{14}CO_2$ incorporated into exogenous peptide substrates was measured in reaction mixtures of 125 µl containing the carboxylase preparation, 0.8 mM vitamin K hydroquinone, 1.5 mM $NaH^{14}CO_3$ (10 µCi = 37 GBq), and 8 mM dithiothreitol (DTT) (38). Assays measuring the carboxylation of FLEEL and proPT28 were carried out at 10 mM substrate and 10 µM substrate, respectively (24). $^{14}CO_2$ incorporation into peptides was quantitated in a Beckman LS1801 liquid scintillation counter. Vitamin K epoxide formation was measured by the method of Sadowski et al.(39).

Preparation of Ammonium Sulfate Precipitated Carboxylase. Crude microsomes from bovine liver were washed, solubilized with detergent, and precipitated with ammonium sulfate as described earlier (32).

Affinity Purification of the Vitamin K-dependent Carboxylase by Using a Propeptide Elution System. The synthetic peptide (40 mg) was coupled to cyanogen bromide-activated Sepharose 4B (5 ml, Pharmacia) according to the supplier's instructions. Two affinity gels were designed, both incorporating the propeptide of prothrombin from residue -18 to residue -1, proPT18 (25). The peptides KKKGGGIGGKAAAAH**VFLAPQQARSLLQRVRR** and KGGH**VFLAPQQARSLLQRVRR**, where amino acids in bold face indicate proPT18, were employed. Columns (1 x 10 cm) of these affinity gels, 5 ml, were equilibrated in buffer A [20 mM sodium phosphate, pH 7.4/0.15 M NaCl/0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sigma) containing 1 mg of L-phosphatidylcholine (type V-E, Sigma) per ml]. Ammonium sulfate precipitated carboxylase (4.0 ml) was applied to the column and the flow was stopped for 3 hr. Then the column was washed with buffer A, buffer A/1 M NaCl and equilibrated with 4 ml of buffer A/10 mM proPT18 for 3 hr. The eluted protein was washed from the column with buffer A and stored at -20°C.

Affinity Purification of the Carboxylase by Reductive Cleavage of the Enzyme-Propeptide Complex. 2-Thiopyridylactivated thiol-Sepharose 4B (Pharmacia) containing 1 μmol of activated sites per ml was swelled in 100 mM Tris/1 mM EDTA, pH 7.0, and washed with 100 mM Tris/1 mM EDTA/ 500 mM NaCl, pH 7.5. Peptide (10 μmol) was dissolved in 6.0 ml of 100 mM Tris/1 mM EDTA/500 mM NaCl, pH 7.5, and the solution was added to 4 ml of activated gel. The reaction proceeded for 3.5 hr at room temperature under N_2. After filtering, the gel was washed with 6 ml of 100 mM Tris/1 mM EDTA/500 mM NaCl, pH 7.5. The coupling efficiency was measured by quantitation of the release of 2-thiopyridone(40). The gel was washed with 100 mM ammonium acetate, pH 4.5, and the unreacted 2-thiopyridyl groups were displaced with a 3-fold molar excess of 2-mercaptoethanol over the original amount of activated sites in 4 ml of 100 mM ammonium acetate, pH 4.5. After 30 min under N_2 at room temperature, the gel was washed with 100 mM ammonium acetate at pH 4.5, 20 mM sodium phosphate, pH 7.4/0.15 M NaCl (PBS), and PBS/0.02% NaN_3 for storage at 4°C.

Ammonium sulfate precipitated carboxylase (2.0 ml) was applied to a 2-thiopyridyl activated thiol-Sepharose column (6 ml of gel; 1 x 10 cm) equilibrated in buffer B [20 mm sodium phosphate, pH 7.4/0.15 M NaCl/ 0.1% CHAPS /15 % (vol/ vol) glycerol/1 mM EDTA/1 mg per ml phospholipid, composed of 91 % (wt/ wt) chicken egg L-phosphatidylcholine (type V-E, Sigma), 4.5 % (wt/ wt) Folch fraction III bovine brain extract, and 4.5 % (wt/ wt) bovine heart L-phosphatidylethanolamine (type VII, Sigma)] at 4°C and

the flow was stopped for 3 hr. The column was then washed with buffer B at 1 ml/ hr. The carboxylase, which did not bind to the column, was collected.

This carboxylase preparation was applied to a CGGHVFLAPQQARS LQRVRR-Thiol-Sepharose column (1 x 10 cm) containing 3.75 ml of affinity matrix equilibrated in buffer B, and the flow-through was recycled through the column for 3 hr at 2 ml/ hr. The affinity gel was then washed at 20 ml/ hr with a series of four buffers: 50 ml of buffer B, 50 ml of buffer B/ 1 M NaCl, 10 ml of buffer B, and 100 ml of buffer C [20 mM sodium phosphate, pH 7.4/0.15 M NaCl/0.25 % CHAPS/15 % (vol/ vol) glycerol/ 1 mM EDTA/1 mg per ml phospholipid]. The affinity gel, at 4°C, was treated with 4 ml of buffer C/35 mM DTT at 4°C. The column was then adjusted to room temperature. Reduction proceeded for 45 min. The carboxylase was eluted at room temperature with an additional 8 ml of buffer C/35 mM DTT at 20 ml/ hr. The partially purified carboxylase was stored at 4°C.

Results

Design of Propeptide-Based Affinity Matrices. In designing an affinity purification of the carboxylase, we have exploited both the specificity and affinity of the propeptides of the vitamin K-dependent proteins for the carboxylase. ProPT18, a synthetic peptide based upon residues -18 to -1 of the human prothrombin propeptide, was the ligand for several affinity matrices (Fig. 1). Although free proPT18 inhibits the carboxylation of proPT28, matrix in which the propeptide was coupled directly to the beads did not. Therefore, we evaluated two peptides that included NH_2-terminal linkersegments for their utility as ligands for affinity chromatography. Matrix A (Fig. 1) has KKKG GGIGGKAAAAHVFLAPQQARSLLQRVRR coupled to CNBr-activated

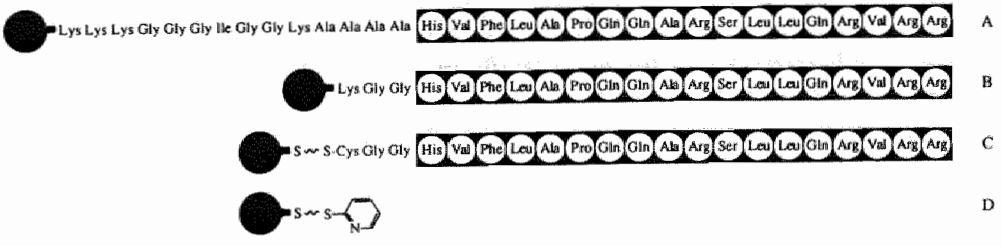

Fig. 1. Propeptide-based matrices for affinity purification of the vitamin K-dependent carboxylase. The highlighted region of the peptide sequences represents residues -18 to -1 of human proprothrombin, the prothrombin propeptide.

Sepharose. Matrix B (Fig. 1) has peptide KGGHVFLAPQQARSLLQRVRR. Both peptides inhibited the carboxylation of proPT28 in their free and Sepharose bound form. Matrix C (Fig. 1) contains a peptide homologous to the peptide in matrix B except that the cystein is substituted for an NH_2-terminal lysine. This peptide was allowed to react with activated thiol-Sepharose. Matrix C allows for the removal of the peptide from the gel by reductive cleavage of the disulfide bond between the thiol on the Sepharose and the cysteine in the synthetic peptide. Matrix D is the activated thiol-Sepharose.

Affinity Purification of Carboxylase. Affinity matrix A was incubated with crude carboxylase that had been detergent-solubilized from bovine liver microsomes and fractionated with ammonium sulfate. After extensive washing, the peptide-Sepharose beads retained high levels of carboxylase activity that remained fully active for 1 week at 4°C. The bound carboxylase activity could be displaced from the matrix by using 10 mM proPT18. Analysis of the eluate by SDS polyacrylamide gel electroforesis revealed a preparation in which a protein with M_r 78,000 (Fig. 2, lane A) was most abundant. Most of the

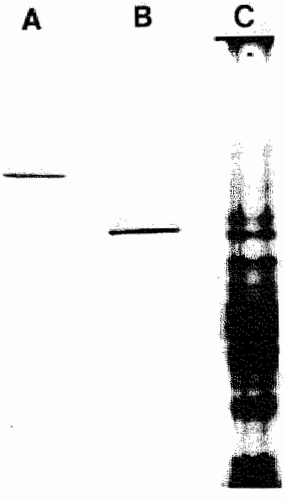

Fig. 2. SDS gel electroforesis of the vitamin K-dependent carboxylase. The gels were stained with Coomassie blue. Lane A, vitamin K-dependent carboxylase prepared by using matrix A and elution with proPT18; 10% acrylamide/SDS gel; M_r 78,000 by comparison to molecular weight markers. Lane B, carboxylase prepared by using matrix B and elution with DTT; 7.5% acrylamide/SDS gel; M_r 78,000. Lane C, detergent-solubilized microsomes subjected to ammonium sulfate fractionation, 7.5% acrylamide/SDS gel.

carboxylase activity was eliminated from the affinity matrix upon elution with proPT18, but minimal carboxylation activity was recovered in the eluate, as monitored by the carboxylation of FLEEL. Although the in vitro carboxylation of FLEEL is stimulated by addition of 1 μM propeptide (26), at high concentrations (10 mM) the propeptide completely inhibits carboxylation. Thus, under the elution conditions described, the vitamin K-dependent carboxylase enzymatic activity cannot be measured. Comparable result were obtained with matrix B.

To isolate the carboxylase in the absence of inhibitory concentrations of propeptide, we developed an alternative elution method. Matrix C, containing CGGHVFLAPQQARSLLQRVRR, was prepared as an affinity gel (Fig. 1C) from which the propeptide-carboxylase complex could be eluted by reductive cleavage with DTT. The carboxylase activity is stable to reducing agents (31,32). Since matrix C contained some excess reactive sulfhydryl groups that were not coupled to peptide, the ammonium sulfate precipitated carboxylase (Fig. 2, lane C) was first applied to a column of activated thiol-Sepharose without a coupled peptide (Fig. 1D) to remove proteins in the preparation with a reactive sulfhydryl. The carboxylase activity was recovered only in the unbound fraction. This carboxylase preparation, with the thiol-binding proteins removed, was applied to affinity matrix C containing the synthetic peptide incorporating the carboxylation recognition site. The enzyme bound to the affinity matrix was extremely stable, hardly any loss of activity could be detected after two weeks at 4°C. Approximately 40% of the applied carboxylase activity remained bound to the column. Since reduction of the column with DTT at 4°C was not associated with the release of carboxylase activity, after extensive washing, several column volumes of elution buffer were applied at this temperature to ensure that the entire bed volume was equilibrated with elution buffer. Then the carboxylase-peptide complex was eluted for 45 min by restoring the column to room temperature. Carboxylase was quantitatively recovered in the eluate and minimal carboxylase activity remained associated with the column matrix. The specific activity of the partially purified carboxylase was 1.3 x 10^7 cpm of $^{14}CO_2$ per hr per mg of protein, an overall purification of 10,000-fold from crude bovine liver microsomes (Table I). SDS gel electrophoresis revealed a preparation with one major protein band corresponding to a molecular weight of 78,000 (Fig. 2, lane B). This band co-migrated with the protein obtained with propeptide elution of the affinity matrix. N-terminal amino acid sequencing identified this protein as BiP, a polypeptide binding protein present in the ER. The partially purified carboxylase was stable for a week at 4°C.

Characterization of the Vitamin K-dependent Carboxylase. The vitamin K-dependent carboxylase and the vitamin KH_2 epoxidase activities may be closely linked (42, 43). The vitamin K-dependent carboxylase, partially purified by reductive cleavage using DTT, was incubated with vitamin K and the substrate FLEEL in the presence of $^{14}CO_2$. Aliquots were removed and assayed for carboxylated FLEEL and for vitamin K epoxide. Linear time-dependent formation of both vitamin K epoxide and γ-carboxyglutamate residues was detected with the partially purified enzyme. The specific activities

	total protein mg	Specific activity cpm x 10^{-4}/hr per mg protein	Total activity cpm x 10^{-4}/hr	Purification, -fold
1. Bovine liver microsomes	ND	ND	ND	1
2. Microsomes detergent-solubilized ammonium sulfate fractionated	83.7	12.5	1043	95
3. Activated thiol-Sepharose	22.6	4.8	108	36
4. CGG-proPT18-thiol-Sepharose	0.035	1342.4	47	10,205

Table I. Partial purification of the vitamin K-dependent carboxylase. The value for the purification of carboxylase from crude microsomes to detergent-solubilized microsomes fractionated with ammonium sulfate is from Soute et al. (32). ND, not determined.

of the ammonium sulfate precipitated microsomes and the partially purified vitamin K-dependent carboxylase were 2.77 and 279.6 nmol of vitamin K epoxide per hr per mg of protein, respectively, a 100-fold purification of the vitamin K epoxidase activity from ammonium sulfate precipitated microsomes. These results suggest that the vitamin K-dependent carboxylase activity and vitamin K epoxidase activity are properties of the same protein.

Discussion

The vitamin K-dependent carboxylase is located in the rough endoplasmic reticulum (RER) (44) and is responsible for the conversion of glutamic acid residues to γ-carboxyglutamic acid residues in the precursor form of the vitamin K-dependent proteins. The carboxylase can be solubilized from the microsomal membrane fraction by using a variety of detergents, including CHAPS (34,38). Purification by traditional methods has not been successful because of the instability of the solubilized membrane protein (45). An active, albeit immobilized, carboxylase preparation can be enriched from bovine liver microsomes by using anti-factor X antibodies bound to Sepharose to bind a putative endogenous profactor X-carboxylase complex in these microsomes (31). Although initial efforts with elution of active carboxylase were unsuccessful, Suttie and colleagues (33) were able to dissociate the

prozymogen-carboxylase complex by elution with 1 mM propeptide and obtain an overall purification of about 400-fold.

The determination of the specific activity and overall purification yield is complicated for this enzyme. There is evidence to support the presence of a carboxylase inhibitor that is removed during the ammonium sulfate fraction step (34). Furthermore, the carboxylase remains active toward small substrates such as FLEEL while bound to profactor X bound to anti-factor X covalently attached to an inert matrix (31), but the effect of immobilization on its specific activity is unknown. Although the propeptide inhibits the carboxylation of synthetic peptides containing the carboxylation recognition site (24), low concentrations of propeptide (1 μM) stimulate carboxylation of FLEEL by 3- to 8-fold (24,26), while high concentrations (10 mM) are inhibitory. Finally, the carboxylase activity is unstable and rapidly decays at room temperature. Therefore, comparison of the current purification with earlier reports is difficult.

We have employed an ammonium sulfate precipitated bovine liver carboxylase as the starting material for the affinity purification (32). Because this enzyme activity is labile, we have developed a rapid affinity purification strategy to obtain a highly purified and active enzyme which is stable for over one week at 4°C.

On the basis of the binding of the carboxylation recognition site of the propeptide to the carboxylase with a K_m of about 4 μM (24), we developed affinity matrices containing synthetic peptides designed about the amino acid sequence of the prothrombin propeptide, incorporating the carboxylation recognition site and a linker domain to eliminate steric effects in the binding of the carboxylase to the affinity matrix. Specific absorption of carboxylase to the gel was realized and specific elution was effected with high concentrations of free propeptide. In these preparations only minimal enzyme activity could be detected due to the presence of inhibitory concentrations of propeptide. Efforts to remove the propeptide from the carboxylase and to regain activity were unsuccessful. For these reasons, we developed an alternative elution system based on the previous observation that the carboxylase is not inactivated by DTT (31,32). The propeptide-linker was modified with the incorporation of an NH_2-terminal cysteine residue and coupled via a disulfide bond to a Sepharose matrix containing an activated thiol. The carboxylase-peptide complex bound to the column was removed after extensive washing by the reduction of the disulfide bond coupling the peptide to the Sepharose. Because some underivatized thiols reacted with contaminating proteins, an activated thiol precolumn was employed to remove any proteins potentially reactive with the

propeptide-Sepharose column and the flow-through applied to the propeptide-Sepharose column. Although thiol columns have been used for the purification of proteins with reactive cysteines, the strategy of attachment of a ligand through a disulfide linkage and elution with a reducing agent has not been previously employed, to our knowledge. We have used a synthetic peptide that includes the primary structure of a recognition element that interacts with another protein. With the ability to synthesize peptides that incorporate this binding structure and other characteristics that maximize specific high-affinity binding and specific elution, we have demonstrated the potential for the rational design of synthetic peptides for affinity purification of proteins containing complementary binding structures. This approach may be usefully applied when the amino acid sequence of a binding domain of one protein is known, and a synthetic peptide based upon this domain may be used to purify the second protein to which it binds.

The protein that migrates as a band of M_r 78,000, and is the major component of the partially purified carboxylase, was identified as BiP. BiP is present in high quantities in the endoplasmic reticulum, and is known to bind strongly to peptides and to the heavy chain of immunoglobulin in the absence of the light chain. BiP is dissociated from the heavy chain upon assembly of this polypeptide with the light chain (46). Other proteins have been found to be dependent on BiP for their multimerisation (47). It has also been reported that mutant polypeptides which fail to assemble properly are bound to BiP permanently (48,49). Based on the techniques described in this paper Wu et al. (50) developed an affinity ligand covering the prosequence as well as the entire Gla-domain of factor IX (FIXQ/S). Using this ligand no BiP was found in the eluate of the affinity column, suggesting that FIXQ/S is more specific for carboxylase than the pro-sequence alone. This higher specificity may be caused by a second recognition site in the Gla-domain of the mature Gla-containing proteins. A second improvement described by these authors was the reduction of the size of the protein/detergent micelles by sonication, before applying the solubilized microsomes to the affinity column. By reducing the number of proteins per micelle the contaminating proteins which bound to the affinity column was minimal. With this procedure extensive washing of the column with high salt buffers and/or high detergent concentrations was not necessary, and a highly purified enzyme was obtained in one step.

On the basis of the analysis of crude and ammonium sulfate precipitated vitamin K-dependent carboxylase and vitamin K epoxidase activities it has been previously proposed that the carboxylation of glutamic residues and the oxidation of vitamin K to vitamin K epoxide are accomplished by the same

enzym (42,43). Indeed, in the experiments described in this paper, we demonstrate that vitamin K-dependent carboxylase and vitamin K epoxidase activity copurify to the same degree. This provides additional evidence for the assumption that both activities are catalyzed by the same enzyme.

With the current availability of a highly purified, enzymatically active bovine liver vitamin K-dependent carboxylase, it will become possible to examine the mechanistic details of this enzyme, with special reference to understanding the role of vitamin K in this reaction. This enzyme is likely to represent a unique carboxylase from a mechanistic point of view. The vitamin K-dependent carboxylase is widely distributed in various tissues, but its functional role in most of these tissues is unknown. Elucidation of the structure of the enzyme should provide insight into structure-function relationships in this integral membrane protein.

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CHAPTER 5

ALTERATION OF THE VITAMIN K HYDROQUINONE BINDING SITE ON VITAMIN K-DEPENDENT CARBOXYLASE BY THE INTERACTION OF PROPEPTIDE-CONTAINING SUBSTRATES

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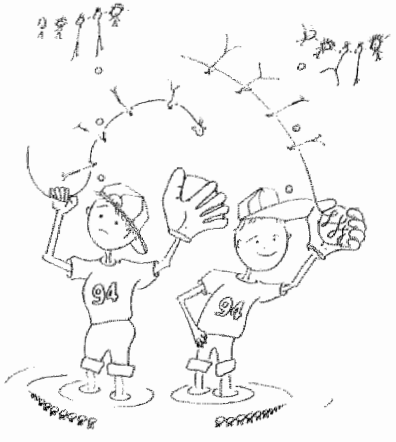

ALTERATION OF THE VITAMIN K HYDROQUINONE BINDING SITE ON VITAMIN K-DEPENDENT CARBOXYLASE BY THE INTERACTION OF PROPEPTIDE-CONTAINING SUBSTRATES

Abstract

The vitamin K-dependent carboxylase recognition site is located in the leader sequence of the precursor molecules of gamma-carboxyglutamic acid-containing proteins. This so called propeptide consists of a 18 to 24 amino acid sequence between the signal peptide and the mature protein. If the propeptide is covalently bound to a synthetic peptide substrate, it enhances the affinity of this substrate for the vitamin K-dependent carboxylase (as measured by its Km^{app}) by three orders of magnitude. Here we describe that in addition to its effect on substrate recognition, the propeptide also creates a high affinity site for vitamin K hydroquinone (KH_2). The affinity of CO_2 for carboxylase and the affinity of vitamin K quinone (K) and vitamin K epoxide (KO) for the DTT-dependent reductases were not affected by the propeptide-containing substrates. A mutation in the propeptide at -16 which is known to have a large impact on the binding of the substrate to the carboxylase did not affect the formation of the high affinity site for vitamin K hydroquinone. Both for the native and the mutant substrates, added in saturating concentrations, the apparent Km values for vitamin K hydroquinone was 40 times lower than that for substrates lacking the propeptide.

Microsomes prepared from the livers of vitamin K deficient animals contain precursors of coagulation factors and displayed the same high affinity site for vitamin K hydroquinone. A propeptide-deficient substrate which is characterized by a low Km^{app}, such as decarboxylated osteocalcin, could not accomplish a change in the affinity of KH_2 for carboxylase even when the propeptide was added to the reaction mixture. These results suggest that, if covalently bound to a substrate, the propeptide induces a conformational change in the enzyme molecule by which a high affinity binding site for KH_2 is displayed.

Introduction

Vitamin K-dependent carboxylase catalyzes the posttranslational modification of glutamic acid residues into gamma-carboxyglutamic acid (Gla) residues (1,2). During the carboxylation reaction KH_2 is converted to KO by the action of epoxidase. Together with KO-reductase and K-reductase this epoxidase forms the vitamin K cycle, by which vitamin K can be recycled effectively and reused in the carboxylation reaction several thousand times before it is degraded. The KO formed is reduced to K by the action of KO-reductase, which subsequently is converted to KH_2. The latter reaction is catalyzed by K-reductase. Several Gla-containing proteins have been isolated from blood plasma and calcified tissues. Among them are the blood coagulation proteins prothrombin, factors VII, IX and X and the anticoagulant proteins C and S. All of these proteins are synthesized in the liver and contain 10 to 12 Gla residues near the NH_2 terminus of the mature protein. The bone Gla proteins, osteocalcin and matrix Gla protein (MGP), are both synthesized in the osteoblasts (3-5), and contain 3 and 5 Gla residues respectively. Sequences obtained from cDNA clones of the Gla-containing proteins revealed that the precursor forms of these proteins, except MGP contain a propeptide located between the signal peptide and the mature protein (6-8). Comparison of the propeptides of the various Gla-containing proteins showed a marked sequence homology (9). MGP is the only known Gla-protein not containing a propeptide in the leader sequence. Instead the mature protein was shown to contain an amino acid sequence homologous with the propeptide (9) and which is regarded as an internal pro-sequence. Based upon the homology of the propeptides of the various Gla-containing proteins, the hypothesis was postulated that the propeptide might contain the vitamin K-dependent carboxylase recognition site. The occurrance of undercarboxylated factor IX

mutants in the blood plasma of patients suffering from hemophilia B, which were shown to contain a mutation in the propeptide region (22-26), strengthened this hypothesis. Jorgensen et al. demonstrated that carboxylation of recombinant factor IX, expressed in mammalian cells, could be effected by mutations in the propeptide (15). The highly conserved phenylalanine at -16 turned out to play a crucial role in the recognition by the vitamin K-dependent carboxylase. In vitro this could also be demonstrated by the use of synthetic substrates containing the propeptide (16,17). The apparent Km of these substrates was 3 orders of magnitude lower than comparable substrates lacking the propeptide, whereas the alteration of phenylalanine at -16 to an alanine increased the apparent Km almost a hundred fold. Although the propeptide was proven to play an important role in substrate recognition by the carboxylase it is not yet certain that this sequence contains the only signal for gamma-carboxylation. It has been suggested by Price (19) that the consensus sequence Glu-X-X-X-Glu-X-Cys in the mature Gla-containing proteins might have an additional role in this process. In vitro studies have shown that substrates obtained from decarboxylated mature Gla-containing proteins lacking the pro-sequence, indeed are readily carboxylated. The apparent Km values for decarboxylated osteocalcin or decarboxylated sperm Gla protein (20) are comparable with those for the synthetic peptides containing the propeptide, suggesting that these proteins do contain a recognition site for carboxylase. The Km^{app} of carboxylase for a 59 residue recombinant peptide substrate based upon the propeptide and the complete Gla-domain of coagulation factor IX (proFIX59) has a Km^{app} slightly lower (0.55 μM) than the Km^{app} for proPT28. On prolonged incubation this substrate was carboxylated completely, suggesting that ProFIX59 contains all information necessary for carboxylation (18). The study presented in this paper shows that the propeptide not only serves as a recognition site for carboxylase but also induces a conformational change in the enzyme, resulting in the exposure of a high affinity binding site for KH_2.

Materials and Methods

Chemical. Vitamin K hydroquinone (Hoffmann-La Roche) was prepared as described previously and stored at -30°C until its use (28). Dithiotreitol (DTT) and 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) were obtained from Sigma. The synthetic substrate FLEEL was obtained from Vega Biochemicals, peptides proPT28 (HVFLAPQQARSLLQRVRRANT

FLEEVRK), proPT28 [FA-16] (HVALAPQQARSLLQRVRRANTFLEEVRK) and proPTFLEEV (HVFLAPQQARSLLQRVRRFLEEV) were from Applied Biosystems, propeptide (HVALAPQQARSLLQRVRRANT) was a gift from Drs. B.C. Furie and B. Furie, New England Medical Center Hospitals, Boston MA, and proFIX59 was a gift from Dr. D.W. Stafford, University of North Carolina, Chapel Hill NC. $NaH^{14}CO_3$ (40-60) Ci/mol) and Formula 989 were obtained from Du Pont-New England Nuclear. All other chemicals were from Merck.

Preparation of Washed Microsomes. Rats were fed with vitamin K- deficient food (Hope Farms, Woerden, The Netherlands) for one week. Livers were obtained from vitamin K-deficient and normal rats that were sacrified under ether anestesia. Fresh, normal cow liver was obtained from the slaughterhouse. Salt washed microsomes were prepared from liver tissue according to a previously described procedure (20).

Carboxylase Assay. The incorporation of $^{14}CO_2$ into synthetic peptide substrates was determined in 125 µl reaction mixtures containing 4 mg crude microsomes, 0.4% CHAPS, 5 µCi $NaH^{14}CO_3$, 6 mM DTT in a buffer containing 0.5 M NaCl, 25 mM Tris-HCl pH 7.5. Synthetic peptides and KH_2 were added as subsequently indicated. The reaction mixtures were incubated in 500 µl sealed tubes at 20°C and stopped at subsequently indicated times by adding 75 µl 0.5 M NaCl to the reaction mixture. 150 µl sample was transfered to a glass vial and 2 ml 5% trichloroacetic acid (TCA) was added. Nonbound $^{14}CO_2$ was removed by boiling the mixture for 1 min. Five ml Formula 989 was added and the samples were counted in a Beckman LS 3801 liquid scintillation counter.

KO Reductase Determination. The KO-reductase activity was measured according to the method of Thijssen et al. (21). Standard reaction mixtures (see carboxylase assay) were incubated at 20°C. Aliquots were taken at 2.5, 5 and 10 minutes, extracted with isopropanol/hexane and analysed directly by HPLC on a Lichrosorb RP 18 reversed phase column (Chrompack).

Results

The kinetic constants for the cofactors of carboxylase were studied in systems in which the following carboxylatable substrates (Fig.1) were used. Substrate 1 (proPT28) is a synthetic peptide based on the amino acid sequence -18 to +10 of human descarboxy prothrombin. It consists of the complete propeptide and the first 10 amino acid residues of the NH_2 terminus of descarboxy prothrombin.

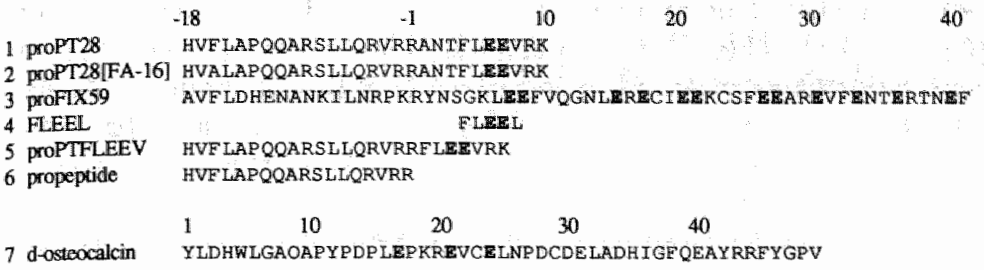

Fig.1. Primary structure of the peptides used as carboxylatable substrates in the vitamin K-dependent carboxylase reaction.

Substrate 2 (proPT28[FA-16]) is the mutant form of proPT28 in which phenylalanine at position 3 is altered in alanine. Substrate 3 (ProFIX59) is a 59 residue recombinant peptide containing the propeptide and the complete non-carboxylated Gla-domain of human factor IX. Substrate 4 (proPTFLEEV) consists of the 18 amino acid propeptide of prothrombin with the pentapeptide FLEEV covalently attached to its C-terminal. Substrate 5 (propeptide) is identical with the 18 amino acid propeptide of prothrombin. Substrate 6 (FLEEL) is a synthetic peptide based upon amino acid residues 4-9 in human factor VII and one of the most frequently used substrates in carboxylase research. Substrate 7 (d-osteocalcin) is the descarboxy form of bovine osteocalcin.

Kinetic constants were determined using various carboxylatable substrates. These substrates were used in saturating concentrations. For the propeptide-lacking substrates, the kinetic constants were also determined in the presence of the propeptide. The initial velocities of the reaction were established from 25 min. time courses in the presence of either 10 mM FLEEL, 20 μM d-osteocalcine, 20 μM proPT28, 1 mM proPT28[FA-16], 10 μM proFIX59 or 100 μM proPTFLEEV. KH_2 concentrations varied from 14 to 444 μM when

substrate	Km^{app} (μM)
FLEEL	41.5
FLEEL + propeptide	22.0
d-osteocalcin	54.0
d-osteocalcin + propeptide	25.2
proPT28	1.1
proPT28 + propeptide	1.4
ProFIX59	0.7
proPT28[FA-16]	1.9
proPTFLEEV	3.9

Table I. Km^{app} values of KH_2 in normal bovine liver microsomes using different substrates. The apparent Km values were calculated from Lineweaver-Burk plots. The initial rates at suturating conditions were determined from the amount of $^{14}CO_2$ incorporated in substrate at 5, 15 and 25 minutes with vitamin KH_2 concentrations varying from 350 nM to 888 μM. Concentrations used were d-osteocalcin: 20 μM, proPT28[FA-16]: 800 μM, proPT28: 20 μM, FLEEL: 10 mM, ProFIX59: 20 μM, proPTFLEEV: 100 μM and propeptide: 10 μM.

the propeptide-lacking substrates were used, and 0.32 to 10 μM when the propeptide-containing substrates were used. Lineweaver-Burk plots show a linear relationship between the reciprocal values of the initial velocity and the substrate concentration. The appararent Km values (table I) for KH_2, determined from the Lineweaver-Burk plots, were 41.5 μM in the presence of FLEEL, and 54 μM when d-osteocalcin was used as a substrate. In the presence of propeptide-containing substrates these values were considerably lower: 0.7 μM with proFIX59, 1.1 μM with proPT28 and 3.9 μM with proPTFLEEV. To evaluate whether the propeptide by itself could accomplish this change in affinity for KH_2 we determined the Km^{app} for KH_2 in a carboxylase system containing 10 μM of propeptide and either FLEEL or d-osteocalcin as carboxylatable substrates. The addition of propeptide to the reaction mixtures did not significantly alter the Km^{app} of KH_2 for carboxylase (22 μM and 25 μM with FLEEL and d-osteocalcin respectively). Through both in vivo and in vitro studies, the amino acid residues -18 to -10 have been determined to be essential for the recognition by the vitamin K dependent carboxylase (15-17). In particularly, a mutation of the highly conserved phenylalanine at -16 greatly affected the carboxylation. If added in saturating concentrations, both proPT28 and the mutant substrate proPT28[FA-16] had the same effect on the Km^{app} value for vitamin KH_2 (table I), however. The affinity of CO_2 for carboxylase did not depend on the carboxylatable substrate used (fig. 2).

We also examined the effect of the propeptide-containing substrate on the binding of the metabolites of vitamin K to the reductases. The results show that affinity of KO and K for the reductases is not changed when propeptide-containing substrates were used (table II).

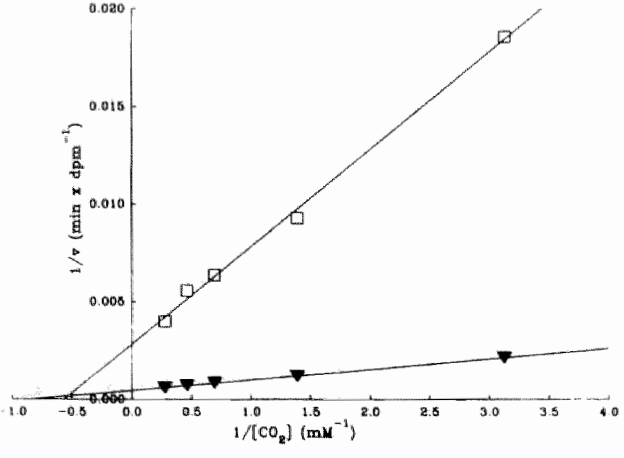

Fig. 2. Lineweaver-Burk plots of $^{14}CO_2$ incorporation in FLEEL (▼) or proPT28 (□).

cofactor	substrate FLEEL	proPT28
	(μM)	(μM)
CO_2	1100.0	1700.0
KO	8.3	8.5
K	22.0	19.0

Table II. Michaelis constants of various cofactors of the vitamin K-dependent carboxylase. Km^{app} values in normal bovine liver microsomes of various cofactors of the vitamin K-dependent carboxylation reaction using proPT28 (20 μM) or FLEEL (10 mM) as the carboxylatable substrates. The Michaelis constants were determined as described in the legends to table I, except for Km for KO which was determined by HPLC technique (see material and methods).

In vitamin K deficiency, the carboxylation process in vivo is stopped resulting in an accumulation of precursor proteins of the coagulation factors in the liver. These propeptide-containing proteins are tightly linked to the carboxylase. They can be used as endogenous substrates for the in vitro carboxylation reaction. The affinity of KH_2 for carboxylase was determined in a system containing endogenous substrates. Microsomes prepared from the livers of vitamin K-deficient and normal rats were used to determine the apparent Km values. The data from these experiments are listed in table III. The apparent Km values for vitamin KH_2 in the normal rat liver with FLEEL as substrate was calculated to be 257 μM. The Km^{app} determined in the liver microsomes containing precursor proteins, was 14 μM.

substrates	Km^{app} (μM) normal microsomes	vit. K-deficient microsomes
endogenous substrate	ND	16
FLEEL	257	14
FLEEL + propeptide	297	ND
proPT28	25	ND

Table III. Km^{app} values of vitamin KH_2 in microsomes derived from livers of normal and vitamin K-deficient rats. Determination of the kinetic constant were performed as described in the legends to table I. ND, not determined.

Discussion

The vitamin K-dependent carboxylase is located at the luminal site of the endoplasmic reticulum of a variety of tissues. The liver has been most often used as a source of the enzyme for in vitro experiments. Despite their limitation the pentapeptides FLEEV and FLEEL have been very helpful in the

study of the kinetics and requirements of this enzyme system. Because of the low affinity of these substrates for the enzyme and the minimal amount of product formed, it was not previously possible to study the enzyme/substrate interaction. The discovery of the propeptide's importance in the recognition of the protein by the vitamin K-dependent carboxylase was a breakthrough in carboxylase research. Synthetic substrates based upon the propeptide and the first 10 amino acids of the N-terminus in the mature coagulation factor were carboxylated readily. The apparent Km values were 3 orders of magnitude lower than those of the small pentapeptides and, under the appropriate conditions, at least 40% of the substrate was converted into product. The data presented here show that the propeptide not only contributes to the substrate recognition, but it also induces a high affinity binding site for KH_2. In all experiments described saturating amounts of carboxylatable substrates were used so that the amount of enzyme/substrate complexes were constant. The highly conserved phenylalanine, that is very important for substrate recognition, was shown to play a minor role in the binding of KH_2 to carboxylase. The data obtained in the experiments in which proPTFLEEV or FLEEL together with the propeptide were used, show that the propeptide has to be covalently linked to the substrate.

A substrate in which the complete Gla-domain in its descarboxy form was present (ProFIX59), was shown to have an affinity for the enzyme that was slightly higher than that for peptides containing the sequence for the propeptide and only the first ten residues of the mature descarboxy protein. Under specific conditions, almost all of the glutamic acid residues (Glu) of ProFIX59 could be converted to Gla. Under the same conditions only 40% of the synthetic propeptide-containing peptides were converted into product. These results suggest that in addition to the propeptide, also the Gla-domain itself contributes to the recognition by carboxylase. This hypothesis is supported by studies performed with substrates derived from decarboxylated Gla-containing proteins. Propeptide-lacking substrates, like a proteolytic fragment of descarboxy prothrombin (27) and descarboxy osteocalcin were shown to have Km^{app} values in the micromolar range, suggesting that indeed, these substrates do contain a recognition site for carboxylase. The specific amino acid sequence in the Gla-domain of these proteins that is responsible for the recognition by carboxylase is not yet clear. From our results, however, it can be concluded that this sequence does not conribute to the development of the high affinity vitamin KH_2 binding site.

The data presented in this paper suggest a dual role for the propeptide in its interaction with carboxylase. Firstly it mediates the binding of the substrate to

the carboxylase and orients the Glu residues to be carboxylated to the active site of the enzyme. In addition it also creates, possibly by a conformational change, a high affinity binding site for KH_2.

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CHAPTER 6

OSTEOCALCIN IN FOSSIL BONES

Based on:

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- Vermeer, C., Ulrich, M.M.W. and Perizonius, W.R.K. Bones, Treasuries of Human Experience in Time and Space 1: 9-21, 1988

EXTRACTION OF OSTEOCALCIN FROM FOSSIL BONES AND TEETH

Abstract

Osteocalcin (also called 'bone Gla-protein') was detected in fossil bovid bones ranging from 12,000 years to 13 million years old and in rodent teeth 30 million years old. Both the antigenic activity and the protein-bound Gla-residues have remained intact. The protein is indistinguishable from recent bovine osteocalcin when analyzed by HPLC using ion exchange and size exclusion columns. If sufficient amounts can be extracted and an adequate purification procedure is established, this would be the first time that amino acid sequences in a protein from fossil bones may be determined. Such sequence data could offer a new aproach to the phylogenetic study of extinct taxa.

Introduction

In recent decades, molecular biology has provided new information about the phylogenetic relationships between organisms (1,2). Whereas the traditional morphological approach to phylogeny is based on comparison of characters from extinct species with those from living ones, molecular biology

has been confined almost exclusively to modern forms. The few exceptions are studies of mummified or frozen soft tissue remains (3,4). The usual remains of extinct vertebrate taxa, fossil bones and teeth, are not known to contain macromolecules of evolutionary interest.

Osteocalcin (also designated as bone Gla-protein (5,6)), is a protein very tightly bound to the mineral phase (hydroxyapatite) of bone. The osteocalcin of most species contains 49 amino acid residues, three of which are γ–carboxyglutamic acid (Gla). The Gla-residues have a high affinity for Ca^{2+} and are located in such a way that strong binding occurs between osteocalcin and crystals of calcium phosphate or hydroxyapatite (7). The primary structure of osteocalcin has been determined for diverse taxa (5) and the function of osteocalcin (especially in relation to a number of bone diseases) is now a subject of investigations in many different institutes.

During the preparation of osteocalcin from fresh human and bovine bones it was found that prolonged heating of the bones for several days at 120°C reduced neither the antigenic activity of the protein nor its Gla-content (8). Other well known properties of osteocalcin, which seem to remain unaffected by heat treatment are its ability to inhibit calcium phosphate precipitation from a supersaturated solution (9) and - after thermal decarboxylation - to serve as a substrate for bovine liver vitamin K-dependent carboxylase (8). This exceptional stability of osteocalcin has lead us to investigate its presence in fossil bones and teeth.

Materials and Methods

Chemicals and Buffers. Buffer A: 1 M EDTA, 50 mM Tris/HCl, pH 8.0, 0.1 M NaCl, 30 mM benzamidine, 100 IU/ml trasylol and 0.02% (w/v) soyabean trypsin inhibitor. Buffer B: 0.1 M NaCl, 50 mM Tris/HCl, pH 7.4. All chemicals were of the highest purity commercially available. QAE Sephadex was purchased from Pharmacia (Sweden). Polyclonal, monospecific antibodies against rat osteocalcin were a kind gift of Dr. I. Gorter-de Vries (University of Brussels). Purified bovine osteocalcin was prepared as described earlier (8) and polyclonal antibodies against it were raised in rabbits.

Fossil Samples. All bone and teeth samples were from geologically well-documented sites (see Table I). The sample weights varied from 1-1000 g.

Crude Bone Extracts. The various bone and teeth samples were carefully cleaned and dried, and subsequently crushed and powdered mechanically using a Retsch centrifugal mill ZM-1 equipped with an 8 μm pore seize sieve. Small

fragments (< 10 g) were powdered with a freezer/mill (Spex 6700). A known dry weight of bone powder was mixed with buffer A (2 ml/g bone) and the slurry was transferred into a dialysis bag (Spectrapor, MW cut of 3500) and dialyzed against the same buffer for 7 days with changes of the buffer every 24 h. EDTA was removed from the samples by a subsequent dialysis against buffer B for 3 days with 3 changes of the buffer. Insoluble material was removed by centrifugation (10 min at 4000 g). The remaining supernatant was designated as crude bone extract.

Osteocalcin Detection. Osteocalcin antigen of bovid origin was measured routinely using a commercial radioimmuno assay (RIA) kit (INCSTAR, USA). In some cases we also used an ELISA (enzyme-linked immuno sorbent assay) in which polyvinylchloride microplates (Titertek, Flow Laboratories) were coated with the osteocalcin-containing samples and subsequently with ovalbumin, incubated with anti-osteocalcin antibodies and with a horse-radish peroxidase-linked second antibody.

Samples nr.	Species	Bone	Site	Period	Collection
1	Bos taurus	tibia	Slaughterhouse Maastricht The Netherlands	recent	
2.	Myotragus balearicus	tibia	Son Muleta, Mallorca Spain	Late Pleistocene	IVAU
3	Bison sp.	metatarsus	North Sea, The Netherlands	Late Pleistocene	IVAU
4	Bison sp.	tibia	Swanscombe, lower gravel, United Kingdom	Middle Pleistocene	BMNH
5	Bubalus palaeokerabau	radius	Trinil, Java,	Early Pleistocene	RMNH
6	Bovidae	metatarsus	Chinji, Pakistan	Miocene	IVAU
7	Caprotragoides sehlini	metacarpus	Manchones I Spain	Miocene	IVAU
8	Theridomorpha	incisors	Olalla, Teruel Spain	Oligocene	RMNH

Table I. Description of bone and teeth samples. Abbreviations used are: IVAU, Institute of Earh Sciences, Utrecht, The Netherlands; RMNH, Rijksmuseum van Geologie, Mineralogie en Natuurlijke Historie, Leiden, The Netherlands; BMNH, British Museum, National History, London, United Kingdom.

Other Assays. Gla was detected by HPLC after alkaline hydrolyzis of the samples, as described by Kuwada and Katayama (10). Protein concentrations were determined as described by Sedmak and Grossberg (11).

Results

Bovid bones of varying ages and from different sources were powdered and extracted with buffer A. The osteocalcin content of the crude extracts was measured by RIA. As is shown in Table II, the osteocalcin content of the fossil material varied considerably, but in all cases its presence could be clearly established. The osteocalcin present in the various crude bone extracts could be recovered quantitatively after incubating the various samples with pre-swollen QAE Sephadex (0.1 ml of slurry per ml) while rotating the tubes end over end. After removal of the supernatant and washing the Sephadex with buffer B, all osteocalcin could be eluted from the Sephadex with 1 M NaCl in buffer B. The results shown in Table II were verified with an ELISA using immuno-purified polyconal antibodies against purified bovine osteocalcin. Also with this technique osteocalcin was detected in the samples (data not shown) and the amounts were comparable with those determined with the RIA. Because teeth are a more dense mineralization, they are more often preserved and are better determinable than bone fragments, we have also tried to detect osteocalin in fossil teeth. These rodent incisors (see Table I, sample 8) were treated in the same way as the bone samples. Because no commercial RIA kit for rat osteocalcin is available, the teeth extract was only analyzed by ELISA, using polyclonal antibodies against recent rat osteocalcin. The amount of osteocalcin detected in this way was 0.8 µg per g of mineral (Table II, sample 8).

Sample	Estimated age	Detection method	Osteocalcin content (µg/g bone)
1	0 y	RIA	26.8
2	12 000 y	RIA	2.4
3	40 000 y	RIA	4.4
4	350 000 y	RIA	0.18
5	1 my	RIA	0.13
6	13 my	RIA	0.03
7	13 my	RIA	5.0
8	30 my	ELISA	0.8

Table II. Osteocalcin content and estimated age of fossil samples. The sample numbers correspond to those described in Table I.

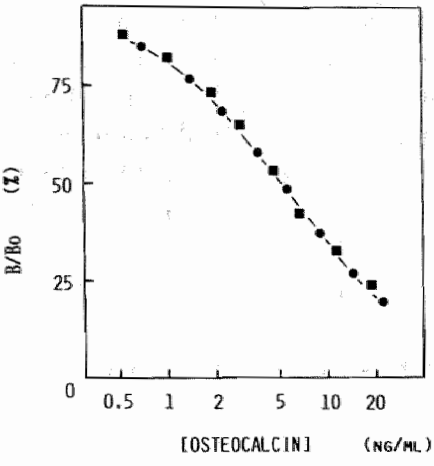

Fig. 1. Radioimmuno assay of osteocalcin. A dose/response curve of purified osteocalcin from recent bovine bone (●-●) was compared with that of the crude bone extract from sample 7 (■-■). B/Bo represents the percentage of label precipitated as compared to the sample containing ^{125}I-labeled tracer only.

To exclude the possibility that unknown processes during the long geologic history of our fossil material would give rise to false-positive results in the various assays, we have performed two control experiments. First, we have compared the behaviour of serial dilutions of the various bone extracts with

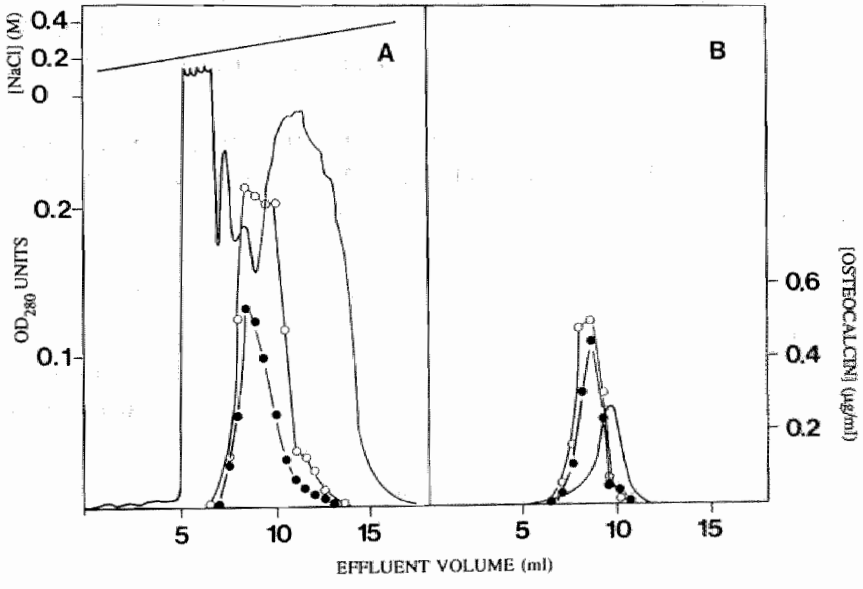

Fig. 2. Fractionation of bone extracts by HPLC. The solid line is the extinct ion profile obtained after chromatography of the samples. The osteocalcin antigen in the various fractions of sample 4 (●-●) and sample 1 (○-○, recent bone) were determined by RIA. A: Three ml of bone extract were loaded on a Mono Q anion exchange column (Pharmacia) and eluted with a linear gradient from 0.1 M NaCl to 1 M NaCl in 20 mM Tris/HCl, pH 7.4. B: The peak fractions shown in A were pooled, concentrated and applied to a TSK-125 size exclusion column in buffer B.

that of purified bovine osteocalcin. A typical experiment is shown in fig. 1 in which we have plotted the dose/response curves in the RIA for extracts from recent bovine bone as well as from 13 my old bone (sample 7). We have tested samples 2, 3, 4 and 5 in a similar way and in all cases parallel dilution curves were obtained (data not shown). The amounts of osteocalcin present in sample 6 was to small to include it in this experiment. A second proof of the authenticity of osteocalcin in the fossil bones was obtained by partly purifying the protein in the extracts from samples 4 and 5. Approximately 50 g of bone were extracted for each sample and the crude EDTA extracts were dialyzed against buffer B, adsorbed to QAE Sephadex (10 ml of slurry) and eluted batch wise with 1 M NaCl. The eluate was fractionated on a Mono Q ion exchange column using a fast protein liquid chromatography (FPLC, Pharmacia) system and the various fractions were tested for the presence of osteocalcin (fig. 2A). The immunoreactive peak was pooled and fractionated further on a TSK-125 size exclusion column. Immunoreactive osteocalcin eluted as a single peak in a position similar to that of recent osteocalcin (fig. 2B). The data displayed in fig. 2 are those for sample 4. Similar results were obtained with sample 5. None of the extractions yielded purified, homogenous osteocalcin, mainly because of the presence of dark coloured material which was distributed uniformly over the various fractions and which partly coeluted with osteocalcin.

In the next set of experiments we investigated the preservation of protein-bound Gla-residues in fossil bones and teeth. The samples used were the same ones listed in Table I. No free Gla could be detected before hydrolysis of the samples. However, protein-bound Gla-residues were 2-4 fold more abundant than calculated from the RIA results. A typical example of the Gla analysis is shown in fig. 3 in which we have compared the presence of Gla in recent and in 13 my old bone (samples 1 and 7). The discrepancy between the observed protein-bound Gla concentration and that expected from the concentration of the osteocalcin antigen, may be caused by the presence of a second Gla-containing protein in the bone extracts (e.g. the matrix Gla- protein (12)) or by a loss of antigenic determinants due to a partial degradation of osteocalcin during the aging of the bones.

Discussion

It has been shown by Huq et al. (13) that osteocalcin antigen has remained preserved in moa (Pachyornis elephantopus) bone approximately 3600 and

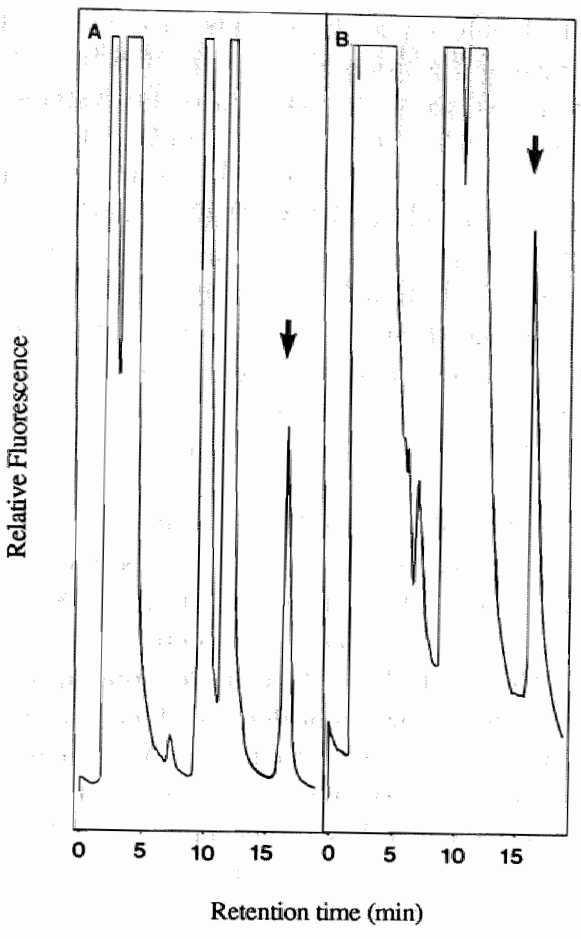

Fig. 3. Gla-detection in crude bone extracts. Samples were hydrolyzed under alkaline conditions and assayed for Gla (arrow) by HPLC analysis. A: extract of recent bone (sample 1). B: extract of 13 my old bone (sample 7).

7400 years old. In the present paper we report that osteocalcin antigen as well as its Gla-residues remain detectable in bovid bones even after 13 my. The osteocalcin extracted from the fossil bones was detectable by both RIA and ELISA. Moreover, on both ion exchange and size exclusion columns, the protein eluted at a position which is characteristic for recent bovine osteocalcin. These results strongly indicate that the osteocalcin molecule has remained intact for millions of years.

The amount of osteocalcin which could be extracted from the various bone samples varied substantially and probably reflects differences in the soil conditions the bones have experienced (humidity, pH, fossilisation, etc.). In all cases the osteocalcin content of the fossil bones was considerably lower than that in recent bone. No strict relation was found between the osteocalcin content and the age of the bone samples. Therefore, it seems likely that

osteocalcin will be found in much older bones, provided that they were preserved under optimal conditions. In this respect, it should be mentioned that all data in this paper are expressed as μg osteocalcin per g of bone (dry weight). The bone samples contained varying amounts of insoluble material, however, and we do not know to what degree the bones contained contaminating cements (phosphates, silicates, carbonates). If the amounts of extracted osteocalcin are expressed per gram of EDTA-soluble material, the figures are generally substantially higher, but again it is unknown to what extent the contaminating cements are dissolved in EDTA. Neither do we know if part of the insoluble material originates from the bone itself and if in that case osteocalcin is washed off from the debris. Therefore, an accurate quantification of osteocalcin relative to the bone matrix is not possible from the data presented here. This does not affect the impact of our observations, however.

The striking stability of osteocalcin during millions of years may lead to a new approach to paleontology: if the extracted osteocalcin may be purified with a reasonable recovery, fragments of 1-10 g of bone or teeth may contain sufficient amounts of extractable antigen to sequence the entire molecule (usually 49 aminoacid residues). This could be of help in determining the relation between extinct taxa on other than morphological characters. However, the differentiation will probably be only at rather high taxonomic levels, because osteocalcin seems to be a very conservative molecule. For example, the amino acid sequence of the cow (5), an artiodactyl, differs from that of the horse, a perissodactyl (Chang and Sandberg, unpublished data), by very little more than it differs from other artiodactyls, such as the goat (5).

Obviously the sequencing of osteocalcin requires a quick procedure for the complete purification of the protein from the crude bone extracts but this turned out to be a serious problem. Because HPLC did not yield a homogenous preparation, other purification procedures (e.g. based on immuno-adsorption and elution) will have to be used. The development of such procedures is one of the topics of our future investigations.

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EXTRACTION OF OSTEOCALCIN FROM EXCAVATED HUMAN BONES

Abstract

Osteocalcin is a protein which mainly occurs in bone, tightly bound to the hydroxyapatite matrix. In this bound form the protein is amazingly stable, even if the bones have been buried for long periods. In a previous paper we (1) showed that osteocalcin antigen is detectable in 13 million years old bovid bones. Here we report that osteocalcin also remains conserved in human bones. The implications of this finding are a) that methods may now be developed to analyse ancient proteins, which may help to establish or confirm phylogenetic relation ships between extinct and living hominids and b) that the quantity and quality of osteocalcin may be compared in excavated normal bones and in excavated deformed or abnormal ones. Because excavated bones of 'ancient' patients' are more readily accessible for physical and chemical investigation than are bones in living patients, this last type of research may contribute to our understanding of the function of osteocalcin in normal and abnormal bone metabolism.

Introduction

Like most other vitamins, also vitamin K acts as a coenzyme in vertebrates, including man. The enzyme to which it belongs is a carboxylase, which is located in the endoplasmic reticulum of many different types of cells (2-4). The function of vitamin K-dependent carboxylase is that it converts distinct glutamic acid (Glu) residues into γ-carboxyglutamatic acid (Gla) residues. This process only occurs in maturating polypeptide chains during the post-translational phase of protein biosynthesis. The structures of Glu and Gla are designated in fig. 1. The function of Gla is the strong binding of Ca^{2+}- ions. Gla-containing proteins are therefore always Ca^{2+}-binding proteins.

Gla was discovered in prothrombin, one of the Gla-containing blood coagulation factors present in blood plasma (27). Another group of Gla-containing proteins was discovered in calcified tissues such as bone, teeth, renal stones, calcified atherosclerotic plaques and coral (5-9). Here we will focus our attention on the bone Gla-protein (osteocalcin), which under laboratory conditions proved to be surprisingly stable as long as it remains bound to the hydroxyapatite matrix of the bone. It was recently discovered still to be present in fossil bovid bones ranging from 12,000 to 13 million years old (1). In the present paper it will be shown that osteocalcin can be extracted from human bones as well.

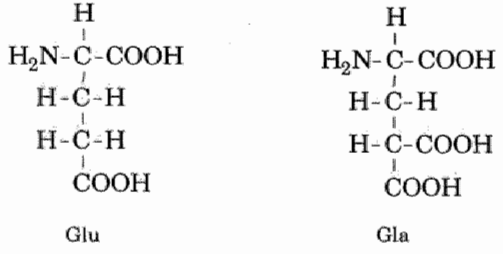

Fig. 1. Structures of glutamic acid (left) and γ-carboxy-glutamic acid (right). The two carboxyl groups at the gamma carbon of Gla form an efficient binding site for Ca^{2+}.

The synthesis of osteocalcin

Osteocalcin is a protein consisting of 49 aminoacid residues, three of which are Gla. Human osteocalcin is an exception in this respect, since it has been reported that part of it may also occur in a 2-Gla form (10). It is a secretory protein which is synthesized by the osteoblasts (bone forming cells). The majority of the newly formed osteocalcin remains bound to the hydroxyapatite

crystal matrix in the bone. After solubilization of the bone, the osteocalcin antigen may be detected by radioimmuno assay (11). Moreover, also the Gla residues (which are probably related to the biological activity of the protein) may be identified with an HPLC-technique (12).

In cultures of osteoblasts and osteosarcoma cell lines (and probably also in bone tissue) the synthesis of osteocalcin antigen cannot be influenced without deregulating the total protein synthesis in the cell. On the other hand, the Gla-content (and probably the biological activity) of osteocalcin is strictly dependent on the presence of vitamin K in the culture medium. This is consistent with the observation that vitamin K-antagonists reduce the production of fully carboxylated osteocalcin (13). Recently it was shown by Price and Kaneda (14) that by using a combination of vitamin K and warfarin, the carboxylation of osteocalcin may be regulated selectively without affecting the synthesis of blood coagulation factors. If such a regulation in vivo may be beneficial for patients suffering from certain bone deseases, has to be elucidated as yet.

The function of osteocalcin

In vitro osteocalcin strongly inhibits the precipitation of various insoluble calcium salts from supersaturated solutions. As is shown in fig. 2 this property

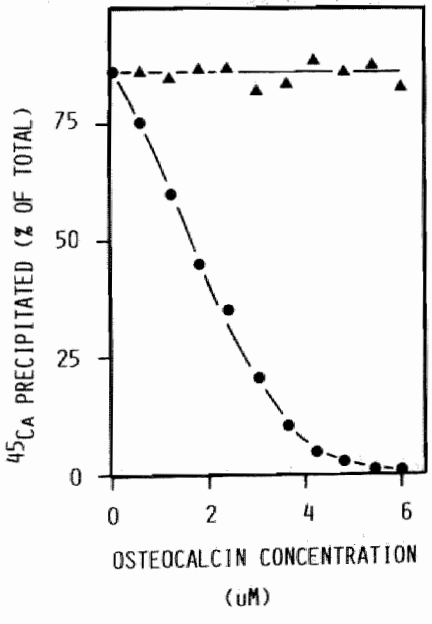

Fig. 2. Calciumphosphate precipitation inhibition by purified bovine osteocalcin. Reaction mixtures (0.5 ml) contained: 0.15 M NaCl, 0.1 M Na-phosphate, pH 6.0, 5 mM $CaCl_2$ and a trace amount (20,000 dpm) of $^{45}CaCl_2$. The mixtures were incubated in Eppendorff tubes for 2 h at 37°C and the precipitate which had formed during this period (if any) was spun down at 2000 x g. The supernatants were counted to measure the amount of residual ^{45}Ca in solution. At a concentration of 5 µM normal osteocalcin (●- ●) was able to keep 5 mM Ca^{2+} in solution (this is a 1000 fold excess), whereas thermally decarboxylated (= Gla-deficient) osteocalcin (▲-▲) had no effect on the precipitation of calcium salts. This experiment therefore demonstrates that Gla-residues are required for the precipitation-inhibitory activity of osteocalcin.

is strictly dependent on the presence of Gla-residues in the protein. Moreover, it has been reported that osteocalcin inhibits the growth of calcium oxalate crystals (15). From fig. 2 it may be calculated that osteocalcin may keep Ca^{2+} in solution, even if the latter is in a 1000-fold excess. This phenomenon may be explained by assuming that osteocalcin binds to the rapid growing site on the surface of forming crystal noduli, which consist at that moment of several thousands of calcium phosphate molecules, and that the binding occurs in such a way that it prevents further accretion of the mineral.

It is not yet known if the inhibition of calcium phosphate precipitation is also the in vivo function of osteocalcin, but two observations favour this possibility. In the first place it has been well documented, that if vitamin K-antagonists (which prevent the Gla-formation) are administered to women during the first trimester of pregnancy, the fetuses are exposed to the risk of defective bone development (16). The defect is characterized by irregular areas of excessive calcification ("stipples"), mainly in the rapid growing parts (e.g. the epiphyses) of the bone. In the calcified areas the growth irreversibly comes to a halt, whereas the growth continues in the non-calcified surroundings, thus causing the deformations.

A second example of bone defects caused by vitamin K-antagonists was described by Price et al. (17), who treated newborn rats with low dosages of warfarin. These investigators observed an excessive mineralization of the bones, leading to a complete closure of the epiphyses in the long bones after 9 months. So it seems that osteocalcin regulates or limits the precipitation of calcium phosphate in rapid growing bone. On the other hand, despite of numerous investigations, the precise function of osteocalcin in adult bone has not been identified thus far.

Osteocalcin and bone diseases

Since the detection of osteocalcin in the blood stream, attempts have been made to to use the serum osteocalcin antigen (SO-Ag) as a biochemical marker of disease activity. It was found that SO-Ag may serve as a marker for osteoblast activity and high levels were found in patients suffering from diseases characterized by rapid bone formation or bone turnover. Examples are: Paget's disease, bone metastases, hyperparathyroidism and several less common bone diseases (18). Less agreement consists about the levels of SO-Ag during postmenopausal osteoporosis, which are reported to be high (19), normal, or even below normal (20). Because SO-Ag represents not more than 0.01% of

our total osteocalcin body supply, it seems to be justified to verify if abnormalities in SO-Ag reflect abnormalities in the bone itself. It would be highly desirable therefore, to take bone biopsies from these patients and to analyze them for bone mass, structure and osteocalcin content.

Osteocalcin in excavated human bones

After it was shown that osteocalcin - provided that it is bound to the hydroxyapatite matrix of bone - is stable for several days at 120°C (21) and that it remains detectable in several thousands years old Moa bones (22) we have recently shown that osteocalcin as well as its Gla-residues remain preserved in bovid bones for at least 13 million years and in rodent teeth even for 30 million years (1). In this study we exclusively used homologous antibodies for the osteocalcin detection, i.e.: antibovine antibodies for bovid bone extracts and anti-rat antibodies for the rodent teeth extracts. The reason for this experimental design was that, although a considerable species cross-reactivity for antibodies against osteocalcin has been described (22,23), the cumulation of species differences and the expected evolutionary mutations might negatively influence the antigen-antibody recognition in the RIA. On the other hand, the primary structure of osteocalcin is conserved remarkably well during evolution, and it is not to be expected that mutations have occured since the development of homo sapiens. Therefore the commercial RIA kit (which contains anti-bovine osteocalcin antibodies, but which is generally applied for clinical use) was used for the osteocalcin detection in several thousand-year-old human bones.

Sample no.	bone part	osteocalcin antigen (ng/g bone)
1	right lower canine (dentine)	10
2	fragment of left temporal	60
3	fragment of a lumbar vertebra	6
4	fragment of the rib	30
5	fragment of a right second metacarpal	40

Table I. Recovery of osteocalcin using different bones from one individual (grave 13, cemetary of the Hospitaller Monastery, Vredenburg, Utrecht, AD 1200-1529). The bones were powdered mechanically and extracted with 1 M EDTA, pH 8.0 (10 ml per g of bone). After 24 h the insoluble material was removed by centrifugation and the supernatant was dialyzed against 0.1 M NaCl in 50 mM Tris/HCl, pH 7.4. The samples were tested for osteocalcin using a commercially available RIA-kit (INCSTAR)

In a first set of experiments we have tried to detect osteocalcin in human bones from 1200-1529 A.D. cemetery (Hospitaler Monastery, Vredenburg, Utrecht) The recovery of osteocalcin from different bones, all belonging to the same individual (grave 13) is shown in table I. It is clear that - if expressed per g bone - the highest yields were obtained from the massive, cortical bones. It cannot be excluded, however, that the lower yields, obtained from trabecular bone were caused by the deposition of silicates in the porous structure of this less massive material. Obviously this would lead to an overestimation of the

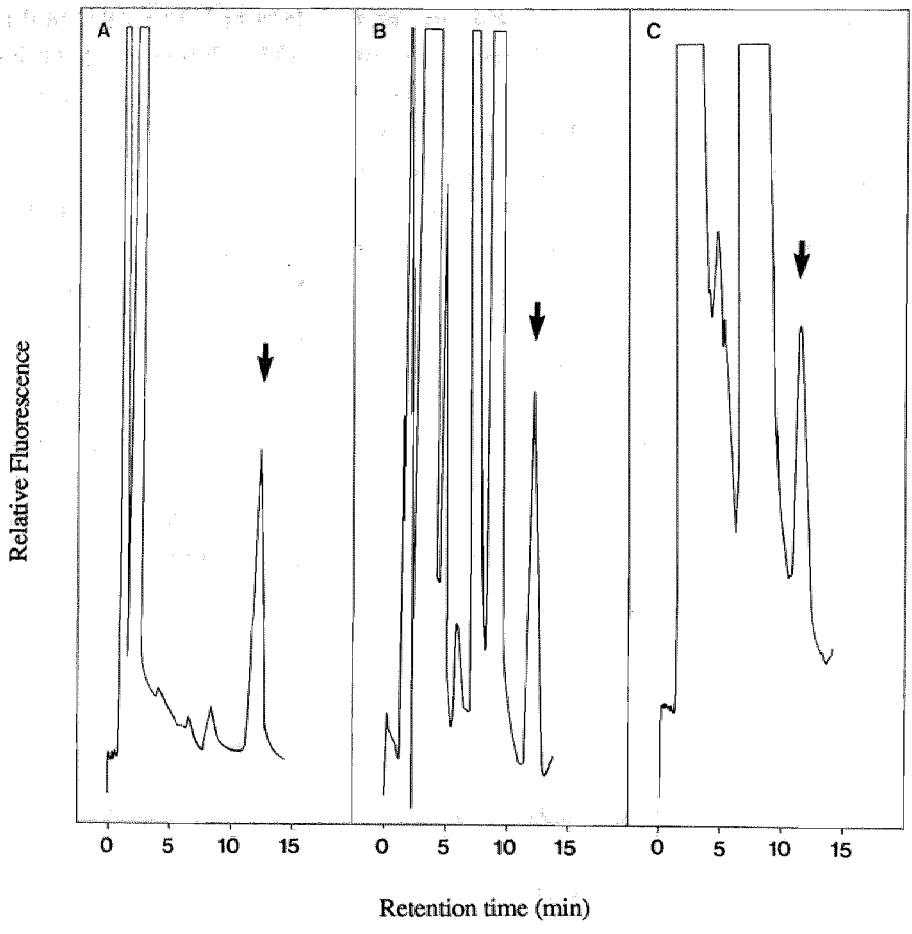

Fig. 3. HPLC chromatograms of Gla-containing samples. Gla elutes after 12.4 min (arrow). Left panel: a commercial Gla preparation (Sigma); middle panel: an alkaline hydrolyzate of purified osteocalcin from fresh bovine femora; right panel: an alkaline hydrolyzate of osteocalcin from an excavated human bone (cemetery Hospitaller Monastery, Vredenburg, Utrecht, grave 13, AD 1200-1529). The analysis procedure was performed as described by Kuwada and Katayama (1983). Osteocalcin from the excavated bone was prepared as described in the legend to table 1.

quantity of bone tissue and thus to an underestimation of its osteocalcin content. The apparent molecular mass of the osteocalcin from excavated bones (as determined by gel permeation chromatography) did not differ from that of osteocalcin prepared from fresh human bones. From these results we have concluded that the osteocalcin had been preserved remarkably well and in such quantities that it could be used for further investigations.

In fig. 3 it is shown that also the Gla residues had remaind intact. This was to be expected since osteocalcin probably owes its stability to its tight binding to calcium phosphate in the hydroxyapatite crystal, which occurs via the Gla residues. In fact slightly more Gla residues were found than could be explained by the osteocalcin antigen recovered. This may be the result of some degradation of the protein during the 400-700 years preservation in the soil, or by the presence of an other Gla-containing protein in bone extracts (e.g. matrix Gla Protein) (24).

Sample (no.)	Estimated age (years)	Osteocalcin antigen (ng/g bone)
1	0	9,800
2	50	300
3	150	200
4	600	50
5	1000	200
6	6000	100

Table II. Osteocalcin in femoral bone of different ages. Recent bone (sample 1) was obtained after authopsy, samples 2,3 and 5 were from excavations in the city of Maastricht, sample 4 was from the cemetery of the Hospitaller Monastery, Vredenburg, Utrecht and sample 6 from Swifterband.

The same type of experiments were repeated with human cortical bone fragments dating from 6 different time periods and ranging from the present to 6000 years old. No substantial differences were found with respect to the recovery of antigen (table II) nor its Gla-content.

Discussion

The data presented in this paper demonstrate that it is possible also to extract osteocalcin from human bones up to 6000 years old. Bovid bones have been

shown to contain osteocalcin even after a preservation period of 13 million years (1) and there is no reason to expect human bones to be different from bovid ones in this respect. It seems justified, therefore, to start the development of special purification procedures for osteocalcin from fossil bone samples. After purification to homogeneity, the protein may be sequenced with the highly sensitive gas phase sequenator techniques presently available. Although the extraction of osteocalcin is destructive to the bone, the potential results warrant the sacrifice of material. Our results indicate that the amounts of osteocalcin required for a complete sequencing (0.5 - 1 μg) may be obtained from less than 10 g of bone. Osteocalcin from a recent monkey (Macacca fascicularis) differs from human osteocalcin in only three amino acid residues (25). Obviously it would be interesting to investigate if the osteocalcin mutations in fossil primates agree with the evolutionary trees extrapolated from osteocalcin differences within recent primates. If osteocalcin extracted from fossil bones may indeed be sequenced, this will offer for the first time the possibility of building molecular (protein)-evolutionary trees directly from fossil bone specimens.

Another aspect of the results in this paper is their clinical relevance. The Department of Anthropo-osteology (Medical Faculty, Utrecht) houses a large collection (Huizinga-collection) of excavated human skeletons (26) and it is to be expected that sufficient bone diseases will be represented to allow a systematic investigation of osteocalcin in the bones of these 'ancient' patients. The characterisation will be physical (bone density), chemical (composition of inorganic matrix) and biochemical (quality and quantity of osteocalcin. Comparison of these data with those obtained from apparently normal, unaffected bones may be of help in our efforts to discover the in vivo function of osteocalcin and to answer the question if regulation of the osteocalcin biosynthesis will have a beneficial effect in one or more bone diseases. These investigations can, of course, only be complementary to medical research in patients, but an obvious advantage of this experimental line is that the excavated bones may be subjected to treatments which are not possible incase of in vivo situations. Even without knowing what the results of these investigations will be, the project alone already illustrates once more the importance of archeologically well-documented collections of excavated skeletons to modern medical science.

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CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

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Vitamin K-dependent carboxylase mediates the post-translational modification of specific glutamic acid residues into γ-carboxyglutamic acid (Gla) residues. The membrane bound enzyme system is located in the rough endoplasmic reticulum (RER) of a wide variety of cells and it utilizes vitamin K as a cofactor. The biologically active hydroquinone is obtained after reduction of vitamin K. This reaction, which is catalyzed by a reductase, takes place in all carboxylase containing tissues known. Two types of reductases are known to catalyze the reduction of vitamin K in vitro, the dithiothreitol (DTT)-dependent reductase(s), and the NAD(P)H-dependent reductase. The physiological counterpart of DTT has not been identified yet. In this thesis we describe the interaction of various substrates and inhibitors with the vitamin K-dependent carboxylase and its accessory reductases. When the work was started, little was known about the presence of the vitamin K-dependent enzyme system in extrahepatic tissues, and neither we knew the mechanism of substrate recognition and the action of inhibitors. Our early attempts to elucidate the characteristics of the vitamin K-dependent carboxylase in various tissues are described in chapter 2. In the subsequent chapters we describe the work that has been done after the discovery of the γ-carboxylation recognition site in the propeptide of the Gla-containing proteins.

The DTT-dependent reductase is inhibited by coumarin derivatives like warfarin. It has been shown that the effect of coumarin derivatives on the Gla-

containing blood coagulation factors can be overcome by high doses of vitamin K quinone. The NAD(P)H-dependent reductase which is sensitive for coumarin derivatives will under these conditions convert the quinone form of the vitamin into the hydroquinone form. The NAD(P)H-dependent reductase is not detectable in osteoblast-like cells (chapter 2) which may explain why the action of warfarin in bone cannot be counteracted by the administration of high doses of vitamin K quinone. Coumarin derivatives do not only have an effect on the Gla-containing proteins synthesized in the liver (blood coagulation factors) and osteoblasts (osteocalcin and MGP) but also on the synthesis of Gla-containing proteins in other tissues. We demonstrated the accumulation of non-carboxylated precursor proteins in liver, kidney and spleen of horses treated with warfarin. We have also shown that upon the administration of salicylate endogenous precursor proteins accumulate in liver as well as in lung and that this drug, similar to coumarin derivatives, blocks the DTT-dependent vitamin K-reductase. The inhibition of the reductase by coumarin derivatives and salicylate has been demonstrated to be cumulative in in vitro carboxylation reactions. Although it is still unclear which Gla-containig proteins are synthesized in the extra-hepatic carboxylase-containing tissues, our results suggest that, the synthesis of these proteins is inhibited by treatment with drugs that inhibit the recycling of vitamin K. Further investigation of the extra-hepatic Gla-containing proteins may elucidate the effect of anti-coagulant therapy on physiological processes other than the blood coagulation. Although salicylate is only a weak inhibitor of the reductase, care should be taken by patients taking oral anti-coagulant therapy because of the cumulative effect of both drugs. Several Gla-containing proteins have been isolated, among them are the plasma proteins prothrombin, factors VII, IX and X, Proteins C, S, and Z and the bone proteins osteocalcin and matrix Gla protein. Substrates based upon sequence homology with these proteins have been synthesized to be able to study the carboxylation reaction in vitro. The pentapeptides FLEEV and FLEEL, based upon amino acids 4-9 in bovine prothrombin and factor VII, are used most frequently. Because of the high apparent Km values, however, we assume that these synthetic substrates do not contain a recognition site. We discovered that, in their descarboxy form, some naturally occurring Gla-proteins may form far better substrates for the enzyme than the pentapeptides. Substrates like fragment Su (amino acid 13 to 29 of descarboxy prothrombin), bovine d-osteocalcin and d-sperm Gla protein were shown to have apparent Km values 2 to 3 orders of magnitude lower than those of FLEEL and FLEEV. Not all substrates derived from naturally occurring proteins did have comparable high affinities for the enzyme. Descarboxy prothrombin, for

instance, is hardly carboxylated and only after digestion with subtilisin a fragment (fragment Su) is obtained which has a low apparent Km value. Also d-osteocalcins derived from bones of monkey, chicken or man were shown to have a lower affinity for the carboxylase than the bovine descarboxy protein, although the amino acid sequence of osteocalcin are highly conserved among these species. Apparently the alteration of two amino acids of opposite charge at positions 3 and 4 in neutral amino acids substantially reduces their suitability as a substrate for carboxylase. Chicken osteocalcin which has one positively charged amino acid and one neutral amino acid at positions 3 and 4 demonstrated intermediate substrate activity. It seems plausible therefore, to suggest that the amino acids of opposite charge at position 3 and 4, about 15 amino acids before the first Gla residue, do play an important role in substrate recognition.

To study whether the recognition site of the substrates in all carboxylase-containing tissues have to fulfil the same requirements, we used the following approach: proteins synthesized in different tissues were decarboxylated and studied in homologous and heterologous enzyme systems and compared with a nonselective substrate pentapeptide FLEEL. The proteins used were osteocalcin (synthesized in the osteoblasts), sperm Gla protein (probably synthesized in testis), and fragment Su (derived from prothrombin which is synthesized in the liver). The enzyme systems used were derived from liver, kidney, lung and testis. The Km^{app} values of FLEEL and d-osteocalcin, which were determined only in heterolog systems, were fairly constant, whereas the Km^{app} for d-sperm Gla protein and d-fragment Su varied more. The affinity for the homologous enzymes turned out to be the highest. From the data described here it is plausible to assume that the mature Gla-containing protein does contain a gamma-glutamyl carboxylase recognition site. However, the actual amino acid sequence important for this role has to be elucidated yet.

Besides the putative recognition site inside the mature protein another sequence has been demonstrated to be important for recognition by carboxylase. The precursor form of the Gla-containing proteins contain a leader sequence. This leader peptide contains a signal peptide which is required for the translocation of the nascent polypeptide through the membrane of the RER, and a propeptide. The latter was shown to direct γ-glutamyl carboxylation in recombinant factor IX. We studied the effect of the propeptide, indicated as amino acid residues -18 to -1 of the two coagulation factors, on the carboxylation of synthetic peptides (chapter 3). Pro-containing peptides based upon amino acid sequences of human prothrombin and factor IX were carboxylated readily, with apparent Km values 3 orders of magnitude

lower than that of FLEEL. Although the propeptides of profactor IX and proprothrombin showed the same affinity for the enzyme, the substrates containing the prothrombin recognition site are carboxylated more efficiently. To determine the size of the recognition site, peptides were synthesized in which alterations were made in the propeptide region. Mutations made at the NH_2 terminus (-18 to -10) had a great impact on the carboxylation of the peptides whereas mutations made at -4 and -1 did not alter the affinity for carboxylase. It is widly accepted now that the amino acids -18 to -10 are important for the recognition of the substrates by carboxylase. The pentapeptide FLEEL which is lacking this recognition site is carboxylated minimally and only the first glutamic acid residue is converted into Gla. To elucidate whether in the pro-containing substrates both adjacent Glu residues are carboxylated or not, peptides were synthesized in which one of them was substituted by alanine. The rate of incorporation of CO_2 into both substrates was the same, and slightly lower than that of the two Glu-containing peptides. When the alanine residues in those peptides were replaced by aspartic acid residues, the same results were obtained, suggesting that aspartic acid residues do not inhibit the carboxylation of adjacent glutamic acid residues. Peptides containing two aspartic acid residues instead of glutamic acid did show some incorporation of CO_2, but only 1% compared to the incorporation of CO_2 in the glutamic acid-containing peptide. An analog of homoglutamic acid (carboxymethylcysteine) could not be carboxylated at all, demonstrating that the vitamin K-dependent carboxylase requires a precise location of the carbon atom to be carboxylated.

In chapter 4 a new method to purify the vitamin K-dependent carboxylase is described. In this method the high affinity of carboxylase for the propeptide is used. Propeptide linked to a resin, CNBr-activated sepharose or activated thiol-sepharose, is able to bind the carboxylase. The enzyme can be eluted from the resins by washing the column with propeptide or in case of the thiol-sepharose, with a reducing agent like dithiotreitol. Both approaches yielded an enzyme preparation in which the contaminating protein BiP formed a substantial amount of the protein present. Nevertheless, this method gave a considerable purification (about 100 times over partially purified microsomes) in one single step. This product can be used as the starting material for further purification of the carboxylase. Recently the group of Stafford has published a paper in which they describe the complete purification of the enzyme with a technique based upon this method.

Besides its effect on the affinity of the substrate for the enzyme, the propeptide induces a conformational change in the enzyme by which a high

affinity site for vitamin K hydroquinone is exposed to the surface (chapter 5). The amino acid at position -16 in the propeptide that was shown to be of major importance for substrate recognition, does not play a role in the exposure of this high affinity site for KH_2. The propeptide by itself can not accomplish this conformational change, it has to be covalently linked to the carboxylatable substrate.

Chapter 6 of this thesis describes the unusual stability of a Gla-containing protein in bone. Osteocalcin is very stable if it is bound to the hydroxyapatite matrix of the bone. We found that the antigen of the protein is still present in bovid bone over 13 million years old, even the gamma-carboxy glutamic acid residues could be detected. Also human bone of younger age has been demonstrated to contain the intact or nearly intact protein. Extraction, purification and amino acid sequence determination of osteocalcin from fossil bones may attribute to study the phylogeny of extinct species.

Samenvatting

Vitamine K-afhankelijke carboxylase is een enzym dat betrokken is bij de post-translationele modificatie van specifieke glutaminezuur residuen in γ-carboxyglutaminezuur (Gla) residuen. Het membraan gebonden enzym-systeem bevindt zich aan de luminale zijde van het ruwwandig endoplasmatisch reticulum (RER) van een groot aantal weefsels. Na reductie wordt het biologisch actieve vitamine K hydroquinon verkregen. Deze reaktie wordt gekatalyseerd door vitamine K quinon reductases. In vitro kunnen deze reductases gestimuleerd worden door NADH of NADPH (NAD(P)H-afhankelijke reductase) of door dithiothreitol (DTT-afhankelijke reductase(s)). De fysiologische tegenhanger van DTT is nog niet geïdentificeerd. De reductie van vitamine K quinon vindt plaats in alle carboxylase bevattende weefsels. In dit proefschrift beschrijven we de interactie van verschillende substraten en remmers met het vitamine K-afhankelijke carboxylase en zijn bijbehorende reductases. Bij de aanvang van het hier beschreven werk was weinig bekend van het voorkomen van het vitamine K-afhankelijke enzym systeem in extra-hepatische weefsels, evenmin was het mechanisme van substraat herkenning en de werking van remmers in de carboxylase bevattende weefsels bekend. Onze eerste pogingen om de karakteristieken van het vitamine K-afhankelijke carboxylase in verschillende weefsels op te helderen staan beschreven in hoofdstuk 2. In de hoofdstukken 3 tot en met 6 staat het werk beschreven dat gedaan is na de ontdekking van de herkenningsplaats voor carboxylase in het propeptide van de Gla-bevattende eiwitten.

Het DTT-afhankelijke reductase wordt geremd door coumarine derivaten zoals warfarine. Toediening van hoge dosis vitamine K kan het effect van de coumarine derivaten op de Gla-bevattende bloedstollings-eiwitten teniet doen. Het NAH(P)H-afhankelijke reductase dat ongevoeliger is voor coumarine derivaten zal vitamine K quinon omzetten in de hydroquinon vorm. Het NAD(P)H-afhankelijke reductase is niet aantoonbaar in osteoblast-achtige cellen (hoofdstuk 2). Dit verklaart waarom het effect van warfarine in botweefsel niet opgeheven kan worden door toediening van grote hoeveelheden vitamine K.

Coumarine derivaten hebben niet alleen een effect op de Gla-bevattende eiwitten welke gesynthetiseerd worden in de lever (bloedstollingsfactoren) en osteoblasten (osteocalcine en MGP), maar ook op de synthese van Gla-bevattende eiwitten in andere weefsels. Wij hebben aangetoond dat niet-gecarboxyleerde precursor eiwitten ophopen in lever, nier en milt van paarden

na behandeling met warfarine. Ook toediening van salicylzuur aan ratten heeft tot gevolg dat endogene precursor eiwitten accumuleren in de lever en de longen. Net als coumarine derivaten blokeerd salicylzuur het DTT-afhankelijke reductase. De remming van het reductase door coumarine derivaten en salicylzuur is cumulatief in de in vitro carboxyleringsreaktie. Ofschoon het nog niet duidelijk is welke Gla-bevattende eiwitten in de extra-hepatische carboxylases bevattende weefsels gesynthetiseerd worden, laten onze resultaten zien dat de synthese van deze extra-hepatische Gla-bevattende eiwitten geremd worden door stoffen die de recycling van vitamine K blokkeren. Verder onderzoek naar de synthese van extra-hepatische Gla-bevattende eiwitten kunnen de invloed van anti-stollings therapie op fysiologische processen anders dan de bloedstolling ophelderen. Ofschoon salicylzuur maar een zwakke remmer van de DTT-afhankelijke reductase is, dienen patiënten op orale anti-stollings therapie voorzichtig te zijn met deze stof vanwege het cumulatieve effect van beide medicijnen.

Verschillende Gla-bevattende eiwitten zijn reeds geïsoleerd, waaronder de plasma eiwitten prothrombine, de stollingsfactoren VII, IX en X, proteïne C, S en Z en de bot eiwitten osteocalcine en matrix Gla proteïne. Om de vitamine K-afhankelijke carboxyleringsreaktie in vitro te bestuderen zijn verschillende substraten gesynthetiseerd gebaseerd op aminozuur sequentie homologie in de verschillende Gla-bevattende eiwitten. De meest gebruikte synthetische substraten FLEEL en FLEEV zijn gebaseerd op de amino zuren 4 tot 9 van respectievelijk bovine factor VII en prothrombine. Op grond van de hoge Km waarden mogen we aannemen dat deze peptiden geen herkenningsplaats voor het carboxylase bevatten. Sommige substraten verkregen uit gedecarboxyleerde Gla-bevattende eiwitten bleken een veel grotere affiniteit voor het enzym te bezitten. Substraten zoals fragment Su (aminozuur 13 tot 29 van descarboxy prothrombine), bovine d-osteocalcine en d-sperma Gla proteïne hebben Km^{app} waarden drie orders van grootte lager dan deze van FLEEL en FLEEV. Niet alle substraten verkregen van natuurlijk voorkomende Gla-bevattende eiwitten hebben dezelfde hoge affiniteit voor het enzym. De gedecarboxyleerde vorm van prothrombine wordt nauwelijks gecarboxyleerd, pas na degradatie met het proteolytische enzym subtilisine wordt een fragment (fragment Su) verkregen dat een lage Km voor carboxylase heeft. Ook de d-osteocalcines verkregen uit botten van apen, kippen en mensen hebben een hogere Km waarde dan het bovine d-osteocalcine ofschoon de aminozuur sequentie van de verschillende osteocalcines grote homologie vertonen. De verandering van twee aminozuren van tegengestelde lading op de plaatsen 3 en 4 in het eiwit in twee neutrale aminozuren heeft een groot effect op de carboxylering. Kippen osteocalcine dat

op deze plaats een positief geladen en een neutraal aminozuur bevat, bleek na decarboxylering een substraat op te leveren met een affiniteit die ligt tussen die van bovine d-osteocalcine (aminozuren 3 en 4 met tegengestelde lading) en van humaan en aap d-osteocalcine (aminozuren 3 en 4 beide neutraal). Deze vinding suggereert dat de twee aminozuren met tegengestelde lading op posities 3 en 4 een belangrijke rol spelen in substraat herkenning. Of de herkenningsplaats van de substraten in alle carboxylase-bevattende weefsels aan dezelfde eisen moeten voldoen hebben we op de volgende wijze bestudeerd: eiwitten die in verschillende weefsels worden gesynthetiseerd werden gedecarboxyleerd en bestudeerd in homologe en heterologe enzym systemen en vergeleken met het niet selectieve substraat FLEEL. De eiwitten die hiervoor gebruikt werden waren d-osteocalcine (gesynthetiseerd in osteoblasten), d-sperma Gla proteïne (waarschijnlijk gesynthetiseerd in testes) en d-fragment Su (afkomstig van prothrombine dat gesynthetiseerd wordt in de lever). Deze substraten werden gebruikt in de enzym systemen afkomstig uit lever, nier, long en testes. De Km waarden voor FLEEL en d-osteocalcine, beide gebruikt in heterologe systemen, waren nagenoeg constant in alle weefsels. De Km voor d-sperma Gla proteïne en d-fragment Su daarentegen varieerde meer. De affiniteit van deze substraten bleek het grootst te zijn voor het enzym afkomstig uit het homologe weefsel. Uit de door ons beschreven resultaten lijkt het aannemelijk dat het Gla-domein van het rijpe eiwit een herkenningsplaats bezit voor het carboxylase. De aminozuur sequentie die van belang is voor deze rol moet echter nog opgehelderd worden.

Behalve de herkenningsplaats in het Gla domein van het rijpe eiwit is er nog een andere aminozuur sequentie waarvan is aangetoond dat het een belangrijke rol speelt in de herkenning door carboxylase. Deze herkenningsplaats is gelegen in de leader-sequentie van het precursor eiwit. De leader-sequentie bestaat uit een signaal-peptide, dat nodig is voor de translocatie van de groeiend polypeptide keten door de wand van het RER, en een propeptide. Experimenten met recombinant stollingsfactor IX hebben aangetoond dat het propeptide nodig is voor de carboxylering van Glu. Wij hebben het effect van het propeptide (aangeduid als aminozuren -18 tot -1) op de carboxylering van synthetische substraten bestudeerd (hoofdstuk 3). Propeptide-bevattende substraten gebaseerd op aminozuur sequenties van humaan prothrombine en factor IX zijn goede substraten met een affiniteit voor carboxylase die ca. duizend maal hoger is dan die van FLEEL. De propeptides van factor IX en prothrombine bezitten dezelfde affiniteit voor het enzym. Substraten die het propeptide van prothrombine bezitten worden echter efficiënter gecarboxyleerd dan substraten die het propeptide van factor IX bezitten. Om de grootte van de

herkenningsplaats te bepalen werden peptides gesynthetiseerd waarbij in het propeptide verschillende aminozuren werden veranderd. Veranderingen in het NH_2 terminale (-18 tot -10) deel propeptide hadden een grote invloed op de carboxyleringsgraad van het peptide terwijl mutaties aangebracht op positie -4 en -1 de affiniteit van het peptide voor carboxylase niet beïnvloedden. Tegenwoordig wordt aangenomen dat de aminozuren -18 tot -10 van belang zijn voor substraat herkenning en dat de aminozuren -4 tot -1 een rol spelen bij de herkenning door het propeptidase. Van het pentapeptide FLEEL, dat de herkenningsplaats voor carboxylase niet bezit, wordt tijdens de in vitro carboxyleringsreaktie slechts een klein gedeelte gecarboxyleerd (minder dan 1%) en van dit Gla-bevattende peptide is ook alleen maar het eerste glutaminezuur residu omgezet in Gla. Om te bepalen of van de propeptide-bevattende substraten beide naast elkaar gelegen glutaminezuur residuen gecarboxyleerd kunnen worden, werden substraten gesynthetiseerd waarbij een van twee Glu's vervangen werd door Ala. De carboxyleringsreaktie verliep even snel met beide substraten, maar de reaktiesnelheid lag iets lager dan de carboxyleringssnelheid voor het substraat dat twee glutaminezuur residuen bevatte. Dezelfde resultaten werden verkregen met substraten waarbij een van de twee glutaminezuur residuen werd vervangen door asparginezuur. In het peptide waarin beide Glu residuen werden vervangen door asparginezuur werd nauwelijks CO_2 geïncorporeerd (ca 1% van de hoeveelheid die wordt geïncorporeerd in Glu-bevattende substraten). Een analoog van homoglutaminezuur, carboxymethylcysteïne, kon geen CO_2 fixeren. Deze resultaten tonen aan dat het proton op het gamma C-atoom, dat tijdens de carboxyleringsreaktie vervangen wordt door CO_2, binnen zeer kleine grenzen gelocaliseerd moet zijn.

Hoofdstuk 4 beschrijft een nieuwe methode om het vitamine K-afhankelijke carboxylase te zuiveren. Deze methode maakt gebruik van de hoge affiniteit van het carboxylase voor het propeptide. Propeptide gebonden aan een vaste drager, CNBr-geactiveerde sepharose of thiol-sepharose, is in staat carboxylase te binden. Het enzym kan, na intensief wassen, van de kolom geëlueerd worden met propeptide of in het geval van de thiol-kolom met reducerende middelen zoals dithiothreitol. Beide methodes leverden een enzym preparaat op dat nog grote hoeveelheden van het contaminerende eiwit BiP bevatte. Desalniettemin geeft deze methode in een stap een aanzienlijke zuivering van het enzym. Het verkregen produkt kan gebruikt worden als begin materiaal voor verdere zuivering. Recentelijk heeft de groep van Stafford een artikel gepubliceerd waarin zij de volledige zuivering van het enzym beschreven met een techniek die gebaseerd is op de door ons beschreven methode.

In hoofdstuk 5 beschrijven we een ander effect dat het propeptide teweeg brengt, namelijk een conformatie verandering in het enzym waarbij een hoge affiniteitsplaats voor gereduceerde vitamine K ontstaat. Voor deze conformationele verandering speelt phenylalanine, dat een belangrijke rol speelt bij substraat herkenning, geen rol. Het propeptide alleen kan deze conformatie verandering niet bewerkstelligen, het propeptide dient covalent verbonden te zijn met het substraat.

In hoofdstuk 6 wordt een Gla-bevattend eiwit uit bot met een ongewone stabiliteit beschreven. Zolang dit osteocalcine gebonden zit aan de hydroxylapatite matrix van botten is het bijzonder stabiel. We hebben het antigen van dit eiwit aan kunnen tonen in bovide botten van 13 miljoen jaar oud. Zelfs de Gla residuen waren nog steeds detecteerbaar. Uit humane botten van jongere leeftijd hebben we het eiwit kunnen isoleren en hebben we aangetoond dat het nog geheel of nagenoeg geheel intact was. Extractie, zuivering en aminozuur sequentie bepaling van dit eiwit uit fossiele botten kan een belangrijke bijdrage leveren tot de bestudering van de fylogenie van uitgestorven soorten.

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Curriculum vitae

De schrijfster van dit proefschrift is geboren op 1 mei 1959 te 's-Hertogenbosch. Na de middelbare school, MAVO en HAVO, doorliep zij de laboratoriumscholen HBO-A Chemie te Eindhoven en HBO-B Biochemie te Oss.
In september 1982 trad zij als analiste in dienst bij de vakgroep Biochemie van de Rijksuniversiteit Limburg. In september 1983 startte zij de part-time doctoraal opleiding Biologie aan de Rijksuniversiteit Utrecht, welke in 1990 werd afgerond met als hoofdonderwerpen Haematologie en Moleculaire Genetica. Van september 1987 tot november 1989 was zij werkzaam als research fellow bij de Division of Hematology/Oncology verbonden aan New England Medical Center Hospitals te Boston, USA. Gedurende de laatste twee jaren is zij als AIO verbonden geweest aan de vakgroep Biochemie van de Rijksuniversiteit Limburg.