Vitamin K-dependent carboxylase : growth to maturity

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GROWTH TO MATURITY

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VITAMIN K-DEPENDENT CARBOXYLASE: GROWTH TO MATURITY

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Bernardus Aloysius Maria Soute

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A journey of a thousand miles
must begin with a single step

Lao Tze

Aan mijn ouders
Aan Anneke, Iris en Marcel

Contents

Abbreviations

BGP	Bone Gla protein (osteocalcin)
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
d-	refers to a decarboxylated protein
DMSO	dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FI	Fibrinogen
FII	Prothrombin
FV	coagulation factor V
FVII	coagulation factor VII
FIX	coagulation factor IX
FX	coagulation factor X
FLEEL	the pentapeptide phenylalanine-leucine-glutamate-glutamate-leucine
FLEEV	the pentapeptide phenylalanine-leucine-glutamate-glutamate-valine
Gla	Gamma-carboxyglutamic acid
Glu	Glutamic acid
HPLC	High performance liquid chromatography
K_1	Phylloquinone
K_2	Groupname for Menaquinones
KH_2	Vitamin K hydroquinone
KO	Vitamin K 2,3 epoxide
MGP	Matrix Gla protein
MK-n	Menaquinone
M_r	relative molecular mass
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) reduced form
$(NH_4)_2SO_4$	Ammonium sulphate
PGP	Plaque Gla protein
Pro	Propeptide
PT	Prothrombin
RER	Rough endoplasmic reticulum
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TF	Tissue factor
TPT	the tripeptide boc-glutamic acid-glutamic acid-valine

CHAPTER 1

Introduction

Blood coagulation

Injury of the vascular endothelium is followed by a series of successive chemical and physical interactions resulting in the generation of thrombin at the site of injury. Thrombin generation will finally lead to an increased platelet deposition and the formation of a stable haemostatic plug. This process has first been described by MacFarlane in the "coagulation cascade" (1). The burst of enzymic reactions is following two distinct routes: the intrinsic and the extrinsic pathway (fig. 1). Formation of enzyme complexes on phospholipid surfaces is essential for rapid delivery of products at the site of vascular injury (2). γ-Carboxyglutamic acid (Gla) residues present in the amino terminal region of some coagulation zymogens are a prerequisite in the Ca^{2+}-dependent interaction with negatively charged phospholipid surfaces (3,4). These Gla-residues are formed during a posttranslational modification, in which the enzyme γ-glutamylcarboxylase and vitamin K are of vital importance (5,6). The carboxylase is tightly bound to the microsomal membranes. Its most important features, as well as the way in which vitamin K fulfils its role as a coenzyme will be discussed below.

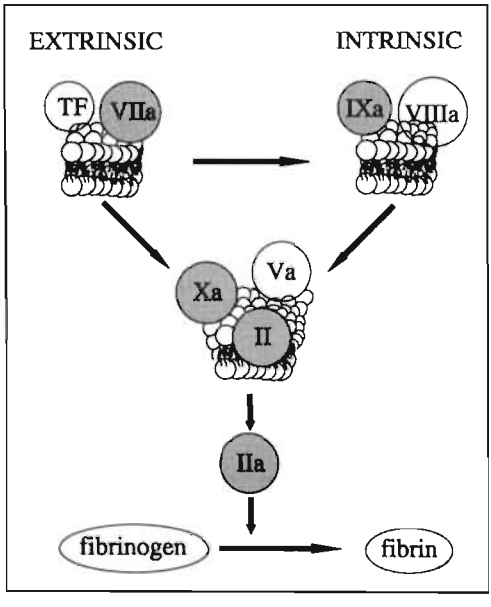

FIG. 1: The blood coagulation cascade. *Schematic representation of the blood coagulation process. The vitamin K-dependent factors are depicted in grey*

History of vitamin K

More than 50 years ago the Danish scientist Henrik Dam discovered vitamin K while measuring the effect of a cholesterol free diet on chickens (7). It turned out that together with the cholesterol a second unknown fat soluble component had been extracted, in the absence of which the animals developed haemorrhages and their blood exhibited a prolonged coagulation time. This unknown factor was identified as a fat soluble vitamin necessary for normal blood coagulation. Therefore it was called "Koagulations vitamin", abbreviated as "Vitamin K".
Already in 1922 Schofield detected comparable bleeding symptoms in cattle on feeding the animals improperly cured sweet clover hay (8). It is now known that these symptoms were provoked by dicoumarol (9), a drug possessing vitamin K antagonistic properties. These and related drugs are nowadays used both as rodenticides, and for the treatment and prophylaxis of thrombo-embolic diseases. In 1963 Hemker et al. (10) already suggested, that blood plasma of anticoagulated patients contained abnormal coagulation factors. However, it lasted until 1974 before Gla was discovered and the real nature of the abnormality could be elucidated.

Function of vitamin K

After protein biosynthesis secretory proteins undergo further post-translational modifications, which in most cases are essential for their biological activity. Subsequently they are transported via the Golgi apparatus and secreted into the extra cellular environment (11). Nowadays many post-translational modifications have been described in the literature (12). Examples are glycosylation, hydroxylation and disulphide bond formation and most of these reactions occur in the rough endoplasmic reticulum of eukaryotic cells. Also γ-glutamylcarboxylation, subject of this thesis, belongs to this category.
Early theories had often postulated a general role for vitamin K in protein biosynthesis, but until 1974 its only known function was being an essential cofactor in the synthesis of the four vitamin K-dependent coagulation factors prothrombin, factor VII, factor IX and factor X. In 1974 Stenflo et al. (5), Nelsestuen et al. (6) and Magnusson et al. (13) reported independently that vitamin K

promotes a unique posttranslational modification of specific glutamic acid residues (Glu) into γ-carboxyglutamic acid residues (Gla). During this conversion an extra carboxyl group is introduced at the γ-position of the glutamate residue (fig. 2). The carboxylation reaction is catalysed by vitamin K-dependent γ-glutamylcarboxylase for which the requirements are the presence of reduced vitamin K, CO_2, O_2 and an appropriate protein precursor. Whereas during the last two decades the mechanism of transcription and translation have been elucidated to a molecular level in procaryotes as well as in eukaryotes, relatively little is known about the mechanism and regulation of post-translational modifications. Especially because the enzymes involved are hydrophobic, membrane bound proteins, their purification raises serious problems. Unfortunately this holds also true for the enzymes involved in the carboxylation process, and only recently γ-glutamylcarboxylase has been purified to near homogeneity (14,15).

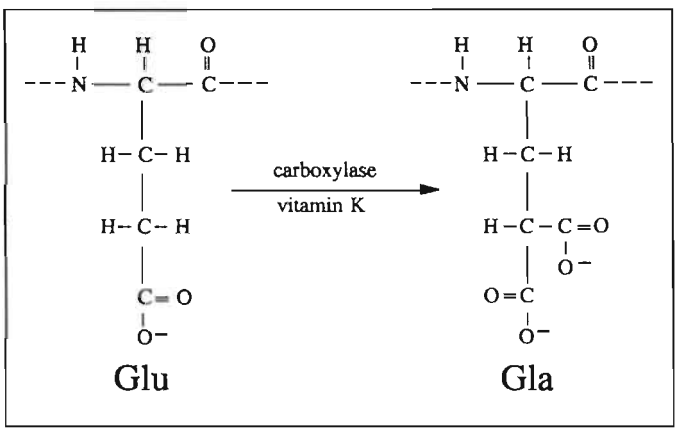

FIG. 2. The vitamin K-dependent carboxylation reaction. *Structures of glutamate (Glu) and γ-carboxyglutamate. (Gla)*

Gla-containing proteins

Since vitamin K-dependent carboxylase has been found in almost all types of eukaryotic cells (16), it was to be expected that a wide variety of Gla-containing proteins would be discovered. Nevertheless, the number of well characterized Gla-proteins has remained surprisingly low. They can be classified in three groups:

a. Gla-containing proteins in blood plasma

Gla was first discovered in prothrombin (5,6,13). At that time the high degree of sequence similarity between prothrombin and the coagulation factors VII, IX and X had already been established. As a logical consequence the latter proteins were also subjected to Gla analysis and all appeared to contain 10-12 Gla residues per molecule (17,18). Some years later three other Gla-containing proteins in plasma were discovered on the basis of their similar chemical properties (e.g. their absorption to and co-elution from insoluble barium salts). The proteins involved were: Protein C (19), Protein S (20) and Protein Z (21). The proteins C and S are known to play an important role in the regulation of the blood coagulation process. Their function is to inactivate the activated coagulation factors V and VIII by limited proteolytic degradation (22,23). Although the function of protein Z has not yet been elucidated there is some evidence that it mediates in the binding of thrombin with phospholipid vesicles in a Ca^{2+}-independent way (24). All four classical coagulation factors and the proteins C, S and Z are typical secretory glycoproteins with molecular masses ranging from 45,000 to 72,000 Da.

b. Gla-containing proteins in calcified tissues

Gla-containing proteins have been found in various calcified tissues such as bone (25,26), dentin (27), renal stones (28), hardened atherosclerotic plaques (29) and coral (30). Except for the two Gla-containing proteins in bone, these proteins have not been well-characterized and their physiological function is not known. Bone is the only extrahepatic tissue in which the products of vitamin K-dependent carboxylation have been unequivocally characterized. The proteins in question are matrix Gla-protein (MGP) and osteocalcin, also known as bone Gla-protein (BGP). MGP was initially discovered in bone (31), but its mRNA has now been detected in tissues varying as much as lung, kidney and vessel wall (32). Hence it must be assumed that this protein is expressed in all these tissues. Osteocalcin is the most abundant Gla-protein in man. Its molecular mass is 5,700 Da. It is smaller than and does not share substantial sequence similarity with the vitamin K-dependent coagulation factors. It is exclusively synthesized by osteoblasts and odontoblasts and after its secretion it is bound to the hydroxyapatite matrix of the bone. Despite substantial knowledge of the bone Gla-proteins, their function remains obscure. Initially it was thought that the coumarin induced bone abnormali-

ties in human fetuses (33) and in young rats (34) were caused by the incomplete carboxylation of osteocalcin. Now these defects can also be explained by assuming a role for MGP during early bone formation.

c. **Other Gla-containing proteins**

In spermatozoa (35), urine (36), lung surfactant (37), and snake (38) and snail (39) venoms Gla-containing proteins have been found. Except for the snail venom, which is a highly powerful neurotoxin (40) and which has been characterized to the level of aminoacid sequence, the proteins have not been well-characterized and their physiological function is not known.

The vitamin K cycle

The active form of vitamin K during the carboxylase reaction is the hydroquinone (KH_2). γ-Glutamyl carboxylation is linked to a vitamin K epoxidase activity (41), which simultaneously converts vitamin KH_2 into vitamin K2,3-epoxide (KO). In theory, the addition of CO_2 to the γ-carbon in a Glu-residue may occur either via an activation of CO_2 or via labilization of a γ-hydrogen. Most evidence presently available supports a mechanism in which carboxylation proceeds by the abstraction of the γ-proton of the Glu-residue followed by a CO_2-attack at the same position (42-44).

The role of vitamin K in this reaction is to act as a cofactor in the labilization of the γ-glutamyl hydrogen, either by an ion- (or radical-) mediated mechanism (43,45). Although the carboxylase and epoxidase activities are not strictly coupled (45-47), it is postulated that the same enzyme (carboxylase) is responsible for both activities (48). It is also thought that the oxidation of KH_2 into KO by molecular oxygen provides the energy required for the CO_2-fixation at the γ-position of a glutamate residue. Via two subsequent reduction steps KO may then be recycled into KH_2 (fig. 3).

The first step in the recycling of KO is the dithiol-dependent reduction of KO into vitamin K-quinone (K) by the enzyme vitamin K epoxide reductase. The cycle is completed by the reduction of K into KH_2 which may be accomplished by a dithiol-dependent as well as by an NADPH-dependent reductase. The importance of this 'vitamin K-cycle' being operative in vivo may be demonstrated by the fact that on a molar base the urinary Gla-excretion exceeds the dieta-

ry intake of vitamin K 200-500 times. Since the intestinal absorption of dietary vitamin K has been estimated to be 30-70% (11) each molecule of vitamin K must be recycled many hundred (and probably several thousand) fold before it is metabolized into inactive degradation products (49).
The enzymes operational in the vitamin K-cycle will be dealt with elsewhere.

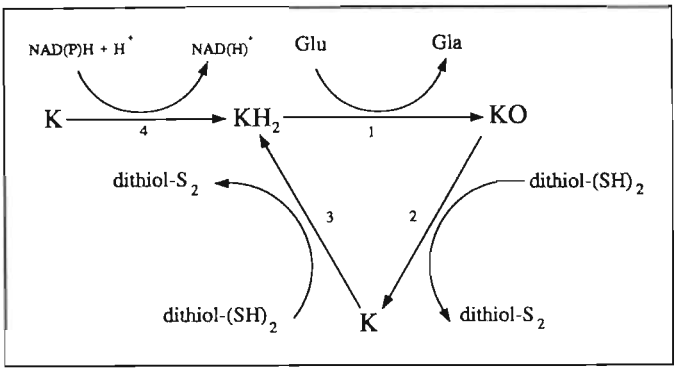

FIG. 3. The vitamin K cycle. *Step 1 is the carboxylase/epoxydase reaction in which Glu is converted into Gla. Step 2 and 3 are dithiol-dependent reductions, while step 4 is NAD(P)H-dependent*

The carboxylase reaction

The cellular function of the enzyme γ-glutamylcarboxylase has been mostly studied using crude and partially purified microsomal liver preparations. In table I the main properties are summarized.
An in vitro system for measuring vitamin K-dependent carboxylase activity was developed by Esmon et al. in 1975 (66). Initial studies, performed in washed rat liver microsomes (54,59,66,67), showed the possibility to incorporate $^{14}CO_2$ into endogenous precursor proteins. The incorporation was dependent on the presence of O_2, vitamin K and HCO_3^- and the system also required NAD(P)H and/or a reduced pyridine nucleotide-generating system. This energy-generating system seemed to be present in the postmicrosomal supernatant, but it was soon established that it could simply be replaced by KH_2 (54,59). The carboxylase activity was then extracted from the membrane by means of detergents or bile salts. Initially Triton X-100 was frequently used (57,68), later it was replaced by 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulphonate (CHAPS) (69). The solubilized system was shown to contain all activities necessary for carboxylation.

Standard conditions	
- Either phylloquinone (K_1) or menaquinone (MK), both either in the presence of reducing agents (NAD(P)H or DTT) or in their hydroquinone form	
- O_2	
- CO_2	
- carboxylatable substrate (exogenous and/or endogenous).	
Stimulating agents	
- Mn^{2+}	(50, 51)
- DTT	(52,53,54)
- $(NH_4)_2SO_4$	(55)
- pyridoxal-5'-phosphate	(52,56)
- DMSO	(51)
- ketones	(51)
Inhibiting agents	
- Chloro-K (2-Cl-3-phytyl-1,4-naphtoquinone)	(57)
- coumarin derivates	(58)
- 2,3,5,6-tetrachloro-4-pyridinol (TCP)	(59, 60)
- CN	(47)
- sulfhydryl reagents	(61, 62)
- peptide substrates analogs	(63,64)
- naphtoquinones	(65)

Table I. Requirements for vitamin K-dependent carboxylase

Although the activity of carboxylase has been measured in the liver of a number of species (70,71) vitamin K-dependent carboxylation has been studied most extensively in microsomal preparations obtained from vitamin K-deficient or warfarin-treated rats and from normal and warfarin- treated cows. During vitamin K deficiency and during warfarin treatment the levels of the vitamin K-dependent proteins in the plasma of the rat are decreased and endogenous precursor proteins accumulate in the liver (72). The hepatic microsomal fractions thus prepared contain both carboxylase and an endogenous substrate (57,59,73). In warfarin-treated cows, on the other hand, non-carboxylated coagulation factors (descarboxy factors) appear in the blood plasma in concentrations similar to those of the normal coagulation factors in untreated animals (10,74). Therefore it was unexpected that warfarin treatment also caused an accumulation of endogenous precursor proteins in bovine liver (75).

In solubilized microsomes the vitamin K- and O_2-requirement seems to be higher than in intact microsomes. Concentrations used were mostly between 50-500 μM for vitamin K. In intact microsomes K_M^{app} values for O_2 of 10-15 μM

have been found (76) where in detergent-solubilized microsomes a K_M^{app} of 45 μM has been reported (49). Available data indicate that the active species in the carboxylation reaction is CO_2 and not HCO_3^- (77). K_M^{app} values for total $[CO_2/HCO_3^-]$ have been reported to be between 0.2 and 0.4 mM (77). Because carboxylase is unstable at 37 °C, most studies were performed at temperatures of 20 °C or lower and at a pH of 7.2-7.4.
At this moment vitamin K-dependent carboxylase activity has been demonstrated in many different tissues such as kidney (78-82), spleen (81-83), testes (82-84), lung (81,83-85), pancreas (82,86), placenta (87,88), vessel wall (82), thyroid, thymus, cartilage, tendon and uterus (82). Activity has also been shown to be present in a wide variety of cultured cells (16,86,88,89) such as osteoblasts, hepatocytes, endothelial cells and tumor cells of various origin.

Detection of Gla-residues

Since it is known that Gla is stable during alkaline hydrolysis, several methods for its detection have been developed. Methods employed vary from a modified aminoacid analysis described by Hauschka (90) to colorimetric methods (91), gaschromatography (92) and mass-spectroscopy (93). Relatively simple procedures have been developed which are based on high performance liquid chromatography (HPLC) using anion exchange or reversed phase columns (94,95). Detection of the amino acids is performed either by pre- or postcolumn derivatization using ortho-phtalaldehyde (94) or phenylisothiocyanate (95). To verify whether the peak eluting at the Gla-position is the correct one, parallel to the alkaline hydrolysis acid hydrolysates of the same samples may be prepared. In the acid hydrolysates the peak eluting at the Gla-position must be absent. By means of a specifically incorporated radiolabel in the Gla-residues, the shift from the Gla- to the Glu-position can also be checked (96).

Various forms of vitamin K

The two most important compounds found in nature having 'vitamin K activity' are phylloquinone and the menaquinones. Active compounds all contain a

naphtoquinone ring system substituted with a methyl group at position 2 and an aliphatic side chain (R) of variable length at position 3 (fig. 4).

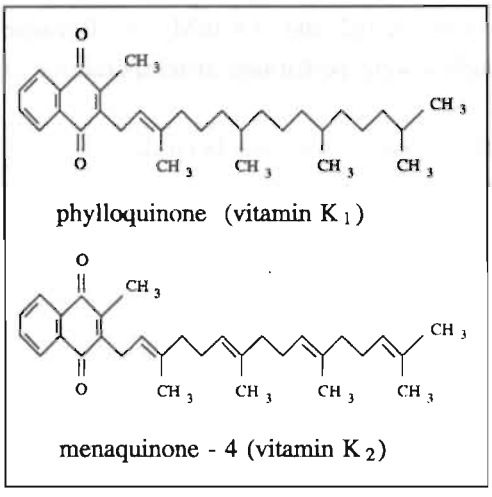

FIG. 4. Various forms of vitamin K. The side-chain of phylloquinone only contains one unsaturated bond where menaquinones have many, dependent on their side-chain length. As an example menaquinone-4 is depicted but the side-chain may vary between 1 and 13 residues

In phylloquinone R is composed of 4 isoprenoid residues of which the first one is unsaturated. In menaquinone R may vary from 1 to 13 residues, each containing one unsaturated bond. Phylloquinone (vitamin K_1) is exclusively found in green plants like spinach, kale, cabbage and broccoli. Also cows' milk contains considerable amounts of phylloquinone, in concentrations between two and three fold higher than those found in humans' milk (97). The phylloquinone content of a wide range of products has been determined presently (98). Menaquinone (vitamin K_2) is a group-name for a number of closely related products of microbial origin. They are generally designated as MK-n, where n stands for the number of isoprenoid residues in the aliphatic side chain. The hydrophobicity of the various menaquinones strongly depends on their chain length, and will probably influence the efficiency of their intestinal absorption, their transport and their binding to carboxylase. Menaquinones occur in various (mainly fermented) foods and also the bacterial flora in the colon produces significant amounts of menaquinones, but the extent to which they are resorbed from the colon is still subject of investigation.

Most data concerning dietary sources, intestinal absorption, plasma transport, tissue concentrations, subcellular distribution and metabolism of vitamin K

regard phylloquinone. The quantitation of menaquinones in food, plasma and tissues has been a problem for many years and only recently detection techniques are being developed which make their quantitation possible (99). The need of a dietary supply of vitamin K has been demonstrated in most species, including man. Even a rather mild limitation of vitamin K-intake (no green vegetables) caused detectable alterations in some blood coagulation parameters after three weeks, both in human volunteers (100) and in rats (101), in spite of the menaquinones produced in the colon. Hence the physiological relevance of the menaquinones is at least doubtful. However, recent evidence has demonstrated that long chain menaquinones account for 80-95% of the total hepatic vitamin K stores (99,101). Their origin, intracellular location and utilization for γ-glutamyl-carboxylation has still to be established. In vitro experiments have demonstrated that like phylloquinone menaquinones may serve as a cofactor in the carboxylase reaction, especially MK-3 and MK-4 (102,103). Because of the above mentioned arguments it cannot be excluded that the hepatic menaquinone stores predominantly originate from the diet, and not from the intestinal flora. An elaborate food analysis for menaquinones levels must be awaited to obtain more conclusive support for this hypothesis.

Enzymes involved in the vitamin K cycle

Vitamin KO, formed during the carboxylation/epoxidase reaction, is recycled into reduced vitamin K by the action of at least two reductases. KO and K reductase require a dithiol as a cofactor; in vitro the synthetic product dithiothreitol (DTT) is frequently used (104). In vivo a possible candidate to fulfil this role is the dithiol protein thioredoxin (105). Thioredoxin abundantly occurs in various subcellular fractions of calf liver, including nuclei, mitochondria and microsomes (106). In vitro experiments demonstrated that the enzyme system thioredoxin/thioredoxin reductase using NADPH as a cofactor, could replace DTT during the reduction of KO and K (105). In a different assay system these results were confirmed by Silverman & Nandi (107).

Two other enzymes require NAD(P)H as a reductant. One is the membrane-bound NAD(P)H-dependent K reductase (108). This enzyme is relatively insensitive to warfarin and during coumarin treatment it can utilize exogenous vitamin

K to bypass the inhibited dithiol-dependent enzyme (109,110). Although unable to complete the vitamin K cycle by itself, it is of vital importance in case of intoxication. This enzyme is membrane-bound and probably of more importance than the other NAD(P)H-dependent reductase DT-diaphorase, an enzyme present in both the microsomal fraction and the cytosol (60,108). It has been demonstrated that DT-diaphorase is able to reduce vitamin K but the importance seems questionable because of its low activity in the liver.

It has been well established that 4-hydroxy-coumarins inhibit the action of the dithiol-dependent KO reductase (111-113). These drugs cause an accumulation of KO in liver, thereby exhausting the supply of KH_2 and preventing further carboxylation events. Coumarins also inhibit the dithiol-dependent K reductase (114,115), but this enzyme may be bypassed by the NADH-dependent reductases mentioned before (116). Therefore KO reductase is the most important chain in the vitamin K cycle. The plasma accumulation of vitamin KO in humans on anticoagulant treatment makes it plausible that indeed vitamin KO-reductase is the target enzyme for warfarin. Recently direct evidence has been obtained for the existence of a microsomal warfarin receptor with binding characteristics specifically related to the activity of KO-reductase (117).

Whether or not the two microsomal DTT-dependent activities are generated by the same protein is still a matter of debate, but the finding that the optimal dithiol concentration (118) and the apparent K_i for warfarin (114) are closely similar does not suggest the occurrence of two different dithiol-dependent reductases. Additionally Fasco et al. (114) brought up very strong arguments in favour of only one reductase, since they found both enzymic activities to be decreased in warfarin-resistant rats. The low probability of a genetic alteration affecting two enzymes in a similar way argues against the presence of two different enzymes.

Coumarin derivatives

The blockade of the vitamin K cycle by coumarins causes the hepatocellular accumulation of coagulation factor precursors (descarboxyfactors).The extent to which descarboxy-factors are excreted into the blood stream is species dependent. The active compounds invariably act by inhibiting the dithiol-dependent

reductases in the vitamin K cycle. In this way the recycling of vitamin K is blocked and the supply of KH_2 is rapidly exhausted. Because only the dithiol-dependent reductases are inhibited by coumarins, the blockade may be bypassed by the administration of 10-125 mg/day of vitamin K, depending on the dose and the kind of coumarin. Under these conditions all KH_2 formed by the action of the NAD(P)H-dependent reductase can only be used once, leading to high circulating KO levels in the plasma.

In 1974 a second generation of 4-hydroxycoumarins became available, which are generally designated as 'super warfarins' (119). These compounds (difenacoum, brodifacoum) are more hydrophobic than is warfarin, and they are characterized by very long half-life times. Because of their extreme toxicity they are only used as rodenticides, especially in areas where rats have become warfarin-resistant (120). The production and application of these drugs include the risks of accidents by human ingestion (121-123).

Several compounds, not being 4-hydroxycoumarins, have vitamin K-antagonistic properties and have been shown to inhibit vitamin KO-reductase. These compounds include salicylate (124-126), lapachol (127) and sulphaquinoxaline (128).

Substrate specificity

Most information available on the mechanism of substrate recognition has been obtained via cDNA research. Initial attempts to find an amino acid sequence in the Gla region of the vitamin K-dependent proteins, known at that time, were not successful. It was demonstrated, however, that a peptide homologous with amino acids 5 to 9 of bovine prothrombin could serve as a substrate in the carboxylation reaction (129). The peptide in question, Phe-Leu-Glu-Glu-Val (FLEEV) is one of the many low molecular-weight peptide substrates that have been synthesised from that time (52,64,73,130). Because they are readily carboxylated these pepides were useful as substrates in the carboxylation reaction, but they all share the disadvantage of having a low affinity for the enzyme. K_M^{app} values reported were all in the millimolar range and it was demonstrated that only the first of the two Glu residues was carboxylated (131,132). The same high K_M^{app} values were found for high molecular weight proteins like decarboxylated coagulation factors (55,136-138). The preparation of smaller protein substrates like

decarboxylated osteocalcin (55) and descarboxyprothrombin fragment 13-29 (135) indicated that some secondary or tertiary structure or some recognition site for carboxylase could facilitate the carboxylation of these substrates.

All Gla-containing proteins are secretory proteins and need substantial post-translational modification for full biological activity. They are synthesized in a precursor form consisting of a signal sequence, a prosequence and the aminoacid residues found in the mature protein. To enter the secretory pathway proteins must be translocated into the endoplasmic reticulum membrane. Targeting to the endoplasmic reticulum membrane occurs through the sequential actions of the signal recognition particle (SRP) and its receptor (docking protein) (136). Shortly after translocation the signal sequence is cleaved by a signal peptidase while the propeptide stays attached to the mature protein probably until it reaches the Golgi body (11). The presence of a propeptide in the Gla-containing proteins was predicted from the cDNA clone of human factor IX by Kurachi and Davie (137). Shortly afterwards Degen et al. demonstrated a propeptide after cloning human prothrombin (138). By the proper alignment of these and other prosequences (139-142) a substantial sequence homology was found indicating a possible important role in γ-carboxylation (see fig. 5).

```
    -18      -10        -1 1        10        20        30        40
      |        |         | |        |         |         |         |
h-PT HVFLAPQQARSLLQRVRR-ANT-FLγγVRKGNLγRγCVγγTCSYγγAFγALγSSTATDVFW
b-PT HVFLAHQQASSLLQRARR-ANKGFLγγVRKGNLγRγCLγγPCSRγγAFγALγSLSATDAFW
h-FX SLFIRREQANNILARVTR-ANS-FLγγMKKGHLγRγCMγγTCSYγγARγVFγDSDKTNγFW
b-FX SVFLPRDQAHRVLQRARR-ANS-FLγγVKOGNLγRγCLγγACSLγγARγVFγDAγQTDγFW
h-PC SVFSSSERAHQVLRIRKR-ANS-FLγγLRHSSLγRγCIγγICDFγγAKγIFQNVDDTLAFW
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FIG. 5. N-terminal sequences in the propeptides and Gla domains of some mammalian vitamin K-dependent proteins. *The sequences are in the one letter code, γ stands for Gla. Abbreviations are: h, human; b, bovine; PT, prothrombin; FX, factor X; PC, protein C*

Additional data were obtained from the mutant forms of factor IX isolated from patients suffering from haemophilia B (140,141,143-145). They were all shown to be biological inactive and to have slightly higher molecular weights. Structural analysis revealed that the propeptide was still attached and that a mutation had occurred in the propeptide region. In all cases substitution had taken place in the Arg-rich region near the cleavage site: Arg-4 → Gln (141,143-145) and Arg-1 → Ser

(140). The importance of the propeptide was also demonstrated by the expression of cDNA coding for human factor IX (146,147) and human protein C (148). When expressed in heterologous mammalian cells the product showed a substantial degree of carboxylation in both recombinant proteins. With the aid of site-specific mutagenesis it was subsequently shown that complete or partial removal of the propeptide dramatically influenced carboxylation (148,149). Recently a rabbit liver microsomal endopeptidase has been purified which cleaves synthetic peptides that mimic the propeptide (150). Proteolytic cleavage was dependent on an arginine residue at positions -4 and -1. Point mutations at position -16 (Phe → Ala) or -10 (Ala → Glu) also severely affected the carboxylation of factor IX (149). After synthesizing a 20-residue peptide homologous to the propeptide region of human factor X, Knobloch & Suttie demonstrated that this peptide stimulated the carboxylation of non-covalently linked small peptide substrates (151). The stimulation was about 3-fold and these results were confirmed by Ulrich et al. with a synthetic peptide homologous to the propeptide region of human prothrombin (152). In the same paper these authors showed a further improvement of the substrate by covalently binding it to the propeptide. A synthetic peptide similar to the sequence -18 to +10 in human descarboxyprothrombin was characterized by a K_M^{app} of 3.6 μM which is at least three orders of magnitude lower than the synthetic peptides lacking the propeptide. These results suggest that the propeptide plays a major role in the recognition of substrates by carboxylase but at this moment it remains unclear whether other sequences contribute to the regulation of carboxylation. It was proposed by Price et al. (153) that also the highly conserved amino acid residues in the Gla-region found in all known mammalian proteins may play a role in carboxylase recognition. This sequence, Gla-X-X-X-Gla-X-Cys at the position +17 to +23 (see fig. 5), is also present in decarboxylated osteocalcin and descarboxyprothrombin fragment 13-29, two low K_M^{app} substrates lacking a prosequence (135,55). By the same author it was suggested that the cysteine residue could be of importance (153) during carboxylation, since carboxylase has been shown to contain an essential thiol group (154).

Introduction to this thesis

Since the discovery of γ-carboxyglutamic acid (Gla) in 1974, the metabolic role of vitamin K and its function in the carboxylation process has been subject of many investigations. The involvement of the enzyme γ-glutamylcarboxylase in the formation of Gla-residues made it necessary to purify the enzymee for proper analysis. However, purification of the enzyme has met with extreme difficulties which make many details of the carboxylation reaction still to be elucidated. Vitamin K-dependent carboxylase, an enzyme having all characteristics of an integral membrane protein and located at the luminal side of the rough endoplasmic reticulum, has been found in a wide variety of tissues. Although initially blood coagulation seemed to be the only physiologically important interest of γ-carboxylation, Gla-containing proteins have been detected in many other, non hepatic tissues and it is still a matter of debate which functions these non-hepatic Gla-proteins might have. The discovery of the new proteins has expanded the role of vitamin K and indicated the need to re-examine required dietary vitamin K intake aside from its traditional role in blood coagulation. Sensitive methods to detect Gla and vitamin K have become available during recent years and will facilitate further research. The discovery of the propeptide and its role in substrate recognition finally lead to the purification of carboxylase to homogeneity. It should be considered, however, that the technique for this purification could only be developed on the basis of data obtained in 15 years of research performed by groups from the USA and Europe.

The work presented in this thesis started in 1975, the same year in which the first carboxylating systems had been developed, and it ends with the complete purification of the enzyme. This thesis describes the contribution of the Maastricht vitamin K group in the discoveries leading to that success.

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CHAPTER 2

Methods for the preparation of artificial Prothrombin and factor X reagents and improved separation of descarboxy and normal coagulation factors by HPLC

Based on:

- Vermeer, C., Soute, B.A.M., and Hemker, H.C. (1977). A new method for the preparation of artificial factor II reagents from normal human and bovine plasma. Thromb. Res. 10, 495-507.
- Vermeer, C., Soute, B.A.M., and Hemker, H.C. (1978). Improvements of the method for the preparation of an artificial prothrombin reagent. Thromb. Res. 12, 713-716.
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Historical background

In vivo there are two ways to retard the vitamin K-dependent carboxylation of proteins. Firstly, a reduced nutritial intake of vitamin K will lead to the exhaustion of the total body stores, and to the subsequent slowing down of all hepatic and extrahepatic carboxylation reactions. Secondly, the recycling of vitamin K may be blocked by the administration of coumarin derivatives. In both cases non-carboxylated precursors of the Gla-containing proteins will accumulate in the RER of a wide variety of cells (1). In a later stage these proteins are also secreted into the extracellular environment, where they are known as descarboxy proteins. Initially, it was thought that the endoplasmic precursor proteins were similar to the circulating descarboxy proteins, but in the early '80s it became clear that the endoplasmic precursors contain a "pro"-sequence directly preceding the N-terminus of the mature protein. This pro-sequence is composed of 18 amino acids with a high degree of similarity for all Gla-containing proteins, and it plays a major role in the substrate recognition by γ-glutamylcarboxylase (2-4).
When we started our investigations on vitamin K-dependent carboxylase in 1976 there were two logical ways to set up an in vitro system in which the posttranslational carboxylation reaction could be investigated:

a. From vitamin K-deficient rats a cell-free microsomal liver system had been described by Esmon et al. (5), who used the accumulated endoplasmic precursor proteins (mainly prothrombin and related coagulation factor precursors) as a substrate for carboxylase. The system was absolutely dependent on vitamin K, whereas $NaH^{14}CO_3$ could serve as a CO_2 donor. By using the radiolabeled CO_2 the progress of the carboxylation reaction could be followed in time. An additional advantage was that the bulk of the radiolabeled bicarbonate was rapidly removed from the solution by adding trichloroacetic acid.
b. Methods had been developed for the purification of descarboxy prothrombin and descarboxy factor X from the plasma of coumarin-treated cows. At that time it seemed plausible that these purified proteins could serve as well characterized substrates for carboxylase. The procoagulant activity of the descarboxyfactors is extremely low, but it was thought that the carboxylation reaction would convert them into normal coagulation factors, with at least substantially improved procoagulant activity. Hence the progress of the Gla

formation should be measurable as the generation of prothrombin and factor X activity estimated by means of a coagulation assay.

For several reasons (one being the long history in blood coagulation of Hemker's group) we decided to start by exploring the second line. New procedures were developed for preparing one-stage prothrombin and factor X reagents, which were essentially free of any prothrombin and factor X, respectively. With these reagents the detection limit for both coagulation factors was decreased several orders of magnitude. However, incubation of the descarboxy proteins with bovine liver microsomes, vitamin K and CO_2 did not lead to the generation of 'normal' coagulation factors. Lateron it became clear that the failure of this system was due to the absence of a pro-sequence in the descarboxy factors, and that the descarboxy factors as such are very poor substrates for carboxylase (2,3). Hence we changed to the system mentioned under a. Yet some positive outcomes of the initial period can be mentioned: i) two new coagulation factor reagents had been developed which were superior to any of the existing ones, and ii) with these reagents the coagulation kinetics initially worked out by Hemker et al. (6) could be proven (7). Presently, chromogenic substrates provide more sophisticated methods for measuring coagulation factor activity (8), but since the one-stage coagulation assays are relatively simple and quick, they are still often used in our lab and elsewhere.

Finally we were the first to demonstrate that normal and descarboxy prothrombin could be separated by ion exchange chromatography. Obviously this was to be expected on the basis of their different charge, but until the introduction of high performance liquid chromatography (HPLC) the differences were too small to form a basis for their chromatographic separation.

As a practical guide the preparation procedures of prothrombin and factor X reagents and their charcterization as well as an HPLC-method for the separation of bovine prothrombin and descarboxyprothrombin are summarized below.

New methods for the preparation of artificial prothrombin and factor X reagents: relation between coagulation time and concentration

Introduction

For the determination of prothrombin generally two procedures are used:

a. The one-stage assay in which prothrombin deficient plasma and thromboplastin constitute a medium in which all factors are present in excess, except prothrombin. The prothrombin content of the sample added is rate limiting. The clotting time upon recalcification thus indicates the prothrombin level (9).
b. The two-stage assay in which first the prothrombin present in the sample is converted into thrombin. In a second stage the sample's thrombin content is assessed by measuring the clotting time with a suitable source of fibrinogen (10).

The disadvantage of the first method is that both artificial and congenital prothrombin deficient plasmas usually contain residual amounts of prothrombin which, in the absence of added prothrombin, are responsible for a rather short coagulation time (the "buffer time", which is generally 50-90 s). Consequently low prothrombin concentrations are difficult to assess. The two-stage method, although more elaborate than the one-stage assay, is especially useful in the determination of more or less purified prothrombin preparations. In plasma and plasma dilutions, however, the assay is hampered by the presence of thrombin inhibitors such as antithrombin III, which necessitates evaluation of the time-course of thrombin generation and degradation (11,12).

An artificial prothrombin and factor X reagent was composed by supplementing oxalate plasma with a concentrated solution, containing either factor VII and X or prothrombin and factor VII. From the oxalate plasma all vitamin K-dependent coagulation factors were first removed by adsorption with $BaSO_4$ and $Al(OH)_3$. To this plasma then either a factor VII/X concentrate or a prothrombin/factor VII concentrate was added. The factor VII/X concentrate was prepared from

citrate plasma by activating prothrombin with Echis carinatus venom. After removal of the clot the factors VII and X were isolated by adsorption on DEAE Sephadex and subsequent elution. The prothrombin/factor VII concentrate, required for the factor X reagent was prepared by purification of these factors on DEAE Sephadex.
In this way plasmas artificially deficient in either prothrombin (prothrombin reagent) or factor X (factor X reagent) could be prepared (13,14). The resulting reagents contained all coagulation factors of the extrinsic pathway in sufficient amounts, except the factors to be tested. It was shown that no clot formation occurred in a mixture in which prothrombin was completely absent and on theoretical grounds neither should clot formation occur in a mixture deficient in factor X. It was found, however, that all factor X-deficient plasma tested or described so far would form a clot in the presence of calcium and thromboplastin. This raised the question whether either trace amounts of factor X are difficult to remove, or other enzymes (c.q. coagulation factors) are able to shortcut or bypass the factor X-dependent step. To solve this question the last traces of factor X were removed from both the X-deficient reagent and thromboplastin with solid-phase antifactor X. With the factor X-deficient coagulation system thus prepared, a better insight was obtained in the relation between coagulation time and factor X concentration.

The bovine prothrombin reagent

Improved recovery of factors VII and X. It was shown that using oxalate plasma for the preparation of the factor VII/X concentrate, reduced the recovery of factor X. The most probable reason for this phenomenon is the partial adsorption of factor VII and X to the calcium oxalate, formed during the collection of blood and after recalcification of the plasma (15). Therefore citrate plasma should be used for the production of the factor VII/X concentrate.

The amount of Echis carinatus venom to be added. Although the concentration of unreacted prothrombin was below the detection range it still impaired the properties of the reagent (a buffer time of 5-15 min was observed). These last traces of prothrombin were removed from the dialysed factor VII/X concentrate by repeating the Echis carinatus treatment, followed by DEAE-Sephadex adsorp-

tion and elution.

Removal of antithrombin III. The $BaSO_4$-adsorbed plasma still contained thrombin inhibitors, the major part of which consists of antithrombin III. As was published earlier (6), a rectilinear curve should be obtained when the clotting time is plotted against the dilution of clotting factor (t-D plot). However, the presence of thrombin inhibitors caused longer coagulation times than expected, especially at prothrombin concentrations lower than 3.10^{-3} U/ml. In the range between 10^{-1} and 3.10^{-3} U/ml hardly any deviation could be detected. Since $Al(OH)_3$ is known to bind antithrombin III (16), it was used to efficiently remove the antithrombin activity from the $BaSO_4$-adsorbed plasma (5% w/v). The prothrombin reagent prepared with this plasma was compared to the normal reagent. This is shown in fig. 1. When plasma, adsorbed with both $BaSO_4$ and $Al(OH)_3$ was used for preparing the prothrombin reagent the t-D plot was rectilinear, whereas the reagent from plasma, adsorbed with only $BaSO_4$, resulted in a curved t-D plot notably at very low concentrations of prothrombin.

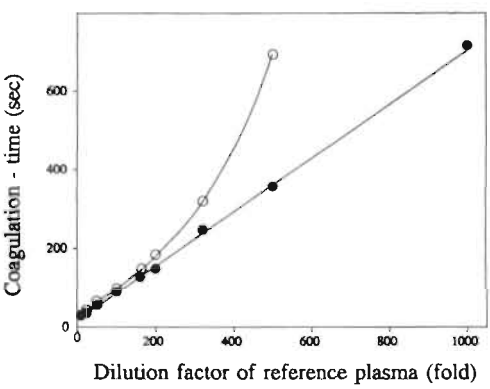

FIG. 1. Reference curves of varying prothrombin reagents. *$BaSO_4$- adsorbed plasma (see text) was adsorbed with $Al(OH)_3$. The plasma before (O–O) and after $Al(OH)_3$ treatment (●–●) was used to prepare a prothrombin reagent*

Characterization of the prothrombin reagent. Before freezing of the reagent, the factors VII en X had been added in amounts of 2.5 and 1 U/ml, respectively. The activity of these factors decreased substantially after freezing and thawing, which should be taken into account when the reagent is composed. Up to very low prothrombin concentrations a linear relationship existed between the coagulation time and the dilution of the reference plasma. When the reference plasma

was replaced by a prothrombin preparation, purified according to ref. 17[1], very short coagulation times were measured at high prothrombin concentrations. The coagulation time of a sample containing 2 U/ml of prothrombin for instance, was found to be 11.7 sec. showing that the reagent contained all other coagulation factors in sufficient amounts (fig. 2). The prothrombin concentration of 0 U/ml in the reagents was concluded from the buffer time (more than 15 hours) and from computer calculations as described in refs. 12 and 18. The samples containing prothrombin were prepared by diluting reference plasma. Experiments in which factor VII was tested with either bovine or human thromboplastin as described by Hemker et al. (19) indicated that, by freezing, the activated fraction of bovine factor VII had lost its activity. The partial activation of factor VII could not be prevented by the omission of $CaCl_2$. Moreover, without $CaCl_2$ the prothrombin was removed less efficiently.

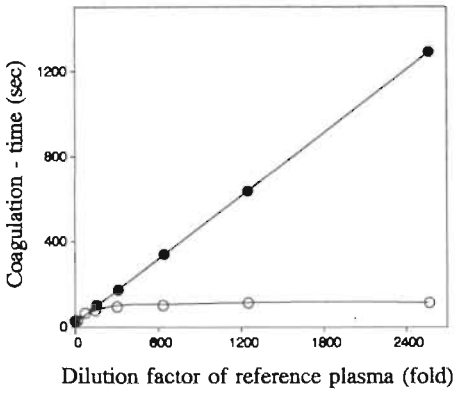

FIG. 2. t-D plot of the reference curves for bovine prothrombin in the coagulation assay.

●–● *reagent B*

○–○ *reagent B to which 5.10^{-3} U/ml of prothrombin has been added*

The preparation of factor II reference curves. With the prothrombin reagent prothrombin was tested in varying dilutions of bovine normal pool plasma. When the coagulation time was plotted against the prothrombin concentration, a convex curve (fig. 3) was obtained on double logarithmic scale (log C/log t plot). Addition of small amounts of prothrombin to the reagent restored the sigmoid shape, which is usual for reference curves of coagulation factors. Obviously, the convex form of the curve was due to the fact that the prothrombin

[1]Purified bovine factor II was kindly provided by Mrs. J.Govers-Riemslag.

reagent was absolutely free of prothrombin: at very high dilutions of the reference plasma the prothrombin concentration in the coagulation mixture becomes negligible and infinite clotting times were obtained. As was proposed by Hemker et al. in 1965 (6) the quasi-linearity of a log-C log t plot is based on the coincidence that most reagents are non-ideal, i.e. they contain traces of the coagulation factor to be tested. In the case of the newly prepared prothrombin reagent, which was not believed to contain even trace amounts of prothrombin, the relation between coagulation time and prothrombin concentration could be studied directly. The results obtained seem to support the hypothesis that a linear relationship exists between the coagulation time and the inverse of the prothrombin concentration (7,20). In a similar way as was described for the bovine system, a reagent was prepared from normal human pooled plasma, which had the same characteristics as the bovine reagent.

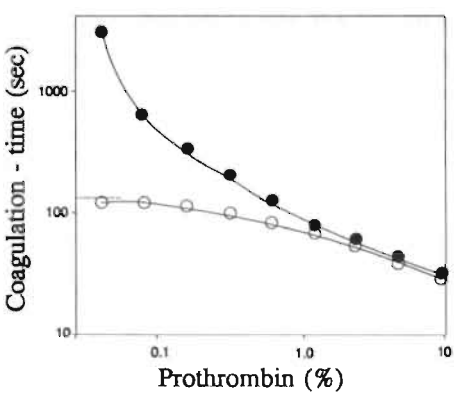

FIG. 3. Double-log plot of the reference curves for bovine factor II in the coagulation assay.

●–● *reagent B*

○–○ *reagent B to which 5. 10^{-3} U/ ml of prothrombin has been added*

----- *buffer time of reagent B containing 5. 10^{-3} U/ ml of prothrombin*

The bovine factor X reagent

Separation of prothrombin and the coagulation factors VII and X on DEAE Sephadex, resulted in the elution of prothrombin and factor VII at about 200 mM NaCl and factor X at 350 mM NaCl (fig. 4a). Nevertheless, the prothrombin/factor VII concentrate (pooled as indicated) was not free from factor X activity. This became clear after the concentrate had been added to $BaSO_4/Al(OH)_3$-adsorbed plasma. The resulting factor X reagent showed a

buffer time of 121 seconds, indicating that still 8.10^{-4} U/ml factor X were present in the reagent. Because the addition of small amounts of antifactor X resulted in very long buffer times, it seemed clear that the buffer time of the reagent was caused by contaminating factor X. Therefore the prothrombin/factor VII concentrate was rechromatographed on DEAE Sephadex which resulted in the separation of a small amount of factor X from prothrombin and factor VII (fig. 4b).

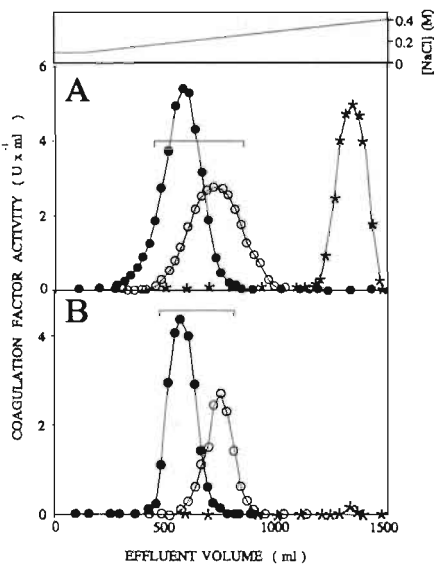

FIG. 4. Separation of factors II (●–●), VII (O–O) and X (★ –★) on DEAE Sephadex

A: The $BaSO_4$-eluate was adsorbed batchwise to DEAE Sephadex, applied to a column which was eluted with a linear salt gradient. Fractions containing factors II and VII were pooled as indicated and dialysed against buffer C.

B: The dialysed factor II-VII preparation was applied to a DEAE Sephadex column and chromatographed at similar conditions as described in fig. 5A. Fractions containing factors II and VII were pooled as indicated and dialysed against 0.15 M NaCl. For further details, see text

If the prothrombin/factor VII concentrate thus obtained was used for the preparation of a factor X reagent, the buffer time was prolonged further (210 seconds) but still factor X (4.10^{-4} U/ml) was present in our coagulation system. In principle the contaminating factor X might be present either in the prothrombin/factor VII concentrate, in the $BaSO_4/Al(OH)_3$-adsorbed plasma, or in the thromboplastin used. To find the origin of the residual factor X, each of these components was filtered separately through columns containing Sepharose-bound antifactor X. Factor X reagents were constituted and the buffer times of combinations of antifactor X-treated and non-treated components were compared (table I). It is clear that factor X is present in all three components, but mainly

in $BaSO_4/Al(OH)_3$-adsorbed plasma and thromboplastin. Therefore, the factor X reagent and the thromboplastin were separately filtered through antifactor X-Sepharose columns immediately before use and a t-D plot was constructed of a number of plasma dilutions. As is shown in fig. 5, a straight line was obtained. The interception with the abscissa (D=0) represents the minimal clotting time and has the same value as that obtained with a prothrombin reagent (22 seconds). However, the slope of the factor X dilution curve was found to be ten times smaller than that of the prothrombin curve. This explains why a small amount of factor X will induce a rather short coagulation time, whereas the same amount of prothrombin will fall below the detection limit.

Components treated with antifactor-X Sepharose	Components non-treated with antifactor-X Sepharose	Buffer time (s)	Factor X left (U/ml)
none	A B C	210	4.10^{-4}
A	B C	232	3.10^{-4}
B	A C	411	1.10^{-4}
C	A B	339	2.10^{-4}
A B	C	241	3.10^{-4}
A B C	none	> 3600	$< 2.10^{-5}$

Table I. The buffer time of various factor X reagents. *Either factor II,VII concentrate (A), or $BaSO_4/Al(OH)_3$-adsorbed plasma (B), or thromboplastin (C) were filtered through antifactor X-Sepharose columns. Factor X reagents were prepared from treated or non-treated components (A and B) and used with thromboplastin in the coagulation assay*

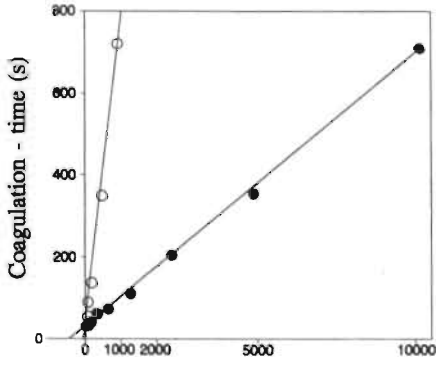

FIG. 5. t-D plots of factor X (●–●) and prothrombin (O–O).
Normal pooled plasma was diluted with buffer (28.6 mM sodium acetate, 28.6 mM sodium barbiturate, 116 mM NaCl, pH 7.4) and tested with factor X reagent and prothrombin reagent

Discussion

With the aid of Echis carinatus venom a prothrombin reagent was prepared which did not contain any detectable prothrombin. The method differs from that described by Koller et al. (9) in the serum preparation procedure. In the classical method, whole blood is collected in thromboplastin, and at least all factors of the extrinsic pathway are activated before prothrombin is converted into thrombin. We were never able to activate prothrombin completely and the best reagent which was obtained contained 5.10^{-3} U/ml of prothrombin.

In this paper a method is described to convert all prothrombin enzymatically into thrombin with only a moderate activation of the other factors. The method is fit for the bovine as well as for the human system. Reasons why these reagents are better than those prepared according to the classical procedures, are:

1. The classical reagents contain at least 5.10^{-3} U/ml (and usually 10^{-2} - 2.10^{-2}) of prothrombin whereas the bovine reagent and the human reagent contained 0 and 10^{-4} U/ml of prothrombin, respectively.
2. The factors I, V, VII and X may be added to the reagents in higher concentrations as compared to the classical ones. It should be noticed however, that an excess of factors VII and X should be added because these factors are partly lost during freezing of the reagent.
3. Since t-D plots of the reference curves are rectilinear, at least between all prothrombin concentrations measured (10^{-1} - 4.10^{-4} U/ml), the position of the reference curves may be determined more accurately.

The hypothesis was proven to be correct in three cases: the one-stage determination of human and bovine prothrombin and that of bovine factor X. Secondly, also a factor X reagent that was prepared completely devoid of factor X. The fact that it was nearly impossible to reduce the buffer time by conventional means raised the question whether a factor X-independent pathway exists in coagulation. To exclude this possibility trace amounts of factor X were rigorously removed from the factor X reagent as well as from thromboplastin. Upon recalcification, no signs of clot formation were observed in mixtures of the factor X reagent and thromboplastin thus prepared. It was used to establish the relationship between coagulation time and factor X concentration. It turned out that, as a consequence of the ten times smaller slope of the factor X dilution curve compared to that of prothrombin, small amounts of factor X will induce a

rather short coagulation time, whereas the same amount of prothrombin rapidly falls below the detection limit. For the same reason small contaminations of the coagulation factor to be tested in the reagent or in thromboplastin will cause considerable deviations in the factor X curve and none in the prothrombin curve. These findings have been confirmed in systems containing purified coagulation factors and phospholipid vesicles composed of phosphatidylcholine and phosphatidylserine: in these systems it was found that at plasma concentrations of prothrombin and factor X (1.4 and 0.17 µmol/ l), dissociation constants for the protein-membrane interactions were 1.04 µmol/l for prothrombin an 0.19 µmol/l for factor X respectively (19-23).

The reference curves presented in this paper strongly support the hypothesis that a linear relationship exists between the coagulation time and the inverse concentration of the coagulation factor and that the sigmoid form of the usual reference curves is caused by the presence in the reagent of a residual amount of the coagulation factor to be tested (20, 6, 7).

The separation of bovine prothrombin and descarboxyprothrombin by high performance liquid chromatography

Introduction

Vitamin K is required as a coenzyme for the post-translational carboxylation of certain glutamic acid residues in proteins (1). The first protein found to be vitamin K-dependent was prothrombin (24), and it could be demonstrated that in the absence of vitamin K or in the presence of vitamin K antagonists (warfarin, dicoumarol, etc.) descarboxyprothrombin is set free in the blood (25). Since each γ-carboxy-glumatic acid (Gla) residue contains an additional negative charge, it might be expected that normal prothrombin (containing 10 Gla residues) can be readily separated from its descarboxy form. It turned out, however, that in the absence of divalent metal ions the electrophoretic mobility of both molecules are closely similar. Only in the presence of Ca^{2+}, prothrombin (which strongly binds Ca^{2+}) is retarded whereas descarboxyprothrombin (which has a low affinity for Ca^{2+}) is not (26-28). Therefore, the detection of descarboxyprothrombin requires a two-dimensional electrophoresis technique, during which Ca^{2+} is present in the first run and antibodies against prothrombin are present in the second dimension (26). With this technique the analysis of a plasma sample usually takes 24 h, which in many cases may be considered as too long. Moreover, it is a qualitative and not a quantitative method to detect descarboxyprothrombin. The concentration of descarboxyprothrombin is generally estimated from the difference between the concentration of prothrombin plus descarboxy-prothrombin and that of prothrombin alone. The total amount of prothrombin-like material is measured by converting it to thrombin with Echis carinatus venom (13). Prothrombin alone is quantified with a coagulation assay (29). Obviously this is an indirect test for descarboxyprothrombin and since plasma contains a number of proteins (antithrombin III, descarboxyfactor X, etc.) which are known to inhibit these coagulation assays, the accuracy of this method is low.

HPLC analysis

Samples (100 µl) were analyzed with an LKB 2150 liquid chromatograph on a Pharmacia Mono Q anion-exchange column. After the samples had been applied to the column the adsorbed proteins were eluted with a linear gradient from 0.1 to 0.5 M NaCl in 20 mM Tris-HCl, pH 7.4. The effluent was recorded at 280 nm with an LKB 2158 Uvicord SD and fractions were collected with the aid of the LKB 2211 SuperRac. The flow rate was 1 ml/min in all cases.

Results and discussion

For more than 15 years electrophoresis in the presence of Ca^{2+} has been the only analytical technique by which prothrombin and descarboxyprothrombin could be separated. Why Ca^{2+} is required for this separation has always remained obscure. Bivalent metal ions are unwanted in many cases because they induce a rapid activation of the clotting factors.

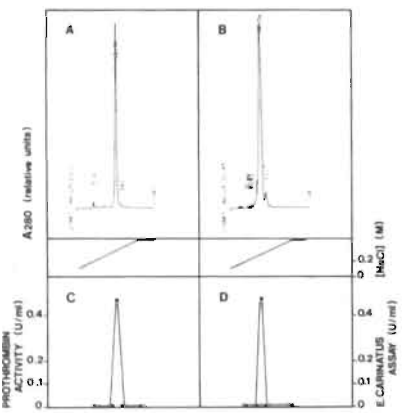

FIG. 6. Elution of purified proteins. *Prothrombin (A, 1.5 U) and descarboxyprothrombin (B, 1.5 U) were adsorbed to the Mono Q anion-exchange column and eluted with a linear salt gradient. The peak fractions were collected and tested with the Echis carinatus assay (closed squares) and with the one-stage prothrombin assay (open squares). C, clotting activity of the fractions obtained in A; D, clotting activity of the fractions obtained in B. Further details are described in the text*

We have tried, therefore, to separate the two proteins using a different technique: high performance liquid chromatography (HPLC). In a first set of experiments we measured the elution times of purified prothrombin (Fig. 6A) and

descarboxyprothrombin (Fig. 6B). Prothrombin was eluted after 12.79 min (0.35 M NaCl) and descarboxyprothrombin after 10.56 min (0.29 M NaCl). The peaks were collected and analyzed for prothrombin activity with the one-stage coagulation assay and with the Echis carinatus assay (Fig. 6C and D). The recovery was 96% for prothrombin and 98% for descarboxyprothrombin. When the two preparations were mixed, the elution time of each component remained unchanged (Figs. 7A and C) and the recovery was 98% for both proteins. Finally, we injected 100-µl samples of full plasma from a warfarin-treated cow. Before injection this plasma contained 0.2 U/ml of prothrombin and 0.7 U/ml of descarboxy-prothrombin. Fractions of 0.5 ml were collected and analyzed as before (Figs. 7B and D). Again the elution times of prothrombin and descarboxyprothrombin remained unchanged and the recovery of these proteins was 95 and 98%, respectively.

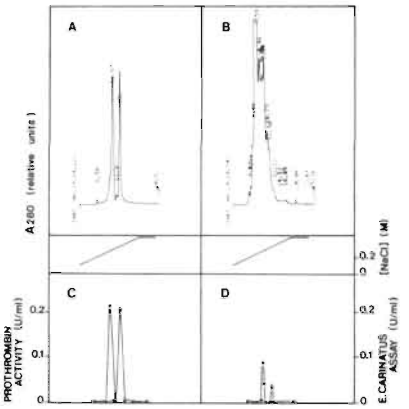

FIG. 7. Elution profile of protein mixtures. *A and C, purified prothrombin and descarboxyprothrombin were mixed in a 1:1 molar ratio and analyzed as described in Fig. 7. B and D, analysis of plasma from a warfarin-treated cow. The starting plasma contained 0.2 U/ml of prothrombin in the one-stage clotting assay and 0.9 U/ml in the Echis carinatus assay. The various tests are represented by the same symbols as in fig. 6*

These experiments demonstrate that prothrombin and descarboxyprothrombin can be separated in the absence of Ca^{2+}. The two proteins could be recovered at nearly 100% from purified mixtures, but also from full plasma which was applied directly to the column. Therefore, this technique might be a helpful tool for the screening of patients under anticoagulant therapy. When used for this purpose, some minor adaptations (such as delipidation of the plasma) might be required. In order to prevent obstruction of the column it may also be advisable to dilute the plasma samples before use.

Acknowledgments

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CHAPTER 3

Efficiency of the vitamin K-dependent carboxylation reaction

Based on:

- De Metz, M., Soute, B.A.M., Hemker, H.C., and Vermeer, C. (1983). Stimulation of the vitamin K-dependent carboxylase from bovine liver. Biochem. J. 209, 719-724.
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Introduction

During the last 15 years many attempts have been made to purify vitamin K-dependent carboxylase. The enzyme is located in the RER (1), and behaves as a typical integral membrane protein, which probably is the reason why the purification proved to be a difficult job. Although substantial amounts of work were directed towards the understanding of its mechanism of action, the lack of purified carboxylase preparations has often been an impediment to draw definite conclusions. It was only recently that the enzyme has been purified to near homogeneity (2,3) and that it even has been cloned (4). The strategies for purification that have been employed during these years are summarized below.

a. Canfield et al.(5) developed a method with which they obtained a 150-fold increase of specific carboxylase activity. The relatively high purification factor was mainly due to the removal of an inhibitor in the microsomal proteins in a rat system. The preparation thus obtained did not contain reductase activity.
b. With ion exchange and size exclusion column chromatography, using CHAPS as a detergent to solubilize the microsomes, Girardot described a method in which a 400-fold purification was reported (6). The preparation had a M_r less than 350,000 Da, still contained an endogenous substrate for carboxylase, but reductase activity was lost during purification.
c. With the aid of sepharose-bound antibodies against normal factor X, enzyme-substrate complexes had been obtained, extracted from detergent-solubilized microsomes (7). The procedure is based on the use of livers from warfarin-treated animals. Microsomes thus obtained contain substantial amounts of non-carboxylated precursors of blood coagulation factors (mainly factor X), which accumulate during warfarin treatment, and at least part of which are firmly complexed to carboxylase. With this method a 100-fold purification could be obtained. The resulting product remained strongly bound to the sepharose beads and was therefore called "solid-phase carboxylase". Attempts to elute the carboxylase from the sepharose in the bovine system have not been successful, but Harbeck et al. (8) reported a method in a rat system in which they were able to elute the carboxylase from an anti-prothrombin antibody column with a synthetic propeptide. In this case the purification achieved was 500-fold.

d. Salt-washed microsomes from normal cow liver were used to obtain partly purified carboxylase (9). This was accomplished by differential detergent extraction followed by $(NH_4)_2SO_4$ fractionation and gelpermeation chromatography. By this procedure the enzymatic activity, expressed per mg of protein, was increased up to 100 fold. The final product resulted in a tight complex of at least 7 proteins and required very low detergent concentrations for maximal activity. Sepharose-bound polyclonal antibodies raised against the partly purified carboxylase have been used to prepare a solid-phase carboxylase. By incubating the sepharose-bound antibodies with detergent-solubilized microsomes from normal bovine liver, a substrate-free solid-phase enzyme system could be obtained. This complex is different from that mentioned under c) in its ability to carboxylate not only the pentapeptide FLEEL but also large exogenous protein substrates like decarboxylated prothrombin and factor X.

e. The propeptide consensus sequence preceding the amino terminus of the vitamin K-dependent proteins, suggested by Pan and Price to be a recognition site for carboxylase (10), was used for the development of an affinity procedure leading to the purification of the vitamin K-dependent carboxylase to near homogeneity (2) and even to homogeneity (3). By the first procedure a 7000-fold purification of carboxylase was obtained with an apparent M_r found by reducing SDS page gel electrophoresis of 94,000 Da. The second procedure resulted in a purification of 350,000 fold and an apparent M_r of 98,000 Da.

Although detailed mechanistic studies and physical characterization of the enzyme can only be reliably performed with the purified enzyme, numerous studies with the crude and partly purified carboxylase have yielded important information about its properties and mode of action. Actually it turned out that part of this knowledge was even essential for the successful strategy leading to the purification of vitamin K-dependent carboxylase to homogeneity. Most of the work presented in this thesis was performed with non-purified material, notably with carboxylase preparations mentioned under c) and d). In this chapter some detailed studies will be discussed in which attempts were made to improve the specific activity of the enzyme, either by removing contaminating proteins or by increasing the rate of the carboxylation events.

Stimulation of the vitamin K-dependent carboxylase from bovine liver by organic solvents

Research on the vitamin K-dependent carboxylation have mostly been performed with liver microsomal fractions of anticoagulated or vitamin K-deficient rats. In our group, however, vitamin K-dependent carboxylase from both non-treated and anticoagulated cows has been used for most studies (11). The enzyme is mainly found in the microsomal fraction from which it is readily solubilized with the aid of detergents. With the livers of anticoagulated cows substantial purification had been achieved by immunoadsorption of solubilized microsomes with sepharose-bound antibodies against blood coagulation factor X. This immune response was possible since precursors of coagulation factors (mainly factor X) had accumulated in the endoplasmic reticulum during anticoagulation, where they had formed tight complexes with the vitamin K-dependent carboxylase (7).

Addition	$^{14}CO_2$ incorporation (% of control)	Formation of vitamin K epoxide (% of control)
None	100	100
DMSO (20%, v/v)	490	500
Acetone (10%, v/v)	440	350
Acetonitrile (5%, v/v)	160	150

Table I. Stimulation of the vitamin K-dependent carboxylation and epoxidation in solid-phase carboxylase. *The vitamin K-dependent incorporation of $^{14}CO_2$ and the formation of vitamin K epoxide were measured in standard reaction mixtures (1 ml), containing 100 μM-warfarin. After incubation for 120 min. at 25 °C, a 0.4 ml sample was used for determining the amount of incorporated CO_2 and 0.4 ml for assessing the amount of epoxide. The control values (without addition) were 260,000 d.p.m. of incorporated $^{14}CO_2$ per mg of protein and 96 nmol of vitamin K epoxide per mg of protein*

The immuno-affinity procedure resulted in a 100-fold purification of the enzyme, which was designated as 'solid-phase carboxylase'. The properties of solid-phase carboxylase are comparable to those of a non-purified solubilized microsomal preparation of bovine or rat liver (12-14). In our attempts to further purify

carboxylase solid-phase carboxylase has been used as a starting material. A wide variety of solvents, such as buffers with high or low pH, chaotropic reagents, high ionic strength and most common detergents has been tested. To increase hydrophobicity of the eluents, mixtures of low salt buffers and various organic solvents have been used. Unfortunately none of these methods was successful in eluting carboxylase from the solid-phase without irreversibly losing biological activity. As an unexpected side-effect, however, we observed that during our efforts to resolubilize the carboxylase some organic solvents stimulated the vitamin K-dependent carboxylation without affecting the K_M^{app} for the various reaction components. The addition of dimethylsulfoxide (DMSO) and acetone to the reaction mixture enhanced not only the vitamin K-dependent carboxylation but also the epoxidation (Table I).

Kinetic analysis demonstrated that the stimulation was the result of a substantial increase of the apparent V_{max}^{app} of the carboxylase reaction. Notably ketones (acetone) and DMSO were powerful stimulators of solid-phase carboxylase activity. A time course of the reaction with and without either DMSO or acetone is shown in fig.1.

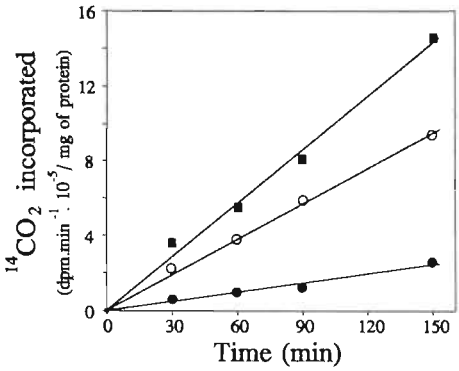

FIG. 1. Time course of the carboxylation reaction.

The incorporation of $^{14}CO_2$ into the pentapeptide FLEEL was measured with solid-phase carboxylase after various incubation times at 25 °C without any addition (●), in the presence of acetone (10%, v/v) (○) and in the presence of DMSO (20%, v/v) (■)

It was demonstrated that the stimulatory effect of organic solvents could be obtained with all vitamin K_1 intermediates and that it was independent of the physical state of the phospholipid. At lower temperatures it was found that the stimulation increased. In detergent solubilized microsomes the influence of organic solvents was far less pronounced and possibly for that reason only

detectable at low reaction temperatures (5 °C), where also in the solid-phase system highest proportional stimulation was reached. The mechanism by which organic solvents stimulate vitamin K-dependent carboxylase has never been understood. It is known from the rat and bovine microsomal system that reducing agents, pyridoxal phosphate and bivalent metal ions (especially Mn^{2+}) stimulated vitamin K-dependent carboxylation (15-17). This is the first time that a stimulation by organic solvents of the vitamin K-dependent carboxylation and epoxidation reaction is reported. The stimulating solvents were all miscible or partly miscible with water and were poor solvents for phospholipids. Solvents like alcohols, which do solubilize phospholipids, may inhibit the carboxylation reaction, because phospholipids are essential for enzyme activity (14). Although the stimulating agents were miscible with water, experiments with ketones having different chain length indicated that hydrophobicity seems to be relevant for their action. With solid-phase carboxylase the stimulation by organic solvents was highest at low temperatures, in detergent solubilized microsomal preparations stimulation was only observed at low temperatures. It is possible that in a non-purified preparation the proteins and lipids surrounding the carboxylase complex interact with the organic solvent and hence suppress the stimulating effect on the carboxylase activity. On the other hand, the difference between solid-phase carboxylase and a non-purified preparation might reflect a difference in the state of the enzyme complex itself, i.e. the activity of solid-phase carboxylase might be suboptimal. However, since most experimental evidence is lacking until now, suggestions that organic solvents improve the exchange of vitamin K between its solvent micelles and carboxylase, or increase the mobility of vitamin K during its recycling remain speculative.

The preparation of partly purified carboxylase

After it had turned out that further purification steps with solid-phase carboxylase were not successful, we returned to the detergent-solubilized microsomes, from which we have tried to isolate protein fractions enriched in carboxylase activity. The procedure comprises of the extraction of salt-washed microsomes with 0.5% CHAPS, the solubilization of carboxylase from the membranes with 1% CHAPS + 1.5 M $(NH_4)_2SO_4$ and subsequent fractionation by $(NH_4)_2SO_4$

precipitation. The recovery of enzymatic activity after the various steps is summarized in table II. The final $(NH_4)_2SO_4$ precipitation step resulted in a 70-fold purification of carboxylase as compared with the salt-washed microsomes. However, this figure is probably an overestimation of the real purification, because in the $(NH_4)_2SO_4$ precipitation step an inhibitor of carboxylase activity (probably haemoglobin) is removed. This phenomenon has also been described to occur during the partial purification of rat liver carboxylase (18).

Step		Protein	carboxylase	recovery	purification
		(mg)	(dpm.10^{-3}/mg)	(%)	(fold)
1.	Washed microsomes	3718	9.5	100	1
2.	CHAPS				
	supernatant (discard)	1871	4.7	25	
	pellet	1320	46.2	175	4.9
3.	CHAPS (1%, w/v) + $(NH_4)_2SO_4$ (25% sat.)				
	pellet (discard)	929	6.3	17	
	supernatant (discard)	357	108	109	11.4
4.	$(NH_4)_2SO_4$ (50% sat.)				
	supernatant (discard)	280	2.5	2	
	pellet	42	682	81	71
5.	Ultrogel A4	9	1756	45	185

Table II. Partial purification of hepatic bovine carboxylase. *Carboxylase activity is quantified as the amount of $^{14}CO_2$ incorporated into FLEEL per mg of microsomal protein in 20 min under standard conditions. Salt-washed microsomes had been obtained from normal cow liver. The data given in this table are averages of duplicated analyses of a single, but representative experiment*

The procedure developed for the partial purification of hepatic carboxylase is equally well applicable for the enzyme from normal as well as from warfarin-treated animals. Whereas microsomes from normal livers mainly contain substrate-free carboxylase, a substrate-bound enzyme complex was obtained from warfarin-treated animals. In the latter case the yields were slightly better, probably because of a stabilisation of carboxylase by the endogenous substrate during the purification procedure. The preparation obtained after $(NH_4)_2SO_4$ precipitation has been used for most of our experiments until now, and has been

designated as "partly purified carboxylase".

Further purification could be obtained if the $(NH_4)_2SO_4$ step was followed by a size exclusion chromatography column (Ultrogel A4). Carboxylase activity was found in a fraction of which the apparent mass was estimated to be 670,000 Da (fig. 2). Analysis on polyacrylamide gels in SDS showed that the preparation still contained at least 7 proteins, ranging from 50,000-100,000 Da.

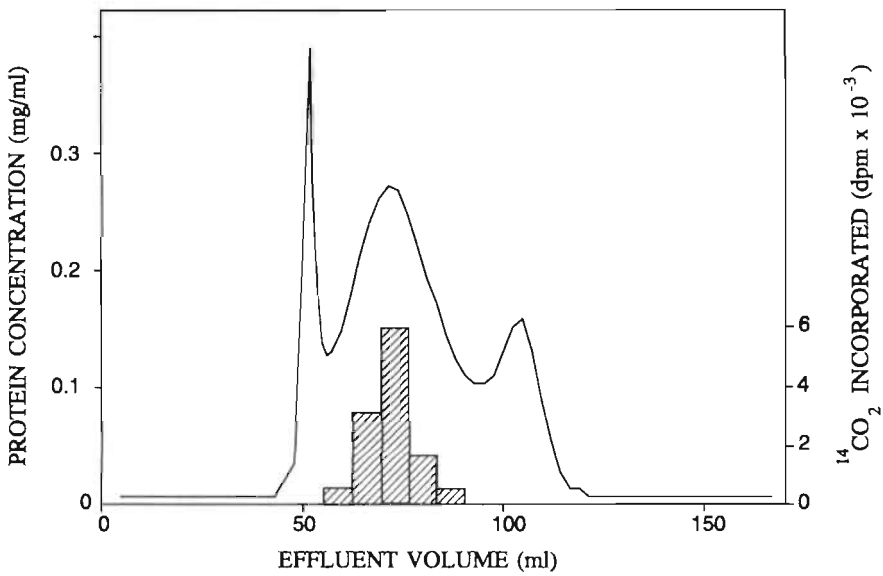

FIG. 2. Fractionation of carboxylase complex on Ultrogel A4. *The protein concentration in the effluent (solid line) was monitored continuously as the optical density at 280 nm and the protein concentration in the various fractions was verified by the method of Sedmak and Grossberg (20). Reaction mixtures contained 60 µl of carboxylase. Vitamin KH_2 was used as a co-enzyme*

Stimulation of carboxylase by $(NH_4)_2SO_4$

In $(NH_4)_2SO_4$ precipitation experiments it was found that high concentrations of $(NH_4)_2SO_4$ may have a strong stimulating effect on the in vitro carboxylase activity of bovine liver microsomes. However, the effect remained restricted to

small peptide substrates lacking the pro-sequence, whereas no effect was measurable on the carboxylation of pro-PT28, a synthetic peptide described by Ulrich et al. (20) and endogenous precursor proteins (21) (table III). Kinetic analysis demonstrated that the effect of ammonium sulphate was mainly the result of an increase of the V_{max}^{app}, whereas the K_M^{app} for a number of substrates was not affected (fig. 3).

Substrate	Concentration (mM)	CO_2 incorporated (mmol/mol of substrate)	
		+ $(NH_4)_2SO_4$	- $(NH_4)_2SO_4$
FLEEL	4	0.9	0.07
pro-PT28	4.10^{-3}	17.9	16.70
d-osteocalcin	4.10^{-3}	55.8	5.00
TPT	4	0.8	0.06

Table III. Carboxylation of various substrates. *All reaction mixtures were incubated for 30 min at 20 °C under standard conditions. Pro-Pt28 is a synthetic peptide consistent with the amino acid residues -18 to +10 in human prothrombin in which the residues 6 and 7 are Glu instead of Gla; TPT is the synthetic tripeptide Boc-Glu-Glu-Val*

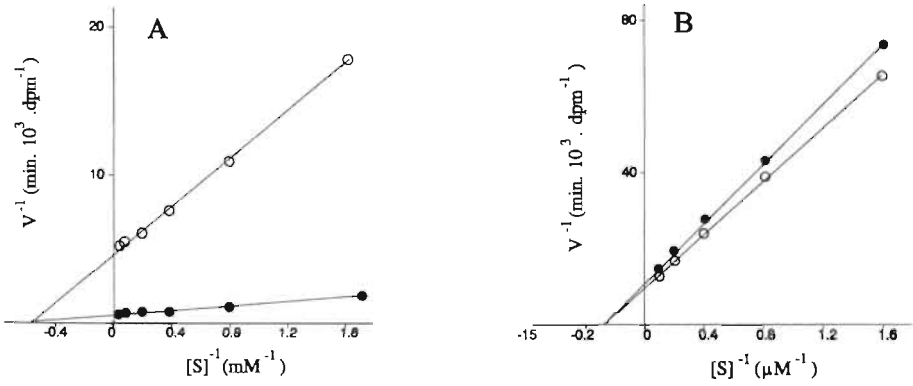

FIG. 3. Lineweaver-Burk diagrams of substrate carboxylation. *Panel A: carboxylation of TPT. Panel B: carboxylation of pro-PT28. Apparent K_M values as calculated from the intersection points were 3 mM for TPT and 6 µM for pro-PT28. Symbols: ●–●, in the presence of ammonium sulphate; ○–○, in the absence of ammonium sulphate*

If the pro-fragment was added as a peptide not covalently bound to a carboxylatable substrate, the carboxylation thereof was only slightly affected and ammonium sulphate remained active as a stimulator of carboxylase activity. It was also demonstrated that in order to substantially decrease the K_M^{app} for carboxylase, the propeptide has to be covalently linked to a peptide substrate (table IV). Therefore the effect of $(NH_4)_2SO_4$ seems to be dependent solely on the absence of the pro-sequence. It has been demonstrated by observations from Knobloch and Suttie (22) and from Ulrich et al. (20) that in the presence of the pro-sequence the efficiency of carboxylation of peptide substrates is considerably improved especially when covalently bound to the peptide substrates, and that this improvement is due to a decrease of the K_M^{app} rather than an increase of the V_{max}^{app}. It has been suggested on several occasions, that the pro-sequence and $(NH_4)_2SO_4$ would have similar effects on carboxylase, namely a conformational change by which Glu-containing substrates would be accommodated better (or quicker) on the active site of the enzyme. Our kinetic data are not consistent with this suggestion: the fact that $(NH_4)_2SO_4$ strongly increased the V_{max}^{app}, whereas the pro-sequence remained restricted to a decrease of the K_M^{app} indicates that these stimulators must work via different mechanisms.

Second peptide or protein present	Kinetic constants for FLEEL carboxylation			
	K_m^{app} (mM)		V_{max}^{app} (nmol/ml)	
	- $(NH_4)_2SO_4$	+ $(NH_4)_2SO_4$	- $(NH_4)_2SO_4$	+$(NH_4)_2SO_4$
None	5.5	4.6	0.12	0.77
Pro-Pt28 (40 µM)	6.1	4.5	0.26	0.82
Propeptide (40 µM)	4.7	4.6	0.31	0.94
Endogenous substrate	3.2	3.1	0.10	0.57

Table IV. Apparent kinetic constants for FLEEL carboxylation in the presence of a second peptide or protein. *Standard reaction mixtures contained constant amounts of either pro-PT28 or propeptide (if added) and varying concentrations of FLEEL. Carboxylated pro-PT28 remained bound to the microsomal proteins after trichloroacetic acid precipitation and was separated from carboxylated FLEEL by centrifugation (5 min, 3000 x g). For comparison, part of the experiment was repeated with microsomes from warfarin-treated animals (last line). In the latter case no pro-containing peptide was added and the reaction mixtures contained the same amount of microsomal proteins as those in which we used normal microsomes*

Improved efficiency by minimized escape of CO_2

In the preceding paragraphs we have seen two classes of compounds capable of increasing the efficiency of the carboxylation reaction: organic solvents (mainly affecting solid-phase carboxylase) and $(NH_4)_2SO_4$ (mainly affecting detergent-solubilized carboxylase). During our studies it turned out, however, that a third factor negatively affected the efficiency of the carboxylation reaction: the escape of $^{14}CO_2$ from our reaction mixtures. Moreover we introduced a procedure by which the incorporated $^{14}CO_2$ may be expressed as nanomoles CO_2 bound, which enabled us to calculate the efficiency of the carboxylation reaction for each substrate used. The procedures were published in a recent paper, which also describes the carboxylating enzyme system as it is presently used in our lab. For that reason it is printed integrally at the end of this chapter.

Conclusions

Our attempts to purify γ-glutamylcarboxylase using classical purification techniques have lead to two forms of partially purified carboxylase, one is bound to a solid-phase, the other one is soluble. Although we did not succeed in purifying the enzyme to homogeneity, these preparations have been helpful in optimizing the conditions for carboxylase activity. This was accomplished with a number of compounds of which $(NH_4)_2SO_4$ is still frequently used in our lab and elsewhere. We have initiated the development of carboxylase in the bovine system and have also contributed to the development and testing of affinity ligands, such as the peptide homologous to the propeptide and a 59-residue recombinant peptide homologous to the propeptide sequence and Gla-domain of human factor IX. This is described in more detail in chapter 6. With these affinity ligands the purification of carboxylase to homogeneity has finally been accomplished by Wu and Stafford (2) and by Berkner and Suttie (23).

Vitamin K-dependent carboxylase : minimized escape of CO_2 from solution may prolong linearity of the reaction rate

Berry A. M. Soute, Ron Budé, Herman Buitenhuis and Cees Vermeer

Abstract

Escape of $^{14}CO_2$ from the reaction mixture into the gas phase may seriously affect the accuracy of in vitro measurement of vitamin K-dependent carboxylase activity (and probably that of other carboxylases as well). In this paper we describe the effect of a) the volume of the test tubes in which the reaction is performed, b) the addition of an excess of $NaH^{14}CO_3$ in parallel with standard amounts of $NaH^{14}CO_3$, and c) the incubation temperature. In this way optimal conditions are defined and used for the carboxylation of various peptide and protein substrates. It is shown that both a presequence and an internal recognition site contribute to the effective recognition of a substrate by carboxylase. The maximal efficiency of carboxylation was 1-2% with substrates lacking both signals and 20-50% if only one was present. This indicates the need for developing peptide substrates containing both recognition signals for vitamin K-dependent carboxylase.

Introduction

It is well known that CO_2 (and not HCO_3^-) is the active component in the vitamin K-dependent carboxylation of peptide-bound glutamate into γ-carboxyglutamate residues (24). To measure the carboxylation reaction in vitro, $NaH^{14}CO_3$ is added to the reaction mixture, so that radiolabeled products are formed (25). The optimal pH for the reaction was found between 7.2 and 7.4 (15,26) and at this pH the equilibrium exists

$$2H^+ + {}^{14}CO_3^{2-} \rightleftarrows H^+ + H^{14}CO_3^- \rightleftarrows H_2O + {}^{14}CO_{2\,aq} \qquad [i]$$

Provided that the reaction is performed in closed tubes, the $^{14}CO_2$ will be distributed between the solution and the gas phase on top of it according to

$$^{14}CO_{2\,aq} \rightleftarrows {}^{14}CO_{2\,gas} \qquad \text{[ii]}$$

The distribution of $^{14}CO_2$ over the liquid and the gas phase is predicted by Henry's Law, which can be expressed as

$$\frac{n^{14}CO_{2\,gas}}{n^{14}CO_{2\,aq}} = k.V_{gas}\,, \qquad \text{[iii]}$$

where *n* is the number of moles, V_{gas} represents the volume of the gas phase, and *k* is a constant, which is still dependent on the ionic strength and temperature. The gas phase does not take part in de carboxylation reaction, which means that escape of $^{14}CO_2$ from the reaction mixtures will decrease the effective concentration of $^{14}CO_2$. Similar equilibria exist for the atmospheric $^{12}CO_2$, but these have been established in the buffers long before the start of the carboxylation reaction. Therefore the $^{12}CO_2$ concentration in the buffers (1-2 mM) may be regarded as constant. $NaH^{14}CO_3$, however, is added to the mixtures shortly before or in parallel with vitamin K, with which the in vitro carboxylation is initiated. Any loss of $^{14}CO_2$ will decrease the ratio between the concentration of $^{14}CO_2$ and that of $^{12}CO_2$ and hence decrease the apparent carboxylation rate. To properly interpret the results obtained in the in vitro carboxylation reaction, it is important that the ratio between $^{14}CO_{2\,aq}$ and $^{12}CO_{2\,aq}$ remains constant throughout the reaction. Second, the loss of $^{14}CO_2$ from solution must be minimized, because it will decrease the sensitivity of the carboxylase assay. Taking into account these considerations we have worked out the optimal conditions for vitamin K-dependent carboxylase and we show how this system may be applied for the carboxylation of a number of peptide substrates.

Materials and methods

Chemicals. Dithiothreitol (DTT) and 3([3-cholamidopropyl]dimethylammonio)-1-propane sulphonate (CHAPS) were purchased from Sigma (St. Louis, MO) and $NaH^{14}CO_3$ (56 Ci/mol) was from the Radiochemical Centre (Amersham, UK). Vitamin K_1 (Konakion®) was obtained from Hoffmann-LaRoche (Basel, Switzerland) and reduced to the hydroquinone form (KH_2) by incubating 6 mM vitamin K quinone in the presence of 150 mM DTT at pH 8.5 and 37 °C overnight in a light-protected tube. All other chemicals were of analytical grade or better.

Peptides and proteins. The pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) was from Vega Biochemical Co (Tucson, AZ) and pro-PT28 (consisting of the amino acid residues -18 to +10 of bovine descarboxyprothrombin) was prepared as described by Ulrich et al (20) and was kindly supplied to us by Dr. B.Furie (Tufts University, Boston). Decarboxylated osteocalcin (d-osteocalcin) was prepared and purified from bovine bones as in (27) and t-Boc-Glu-Glu-Val was a kind gift from Drs. F.Azerad and F.Acher (Université R.Descartes, Paris, France). Salt-washed microsomes were prepared from normal cow liver as described earlier (26).

Carboxylase assay. Standard reaction mixtures (0.125 ml) contained 2 mg of microsomal proteins, 0.4 % (w/v) CHAPS, 0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), 1 M $(NH_4)_2SO_4$, 6 mM DTT, 0.1 mM KH_2, 1.5 µCi $NaH^{14}CO_3$ and 4 mM FLEEL. Incubation periods and temperatures are indicated in the text. Incubations were stopped by adding 75 µl 1 M NaOH to the reaction mixtures. At the various time points two samples were taken from each tube: 10 µl was transferred into 100 µl Tris-HCl (pH 10.2) and diluted 100-fold with distilled water and 10 µl of this solution was counted as such. The other sample (150 µl) was transferred into a vial containing 1 ml of 5% (w/v) trichloroacetic acid. Trace amounts of nonbound label were removed by shortly heating the sample and peptide- or protein-bound $^{14}CO_2$ was quantitated in a Beckman LS 1301 liquid scintillation counter. Formula-989 (Dupont de Nemours, Dreieich, FRG) was used as a scintillation liquid for all samples. All data are given as the means of triplicate experiments and blank values (less than 200 dpm) are subtracted throughout this paper.

Results

As can be seen from Eq. [iii] the loss of $^{14}CO_2$ into the gas phase may be decreased by reducing either V_{gas} or k. The limits to which V_{gas} may be reduced are set by practice, and therefore we have investigated the extent to which the efficiency of the $^{14}CO_2$ incorporation could be improved by a reduction of the tube volume. Standard reaction mixtures (0.125 ml) were pipetted in tubes of varying capacity (7, 1.5 and 0.15 ml respectively) and subsequently incubated at 25 °C. The disappearance of $^{14}CO_2$ from solution is shown in figure 1A. In the large, wide vessels an equilibrium between $^{14}CO_{2\ aq}$ and $^{14}CO_{2\ gas}$ was reached after about 40 min and at that stage not more than 40% of the original label was left in solution. In the midsize tubes (which were also narrower) the equilibrium state was reached after about 1 h. The final concentration of $^{14}CO_{2\ aq}$ was 55% of the original value. In the small tubes the loss of $^{14}CO_2$ from solution was less than 5%. In parallel tubes we also measured the vitamin K-dependent incorporation of $^{14}CO_2$ into the pentapeptide substrate FLEEL (figure 1B).

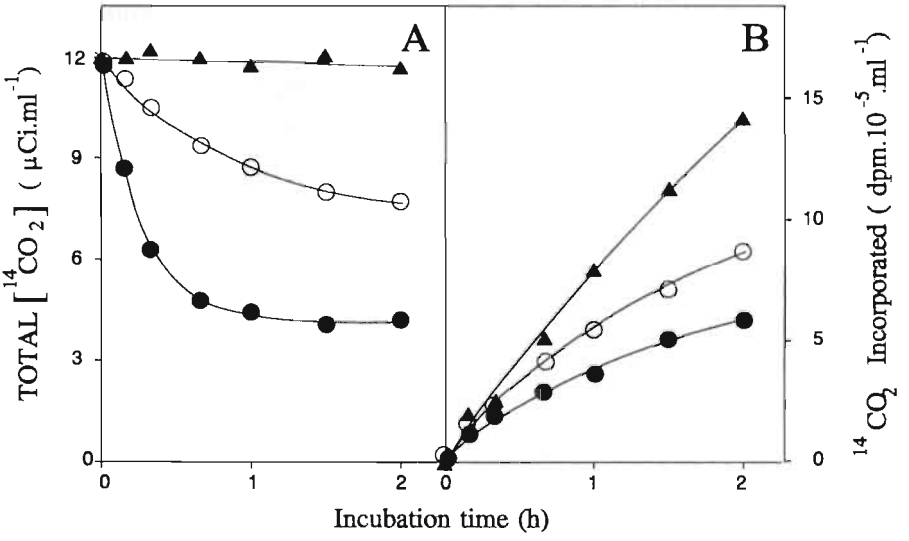

FIG. 1. Vitamin K-dependent $^{14}CO_2$ fixation: effect of tube size. *Standard reaction mixtures were incubated in tubes with a capacity of 7 ml (●-●), 1.5 ml (O-O), and 0.15 ml (▲-▲). a) Total $^{14}CO_2$ concentration in the reaction mixtures at various time points. b) $^{14}CO_2$ incorporated into the pentapeptide substrate FLEEL*

In the largest vessels the carboxylation rate declined during the first 40 min and remained constant for 1 h thereafter. Also in the midsize tubes the curve became linear only after the $^{14}CO_2$ distribution had reached its equilibrium. Only in the smallest tubes the initial curvature was negligible and a straight line could be drawn from the origin. It is also clear that the slopes of the linear parts of the three curves in figure 1B are different. Probably this is a reflection of the different $^{14}CO_2$ /$^{12}CO_2$ ratios at that phase of the reaction. From these results we have concluded that optimal results were obtained in the smallest tubes only. In all other cases the incorporated $^{14}CO_2$ will give rise to an underestimation of the real amount of carboxylation events.

A second possibility to avoid loss of $^{14}CO_2$ in the gas phase would be to decrease k in Eq. [iii], either by decreasing the incubation temperature or by increasing the ionic strength. Because the optimal conditions for the carboxylase assay already include an unusual high ionic strength we have chosen to focus our attention to decreasing the incubation temperature. Several other groups have chosen this strategy (15,28). The relation between incubation temperature and the $^{14}CO_2$ distribution was investigated in large vessels only, and the results are shown in figures 2A and 2B. It is clear that at increasing temperature both the equilibrium levels of $^{14}CO_{2\ aq}$ and the time interval required to reach these levels decrease. In this case the peptide carboxylation was also studied in a parallel experiment and again it was clear that linear reaction rates could only be obtained after the distribution of $^{14}CO_2$ had reached its equilibrium (figures 2C and 2D).

During the first phase of the reaction the curves were nonlinear at all temperatures investigated. It may be concluded, therefore, that decreasing the volume of the gas phase over the reaction mixtures is the most effective way to minimize degassing of the reaction mixtures. Only the smallest tubes Eq. [ii] may be neglected.

If a mixture of labelled and nonlabelled $NaHCO_3$ is added to the reaction mixtures in concentrations which are high relative to the amount of $^{12}CO_{2\ aq}$ originating from the atmosphere, the $^{14}CO_2$ /$^{12}CO_2$ ratio will not markedly change during the course of the incubation. In that case the incorporation of $^{14}CO_2$ is expected to be constant from the onset of the reaction, irrespective of the capacity of the tubes in which it is performed. This was confirmed in an experiment in which we added increasing amounts of $NaH^{14}CO_3$ to standard reaction mixtu-

res. Incubations were performed in large vessels at 20° C. As is shown in figure 3 the loss of $^{14}CO_2$ is independent of the $NaH^{12}CO_3$ concentration in the mixtures. Linear carboxylation rates were only achieved at relatively high bicarbonate concentrations, however.

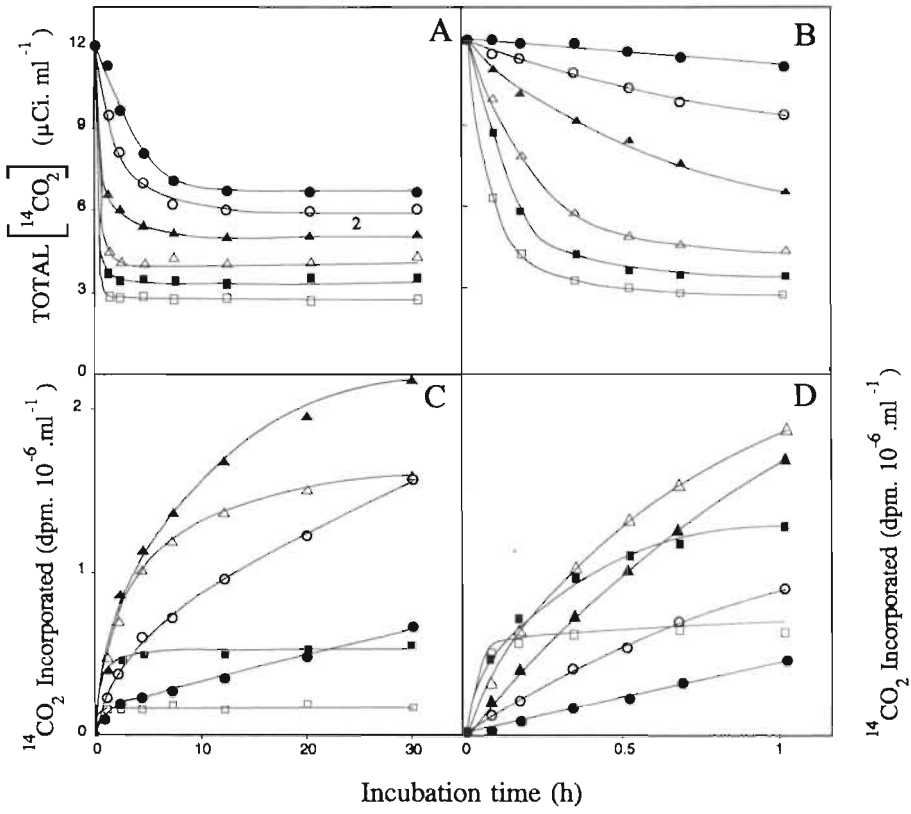

FIG. 2. Vitamin K-dependent $^{14}CO_2$ fixation: temperature dependence. *Standard reaction mixtures (○-○), were incubated in large vials (7 ml) at the following temperatures: 0 °C (●-●), 10 °C 20 °C (▲-▲), 25 °C (△-△), 30 °C (■-■) and 35 °C (□-□). (A and B) Total $^{14}CO_2$ concentration in the reaction mixtures at various time points. (C and D) $^{14}CO_2$ incorporated into FLEEL. B and D represent the first parts of A and C, the points were taken from the same experiment*

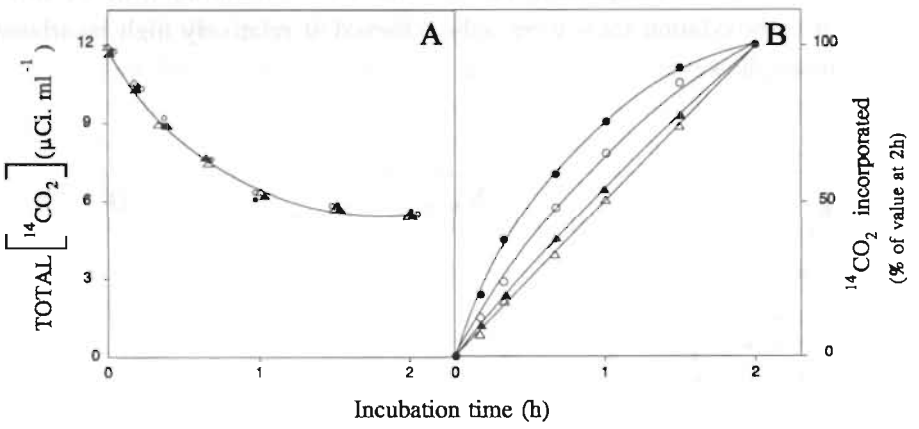

FIG. 3. Vitamin-K dependent $^{14}CO_2$ fixation: effect of $NaH^{12}CO_2$. Standard reaction mixtures were incubated in the presence of 0 (●-●), 2 (○-○), 8 (▲-▲) or 32 (△-△) μM nonlabelled $NaH^{12}CO_2$. (A) Total $^{14}CO_2$ concentration in the reaction mixtures at various time points. (B) $^{14}CO_2$ incorporated into FLEEL. To be able to compare the shape of the different curves in B they are plotted as a percentage of the 2-h value. One hundred percent stands for 239 x 10^3 (●-●), 168 x 10^3 (○-○), 81 x 10^3 (▲-▲) and 40 x 19^3 (△-△) dpm, respectively

To measure the enzyme stability the carboxylation reaction was performed in small tubes containing standard reaction mixtures supplemented with 10 mM $NaH^{12}CO_3$. Time course experiments were performed at seven different temperatures. Figure 4B shows the initial phase of the reaction and it is clear that the highest reaction rates were obtained at the highest temperatures. A disadvantage, however, is that the enzyme is labile at these temperatures. This is demonstrated in figure 4A. At lower incubation temperatures the linearity of the time courses was substantially prolonged, resulting in very high levels of total CO_2 fixation after 24-48 h. Like the other experiments described in this paper the experiment shown in figure 4 was performed with salt-washed microsomes from normal cow liver. Similar results (data not shown) were obtained, however, with salt-washed microsomes from warfarin-treated cows and with partly purified carboxylase, prepared from these two types of microsomes in the way described by Soute et al. (29). Hence the stability of all four carboxylase-containing preparations were closely similar. From these results we also concluded that the

maximal level of CO_2 incorporation was achieved between 10 and 15 °C after an incubation period of 48 h.

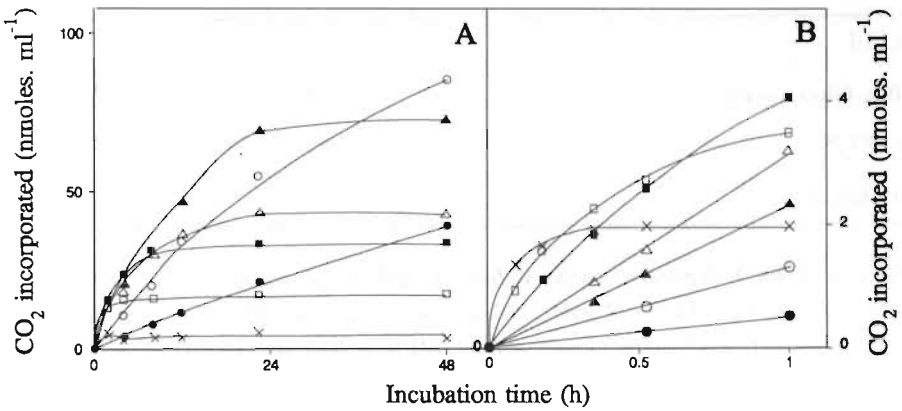

FIG. 4. Vitamin K-dependent CO_2 fixation: *stability of carboxylase at various temperatures. Standard reaction mixtures were supplemented with 32 mM $NaHCO_3$ and incubated in small test tubes (capacity 0.15 ml) at the following temperatures: 0 °C (●-●), 10 °C (○-○), 15 °C (▲-▲), 20 °C (△-△), 25 °C (■-■), 30 °C (□-□) and 35 °C (x-x). (B) The first phase of A, the points were taken from the same experiment*

Under these conditions we have measured the degree to which a number of substrates were carboxylated. The substrates chosen were FLEEL, t-Boc-Glu-Glu-Val, pro PT28 and d-osteocalcin and the results are shown in table I. It is obvious that the tri- and pentapeptides are very poor substrates: the efficiency with which they are carboxylated is low, and high concentrations are required to see any carboxylation at all. The two polypeptide substrates pro-PT28 and d-osteocalcin, on the other hand, were carboxylated to a reasonable extent: in the case of d-osteocalcin even more than 1 mol of Gla was formed per mole of substrate.

Substrate	Gla residues formed (residue/ molecule)	Efficiency of carboxylation (% of theoretical maximum)
FLEEL	0.02	1
t-Boc-Glu-Glu-Val	0.04	2
pro-PT28	0.42	21
d-Osteocalcin	1.47	49

Table I. Vitamin K-dependent carboxylation of various substrates. *Note: Standard reaction mixtures containing FLEEL (4 mM), t-Boc-Glu-Glu-Val (3 mM), pro-PT28 (4μM), or d-osteocalcin (4 μM) were supplemented with 32 mM $NaH^{12}CO_3$ and incubated in small tubes at 10 °C for 72 h. The concentrations of the various substrates represent their K_M^{app} values. The theoretical maximum of Gla formation is three residues per molecule of osteocalcin and two residues per molecule in the other substrates*

Discussion

Kinetic constants are generally defined at 25 °C and most enzymatic reactions are therefore performed at that temperature. A prerequisite for a correct interpretation of the results, however, is that measurements are made during a phase at which the reaction rates are constant. Due to the fact that vitamin K-dependent carboxylase is rather labile at 25 °C, all data should thus be obtained during the first hour of incubation. Unfortunately, in the same period equilibrium [ii] is established, so that the $^{14}CO_2$ /$^{12}CO_2$ ratio decreases continuously. Hence linear reaction rates (expressed as $^{14}CO_2$ incorporated) may not be expected.

In this paper we describe two ways to approximate linearity of the curves during the first hour of incubation at 25 °C. This first method consists of using small test tubes with a capacity barely more than the volume of the reaction mixtures. If the tubes are tightly stoppered, the volume of the gas phase is minimized, and the loss of $^{14}CO_2$ is negligible. This technique will give the highest amount of $^{14}CO_2$ fixation, but the total CO_2 concentration is of the same order of magnitude as the K_M^{app} value. Hence the assay is sensitive to unwanted fluctuations. In

general the increase of $NaH^{14}CO_3$ concentrations to values as high as 10 times the K_M^{app} is not possible because of the costs and safety regulations. The second technique is the addition of an excess (32 mM) of nonlabelled $NaHCO_3$ to the reaction mixtures. In the latter case not only $^{14}CO_2$, but also $^{12}CO_2$ will redistribute itself between the liquid and the gas phase. Hence the ratio between the $^{14}CO_2$ and the $^{12}CO_2$ concentrations will remain nearly constant, even if relatively large incubation vessels are used. Because the final bicarbonate concentration in solution remains far above the K_M^{app} (0.4 mM (24)), the escape of CO_2 will hardly affect the rate of carboxylation. An additional advantage of using an excess of $^{12}CO_2$ is that the extent of carboxylation may be expressed on a molar base (29). A disadvantage, however, is that the total amount of radiolabel incorporated will remain rather low.

For many types of experiments a temperature of 25 °C is not an absolute requirement, so that the experiments may be performed at lower temperatures (e.g. 10 or 15 °C). Under these conditions carboxylase is surprisingly stable, and a linear reaction rate may be maintained for over 24 h. The reason why carboxylase activity rapidly decreases at 25 °C is presently unknown, but it has been described that in a more purified form (like solid-phase carboxylase (7,28)) the enzyme is far more stable.

From these data it may be concluded that a maximal incorporation of CO_2 into a suitable substrate is achieved in small tubes, at a high bicarbonate concentration at 10 or 15 °C. Under these conditions we have compared the efficiency of carboxylation of a number of substrates, and it appeared that the low molecular weight peptides were poorly carboxylated. The importance of the pro-sequence becomes clear from the fact that the extent of carboxylation of pro-PT28 is over 20-fold higher than that of the pentapeptide. This is consistent with earlier observations, which showed that the K_M^{app} for pro-PT28 is about 100-fold lower than that of FLEEL (21). Yet pro-PT28 is not fully carboxylated, possibly because it lacks the "internal recognition site". The latter sequence consists of the amino acid residues Glu-X-X-X-Glu-X-Cys, and its presence in all mature mammalian Gla-containing proteins was initially discovered by Price et al. (30). Decarboxylated osteocalcin contains this internal recognition site, and although it lacks the pro-sequence, it is a good substrate for carboxylase (27). Under the conditions mentioned above, even 1.5 mol of CO_2 could be incorporated per mole of substrate. It is to be expected, therefore, that substantial improvements

may be achieved by constructing polypeptides which contain both the pro-sequence and the internal recognition sequence. Whether these substrates will be fully carboxylatable in vitro remains to be awaited, however.

Acknowledgments

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CHAPTER 4

Vitamin K : Its role as a coenzyme in the carboxylation reaction

Based on:

- Soute, B.A.M., de Metz, M., and Vermeer, C. (1982). Characteristics of vitamin K-dependent carboxylating systems from human liver and placenta. FEBS Lett. 146, 365-368.
- Buitenhuis, H.C., Soute, B.A.M., and Vermeer, C. (1990). Comparison of the vitamins K_1, K_2 and K_3 as cofactors for the hepatic vitamin K-dependent carboxylase. Biochim. Biophys. Acta 1034, 170-175.
- Soute, B.A.M., Watson, A.D.J., Maddison, J.E., Ulrich, M.M.W., Ebbering, R., and Vermeer, C. (1992). Congenital deficiency of all vitamin K-dependent blood coagulation factors due to a defective vitamin K-dependent carboxylase in Devon Rex cats. Thromb. Haemostas. 68, 521-525.

Introduction

On a molecular level many details of the carboxylation reaction still have to be clarified. In principle the chemical possibilities for the addition of a CO_2-molecule to the γ-carbon in a substrate-bound Glu-residue may proceed either via a) activation of oxygen, b) activation of CO_2 or c) activation of glutamic acid. Since it has been shown that both superoxide dismutase and catalase did not inhibit carboxylation (1,2), oxygen activation seems not likely to occur. It has been established by Jones et al. (3) that the active species in the carboxylation reaction presumably is CO_2 and not HCO_3^-, but a CO_2-carrier role for vitamin K hydroquinone has not been demonstrated (4). Although activation of CO_2 by a radical exchange reaction has been proposed by Gallop et al. (5), most evidence strongly supports a start of a carboxylation event by labilization of a γ-hydrogen. Many investigators have suggested that hydrogen removal precedes addition of CO_2 (6,7). Moreover, Azerad et al. (8) and Dubois et al. (9) have established that the enzymatic γ-abstraction step is stereospecific and corresponds to the elimination of the pro-S hydrogen of glutamic acid. This justifies the expectation that CO_2-addition also occurs in a stereospecific way. Both radical formation followed by a one-electron reduction and proton abstraction have been proposed as the pathway leading to a carbanion (7,10).

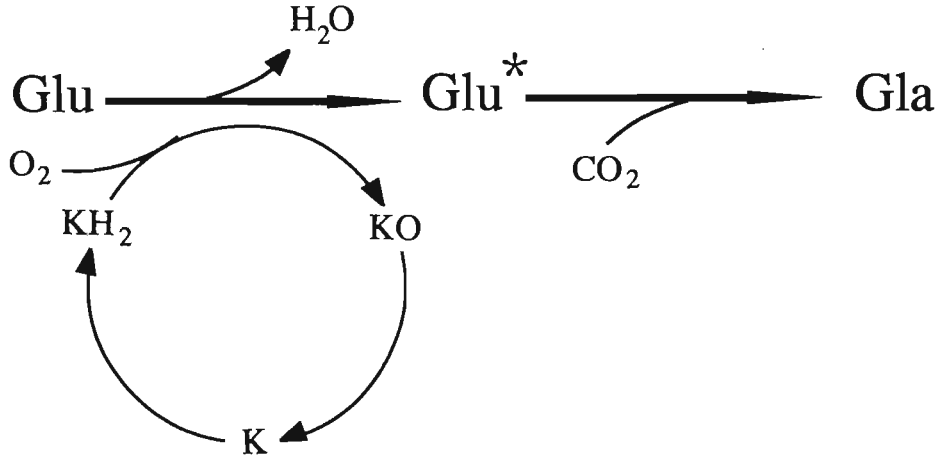

FIG. 1. Proposed pathway for vitamin K-dependent γ-glutamylcarboxylation. *Glu* stands for a carbanion formed either by radical formation or by proton abstraction.*

A carboxylation event would then be completed by the electrophilic attack of this carbanion by CO_2. The precise reaction mechanism by which this complicated system proceeds needs further investigation, preferably in purified systems. Although it is not clear at this moment how epoxide formation and γ-hydrogen abstraction are coupled, there is general consensus about the way in which the vitamin itself is metabolized in the liver during and after the reaction. The active cofactor for carboxylase is vitamin K hydroquinone (KH_2), and all data presently available indicate that the oxidation of (KH_2) to vitamin K epoxide (KO) provides the energy required for the carboxylation of Glu residues (11,12). Subsequently KO is reduced by the action of at least one reductase (see Chapter 1). In this way vitamin K is efficiently recycled, and it may be calculated from the difference between daily vitamin K intake and urinary Gla-excretion that the vitamin is re-used several thousand times before it is degraded via lactone and glucuronide formation (13).

Recycling of vitamin K

The involvement of γ-glutamylcarboxylase in the vitamin K-cycle has been unequivocally demonstrated and all enzymes participating in the recycling of vitamin K have been identified in liver tissue. Inspection of a wide variety of tissues has revealed that γ-glutamylcarboxylase is distributed in tissues other than the liver. In the recent past our group has contributed significantly to the discovery of extra-hepatic tissues and cell lines (14-16), and it is now generally agreed that γ-glutamylcarboxylase belongs to the standard machinery of most cell types. Whether the same holds true for the reductases involved in the vitamin K-cycle is not clear, however. It has been reported by Price et al (17) that the effect of warfarin on the γ-carboxylation of circulating osteocalcin could not be counteracted by high doses of vitamin K. One possible explanation for this phenomenon is that the warfarin-insensitive NADH-dependent reductase is inactive or absent in bone cells. The hypothesis was supported by Ulrich et al. (18) who reported that the NADH-dependent K reductase activity was absent in the osteoblast-like osteosarcoma cell line UMR 106. As early as in 1982 we have described another extra-hepatic carboxylating system from human placenta in which dithiol-dependent reductase activity could not be demonstrated. The

importance of this reductase becomes clear when the carboxylation reaction in vitro is started with vitamin K quinone as a cofactor, since only the reduced cofactor is able to catalyse the carboxylation reaction. The results presented in table I clearly demonstrate the presence of substantial amounts of carboxylase in placental microsomes. The K_M^{app} for KH_2 was comparable with that in human and bovine liver systems. However, if vitamin K quinone was used as a cofactor the placental microsomes had no activity whereas normal activity was found in the hepatic systems. Also replacement of dithiothreitol by other reducing agents like NAD(P)H or β-mercaptoethanol did not stimulate the vitamin K-dependent carboxylation reaction.

	Source of carboxylase		
	Bovine liver	Human liver	Human placenta
Carboxylase ($dpm.min^{-1}.mg^{-1}$)	31	95	12
K_M for vitamin KH_2 (μM)	10	22	16
K_M for vitamin K (μM)	13	16	--

Table I. Characteristics and kinetic parameters of carboxylase from different tissues. *All tests were performed under standard conditions. The amount of carboxylase was measured as the carboxylation rate in endogenous + exogenous substrate (FLEEL, 10 mM) and is expressed as dpm per min per mg protein.*

Obviously the detection of reductase activity via the K quinone + carboxylase route is an indirect test, but it should be kept in mind that the direct measurement of reductase activity was not possible at that time. Therefore it is not clear whether the absence of all vitamin K reductase activity is a real reflection of the situation in vivo or merely a result of our inability to detect the enzyme activity. Alternative explanations for the data presented are that either the placental reductase is inactivated or separated from carboxylase during the preparation and solubilization of the microsomes. At this moment direct tests for both KO and K reductase are operational in our lab, and it would be interesting to repeat these early experiments using the new and sensitive techniques. A physiological result

of the absence of reductase in placenta would be that all vitamin K entering the placenta is converted into KO. Direct evidence for a placental barrier for vitamin K has been obtained by Hamulyák et al. (19) using radiolabelled phylloquinone in rats. In addition, Shearer et al. (20) have demonstrated the presence of vitamin K_1 in plasma of neonates, although the levels were 2 orders of magnitude lower than those in maternal plasma. Since KO is more hydrophillic than is the quinone form this conversion might facilitate the vitamin K transport over the placenta. As long as no placental Gla-proteins have been identified, it even cannot be excluded that the conversion of vitamin K quinone into KO is the main function of placental carboxylase.

Various forms of vitamin K

In nature two forms of vitamin K are found, both characterized by a functional naphtoquinone ring and an aliphatic side chain. The main difference between vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone) is the composition of their side chains (see chapter 1). Vitamin K_1 is mainly found in green plants (21,22) whereas the menaquinones are produced by the intestinal flora (23). The extent to which each of the K vitamins contributes to the carboxylation of blood coagulation factors is still a matter of dispute. It has been shown that 92% of the vitamin K store in liver consists of menaquinones (24). Yet a significant effect on blood coagulation parameters was found in human volunteers after a short period of reduced nutritional vitamin K intake (25,26). No changes had occurred in the faecal menaquinone concentrations during the experiment. These results suggest that menaquinones are of minor importance for the production of coagulation factors. On the other hand vitamin K_2 is successfully used in Japan for preventing haemorrhagic disease in newborns (27,28). Various menaquinones have been tested in early systems, showing maximal activity for MK-3 or MK-4 (29-34). The activity of vitamin K_1 was only slightly lower (29-32). During the last 15 years in vitro carboxylating systems have been substantially improved (35,36) resulting in a more accurate comparison of apparent kinetic constants of the various vitamins. In this way we have established the in vitro activity of phylloquinone and various menaquinones. It was found that the cofactor activity of the menaquinones varied with the length of the aliphatic side chain with an

optimum at MK-3. Menadione was not active at all, which is consistent with data reported by others (16-19). Menaquinones containing more than 10 isoprene subunits were not tested, but if our results may be extrapolated to the higher menaquinones, it is doubtful whether these forms of vitamin K are of physiological relevance. The activities of both MK-3 and MK-4 were comparable to that of phylloquinone. Menaquinones with shorter side chains were characterized by a high K_M^{app}, those with longer side chains by low V_{max}^{app} values. In human liver and plasma the most abundant menaquinones are the menaquinones 7 and 8 whereas the menaquinones 9 and 10 are generally found in slightly lower amounts (24). Because in vitro all these forms are less active than phylloquinone is, their physiological importance remains unclear. Since no data exist about the menaquinone absorption from the large intestine in humans, the source of liver menaquinones remains to be established. More information will be obtained from in vivo experiments with a vitamin K-deficient rat model system recently developed in our lab. Experiments on this subject are in progress at this time.

	K_M^{app} (μM)	V_{max}^{app} (μmol CO_2 bound/h)	V_{max}^{app} (%)
K_1	12.3	25.4	100.0
MK-1	30.0	4.0	15.9
MK-2	9.7	24.9	98.1
MK-3	5.8	30.5	120.0
MK-4	3.6	24.7	96.7
MK-5	2.5	22.2	87.7
MK-6	1.6	19.6	77.0
MK-7	1.9	18.0	70.7
MK-8	1.4	15.8	62.0
MK-9	1.4	14.0	55.8
MK-10	1.4	13.0	51.2

Table II Kinetic constants of menaquinones 1 to 10. *Vitamins were added to the reaction mixtures in a triton - solubilized form, and the initial reaction rates were determined after incubation of the mixtures for 0, 10 and 20 minutes at 25 °C.*

It has been confirmed that all metabolites of both phylloquinone and menaquinone (in this case MK-4) were active as a cofactor in the carboxylase reaction.

All kinetic data is summarized in table III and it was demonstrated that the K_M^{app} value for K_1 was three times higher than that for MK-4, whereas the V_{max}^{app} value was closely similar.

	K_M^{app} (μM)	V_{max}^{app} (μmol CO_2 bound/h)	V_{max}^{app} (%)
K_1H_2	13.3	35.3	100
K_1	12.3	25.4	72
K_1O	11.3	16.2	46
K_2H_2	4.2	36.9	100
K_2	3.8	25.1	68
K_2O	3.7	17.4	47

Table III. Kinetic constants of vitamin K_1 and K_2. *Vitamins were added to the reaction mixtures in a triton - solubilized form, and the initial reaction rates were determined after incubation of the mixtures for 0, 10 and 20 minutes at 25 °C. V_{max}^{app} is expressed in two ways: as μmol CO_2 bound per hour and as a percentage of the K_1H_2 - stimulated reaction.*

No difference was found in the relative amount of carboxylation obtained with the various metabolites of vitamin K_1 and MK-4. Another observation was that in reaction mixtures containing solubilized microsomes, independent of the starting metabolite form, both phylloquinone and menaquinone reached an equilibrium after about 15 min. Hence, irrespective whether the carboxylase reaction was started with KH_2, K or KO, these metabolites were found in a constant ratio after this incubation period. These observations were made at 20 °C and in the equilibrium stage the reaction mixtures contained 2 % KO, 30 % KH_2 and 70 % K quinone (figure 1). The observation that equilibria were not reached before 15 minutes is inconsistent with a linear carboxylation rate for K and KO found at 25 °C. During the initial phase of the K- and KO-stimulated carboxylation reaction, KH_2 cannot be present in excess. Instead it will increase during the first 20 minutes from zero to the equilibrium value. Yet linear time curves were found. A plausible explanation may be that only a small fraction of the vitamin K added to the reaction mixture is directly available for the carboxylase/reductase complex, and that the KH_2 formed during the first minutes of the reaction is not distributed uniformly over the phospholipid micelles, but remains concentrated on or around the carboxylating enzyme system. In this

way high local concentrations of KH_2 would be generated without notably affecting the total vitamin K pool. The rapid oxidation of KH_2 was not only found in solubilized microsomes but also in reaction mixtures containing purified carboxylase (kindly supplied by Drs. Wu and Stafford). However, reaction mixtures from which carboxylase was omitted were far more stable. These data should be taken into account when KH_2 is thought to be added in excess to a carboxylating reaction mixture, and they probably explain why several authors have found only very short periods of linear carboxylation (37).

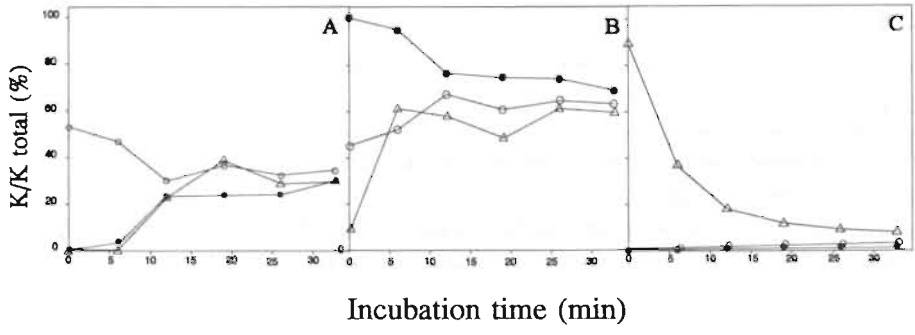

FIG. 1. Analysis of the vitamin KH_2, K and KO concentration during the epoxidase/reductase reactions. *At different time intervals the relative concentrations of KH_2, K and KO were determined by HPLC. The data are represented as the formation of K_1H_2 (fig. 2A), K_1 (fig. 2B) and K_1O (fig. 2C). Starting material at time zero is respectively K_1H_2 (○-○), K_1 (●-●) and K_1O (△-△)*

Allosteric interaction between propeptide-binding site and vitamin K-binding site on carboxylase

Recently we were confronted with two Devon Rex cats exhibiting an unexplained bleeding problem. With plasma and liver samples from affected cats we could not only explain the defect by postulating a mutation of the propeptide-binding site, but we also demonstrated the allosteric interaction between the propeptide- and the vitamin K-binding sites on carboxylase in unaffected cats. This observation was confirmed in the well characterized rat and bovine systems. At the time of writing this thesis the paper reporting these data is still in press, which is the reason of integrally reproducing it in this chapter.

Conclusions

All enzymes of the vitamin K cycle are active in our cell-free liver system. After addition of KH_2 to the reaction mixtures, it is rapidly oxidized until after about 15 min a steady state is reached in which not more than 30 % of the original amount of KH_2 is left. This conversion even takes place if the number of carboxylation events is reduced to a minimum, e.g. by omitting a carboxylatable substrate in the reaction mixture. Also the presence of DTT is of no influence on the auto-oxidation of KH_2. Therefore the actual concentration of KH_2 may only be a fraction of the original value and may influence linear reaction velocity. On the other hand we have found that the binding of propeptide-containing substrates gives rise to the formation of a high affinity site for KH_2 on carboxylase, resulting in a decrease of the K_M^{app} for KH_2 by at least one order of magnitude. Since the decrease of both the actual KH_2 concentration and its K_M^{app} are expected to have opposite effects, these data may explain why in the presence of propeptide-containing substrates, linearity of the carboxylation reaction was observed for several hours.

Congenital deficiency of all vitamin K-dependent blood coagulation factors due to a defective vitamin K-dependent carboxylase in Devon Rex cats

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Abstract

Two Devon Rex cats from the same litter, which had no evidence of liver disease, malabsorption of vitamin K or chronic ingestion of coumarin derivatives, were found to have plasma deficiencies of factors II, VII, IX and X. Oral treatment with vitamin K_1 resulted in the normalization of these coagulation factors. After taking liver biopsies it was demonstrated that the coagulation abnormality was accompanied by a defective γ-glutamylcarboxylase, which had a decreased affinity for both vitamin K hydroquinone and propeptide. This observation prompted us to study in a well defined in vitro system the possible allosteric interaction between the propeptide binding site and the vitamin K hydroquinone binding site on carboxylase. It was shown that by the binding of a propeptide-containing substrate to γ-glutamylcarboxylase the apparent K_M for vitamin K hydroquinone is decreased about 20-fold. On the basis of these in vitro data the observed defect in the Devon Rex cats can be fully explained.

Introduction

Vitamin K hydroquinone (KH_2) is an essential cofactor in the posttranslational proces-sing of a number of proteins including the coagulation factors II, VII, IX, and X. The vitamin K-dependent step is the conversion of the first 10-12 aminoterminal glutamate residues into γ-carboxyglutamate (Gla) residues. During this reaction, which is mediated by the microsomal enzyme γ-glutamylcarboxylase (37), KH_2 is oxidized into vitamin K 2,3-epoxide (KO). At least one, but probably several, reductases are involved in the subsequent recycling of KO into

KH_2 (38). This sequence of events is generally known as the 'vitamin K cycle'.
Besides vitamin K deficiency there are two major reasons why Gla-containing proteins may occur in an undercarboxylated form. Firstly, the vitamin K cycle may be interrupted by the action of 4-hydroxycoumarin derivatives, which are potent inhibitors of the enzyme KO-reductase (39). The blockade of this enzyme hampers the recycling of KO, leading to an increased circulating KO concentration, and rapid exhaustion of the KH_2 store. This results in an accumulation of non-carboxylated precursor proteins in the endoplasmic reticulum and, if the blockade is continued, in the cellular secretion of maturated Gla-proteins in a non- or undercarboxylated form (the so-called 'descarboxyproteins'). Whether or not the descarboxyproteins are secreted depends on the dose and the duration of coumarin intake, as well as on the species (40).
A second reason for undercarboxylation of Gla-proteins is a poor recognition of the precursor proteins by carboxylase. The so-called 'pro-sequence' in these precursors consists of 18 amino acid residues directly preceding the amino terminus of the mature proteins, and has been demonstrated to play a major role in their affinity for carboxylase (41,42). Mutations in the pro-sequence may lead to impaired substrate recognition and hence to poor substrate carboxylation, both in vivo and in vitro (43,44). Blood coagulation disorders of this type are rare, however (45).
Recently we encountered with two Devon Rex cats from the same litter, which were affected by a bleeding tendency (46). Blood coagulation factor analysis demonstrated substantially decreased levels of factors II, VII, IX, and X, whereas the plasma concentration of factors V, VIII, XI, and XII were normal. There was no evidence for exposure to coumarin derivatives, liver disease or fat malassimilation. Yet oral treatment with vitamin K_1 resulted in normalization of all vitamin K-dependent coagulation factor levels. Inspection of the family history of the cats gave evidence that the defect was inherited as an autosomal trait (46).
We have further investigated these cats, and our results indicate that the low procoagulant activity is the result of a mutation in the vitamin K-dependent carboxylase, leading to poor recognition of the pro-sequence and hence to a poor carboxylation of the various coagulation factors. A model explaining why this defect may be corrected by an increased vitamin K intake is discussed.

Materials and methods.

Chemicals. Vitamin K_1 (Konakion®) was obtained from Hoffmann-La Roche (Basle, Switzerland) and vitamin K hydroquinone (KH_2) was prepared by incubating 6 mM vitamin K quinone in the presence of 150 mM dithiothreitol (DTT) at pH 8.5 and 37 °C overnight in a light-protected tube. Vitamin K epoxide (KO) was prepared according to the method of Tishler et al. (47). The chromogenic substrates S 2238 and S2337 were obtained from Kabi Diagnostica (Stockholm, Sweden). Russell's viper venom, Echis carinatus venom, DTT and 3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulphonate (CHAPS) were from Sigma (Saint Louis, MO). The pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) was purchased from Vega Biochemical Co. (Tucson, AZ), $NaH^{14}CO_3$ (56 Ci/mol) and Formula 989 from New England Nuclear (Dreieich, F.R.G). ProFIXPT28 and propeptide were kind gifts from drs. B.C. and B. Furie (Tufts University, Boston, MA). ProPT28, proPT28(FA-16) and proPTFLEEV were prepared at Applied Biosystems (Maarssen, The Netherlands), proFIX59 was a kind gift from dr. D.W. Stafford (University of North Carolina, Chapel Hill, NC). The structures of these substrates are given in Fig. 1. Osteocalcin was prepared from bovine bone, and thermally decarboxylated as described earlier (48). All chemicals were of analytical grade or better.

Experimental animals. Blood required for the various tests was taken by venipuncture and collected into 0.1 volume of 3.8% sodium citrate. The blood sampling from two normal and both affected cats was performed before and after a single, oral dose of 5 mg of vitamin K_1. Liver biopsies were taken from the same cats before vitamin K administration only. Vitamin K-deficiency was generated in male Lewis rats (12 weeks old) by feeding them a vitamin K-deficient diet (Hope Farms, Woerden, The Netherlands) for one week prior to sacrifice.

Carboxylase assay. Salt washed microsomes from normal and affected cats and from normal and vitamin K deficient rats were prepared from liver according to earlier described methods (49). Standard reaction mixtures (0.125 ml) contained: 1 mg of microsomal proteins, 0.1% (w/v) CHAPS, 0.5 M NaCl, 25 mM Tris/HCl (pH 7.5), a carboxylatable substrate (concentration as indicated), 1.5 µCi $NaH^{14}CO_3$ and either vitamin K, KO or KH_2 (as indicated). When propeptide-containing substrates were used, no $(NH_4)_2SO_4$ was added to the reaction

mixture. Incubations were performed for 30 min at 20 °C. For kinetic studies, the reaction mixture without peptide or KH_2 was preincubated at 20 °C for 3 min and equal amounts were added at 20 s intervals to tubes containing varying amounts of peptide or KH_2. Kinetic data were calculated from time courses obtained for at least five different substrate concentrations after incubations at 0, 5, 10, 20 and 30 min. All reactions were stopped by the addition of 50 µl of 1 M NaOH. Non-bound $NaH^{14}CO_3$ was removed by adding 1 ml of 5% (w/v) trichloroacetic acid and boiling for 5 minutes. Samples were counted in Formula 989 using a Beckman LS 1801 liquid scintillation counter. Data are expressed as pmol CO_2 fixed per min and per mg of microsomal proteins.

KO-reductase assay. For the in vitro test, standard reaction mixtures were incubated under similar conditions as described for the carboxylase assay. Aliquots were taken after 2.5, 5 and 10 min, extracted with isopropanol/hexane and analyzed for KO and K by HPLC using a Lichrosorb RP 18 reversed phase column (Chrompack) (50). Data are expressed as pmol K formed per min and per mg of microsomal proteins. The in vivo KO-reductase activity was deduced from circulating plasma KO levels, which were determined according to the method of Shearer (51).

Coagulation assays. Prothrombin and factor X concentrations were determined with a one stage assay (52) in which prothrombin- and factor X-deficient bovine plasma were used as a reagent, respectively. The total amounts of plasma prothrombin + descarboxyprothrombin were determined in a slightly modified two-stage assay initially described by Kirchhoff et al. (53). In this assay, which is based on thrombin generation by Echis carinatus venom, plasma samples (25 µl) were diluted in 420 µl of a buffer containing 175 mM NaCl, 5 mM $CaCl_2$ and 0.5 mg/ml human serum albumin, and supplemented with 5 µl of a solution containing 0.5 mg/ml E. carinatus venom. The samples were preincubated for 10 min at 37 °C, after which 50 µl of a 1.5 mM solution of the chromogenic substrate S2238 were added. The rate of thrombin generation was monitored by the absorbance at 405 nm and compared with a reference curve. Descarboxyprothrombin concentrations were calculated from the difference between the two-stage and the one-stage prothrombin determinations. Descarboxyfactor X concentrations were determined in a similar way but using Russell's viper venom (5 µl, 0.2 mg/ml) for the two-stage generation of factor Xa and S2337 as a chromogenic substrate (54).

Other assays. Protein concentrations were determined according to the procedure described by Sedmak and Grossberg (55).

Results

Effect of vitamin K administration on prothrombin and factor X concentrations.

Citrated plasma from normal and affected cats was taken before, and 12 and 24 h after vitamin K treatment. In the affected cats the circulating prothrombin levels before treatment were about 10% of those in the control cats. In these samples the two-stage assay was even slightly, but not significantly, lower than the one-stage values (table I, lanes 2 and 3). Hence no descarboxyprothrombin could be detected in this way. Twelve h after vitamin K administration to the affected cats their plasma prothrombin concentration had increased to the normal levels, and remained constant for at least another 12 h period. Also in these samples the differences between the one-stage and the two-stage assays were small and insignificant.

	Coagulation factor concentration (% of control rats)			
Time (h) after oral dose of K	Prothrombin		Factor X	
	one-stage	two-stage	one-stage	two-stage
0	10	8	5	9
12	89	111	115	134
24	99	106	92	117

Table I. Effect of vitamin K administration on blood coagulation factors in normal and affected Devon Rex cats. *One stage and two stage prothrombin and factor X determination in citrated plasma before, and 12 and 24 h after the administration of an oral dose of 5 mg vitamin K (Konakion). All data are the means of triplicate experiments in each of the affected cats, and they are expressed as a percentage of parallel data obtained in the control animals*

Similar results were obtained with factor X (table I, lanes 4 and 5): in the affected cats the factor X concentration before treatment was less than 10% of normal and descarboxyfactor X was insubstantial (if any). Vitamin K administration resulted in a strong increase of the factor X concentration (both in the one-stage and in the two-stage assay) up to levels which were comparable with those in the control cats.

Characterization of carboxylase/reductase content in normal and affected cats.

Because the bleeding problem in the affected cats was clearly related to vitamin K, we have investigated whether it could be ascribed to an alteration of one of the enzymes of the vitamin K cycle. Firstly we have tested the ratio between the plasma KO and K concentration before and 12 h after vitamin K administration. In normal mammalian liver any KO formed is readily reduced by the enzyme KO-reductase, and no KO is detectable in blood plasma. It is well known, however, that in the case of a decreased KO-reductase activity substantial amounts of KO are set free in the blood stream, especially after the administration of high doses of vitamin K (56). No elevated KO concentrations were found, however (data not shown). Subsequently we have analyzed the various liver biopsies for their carboxylase and reductase content. The results of these in vitro experiments are summarized in table II. In agreement with the in vivo measurements, no difference was found between the KO-reductase activity in affected and that in control cats. The KH_2-dependent carboxylase in the affected cats seemed to be strongly impaired, however. The routine carboxylase assay is performed at 4 mM of the pentapeptide FLEEL, which serves as a carboxylatable substrate. Under these conditions the enzyme activity in the affected cats was not more than 16% of that in the control ones. Hence the problem seemed to be related to γ-glutamylcarboxylase, not to KO-reductase. It also seemed interesting to know whether the relatively low carboxylase activity would be accompanied by increased levels of non-carboxylated precursor proteins. The fact that at least seven of these precursor proteins, each of them in varying stages of maturation, are expected to be present in the liver microsomes places severe constraints to their direct quantification. However, the pool of uncarboxylated proteins generally forms an 'endogenous substrate' for carboxylase during the in vitro carboxylation reaction, and by having the carboxylation

reaction proceed in the absence of exogenous substrate, a rough estimate about the amount of precursor proteins may be obtained. No indication for elevated amounts of endogenous substrate was found in the affected cats, however (table II, line 3).

Hepatic marker tested	Affected cats	Normal cats
KO-reductase (pmol K formed/min	120 ± 18	100 ± 15
γ-Glutamylcarboxylase (pmol CO_2 bound/min)	11.2 ± 3.8	73 ± 14
Endogenous precursor proteins (pmol CO_2 bound)	11.4 ± 2.2	21.6 ± 3.1

Table II. Comparison of vitamin K-dependent proteins in normal and affected Devon Rex cats. *All reactions were performed under standard conditions. γ-Glutamylcarboxylase was quantified from the initial reaction rate in the presence of 4 mM of the pentapeptide FLEEL, the microsomal content of endogenous precursor proteins was estimated from the amount of CO_2 incorporated during incubation for 1 h in the absence of exogenous substrate. The data (± S.E.M.) are presented as the means of triplicate measurements in each of either two affected or two normal cats.*

Comparison of the apparent kinetic parameters for substrates and cofactors of γ-glutamylcarboxylase.

In order to further identify the nature of the abnormality in the affected carboxylase, we have measured the apparent kinetic constants for several substrates involved in the carboxylation reaction. The results are summarized in table III. The K_M^{app} and the V_{max}^{app} for KH_2 were measured in the presence of an excess of CO_2 and 10 mM of the pentapeptide FLEEL, and it is clear that in normal and affected animals the K_M^{app} values for this substrate were closely similar. Consistent with the data shown in table II, the V_{max}^{app} for KH_2 in the affected system was found to be about 5.5 fold lower than that in the control one. We also compared the kinetic constants for a propeptide-deficient (FLEEL) and a propeptide-containing substrate (proPT28), obviously these tests were performed in the presence of an excess of KH_2 (0.1 mM) and CO_2. It turned out that in carboxylating systems from normal cats the K_M^{app} for the propeptide-containing substrate was 2300 fold lower than that for FLEEL. This is consistent with earlier observations in bovine systems (41). In the affected cats, however, the effect of the

pro-sequence on the K_M^{app} was less prominent and resulted in not more than a 115-fold difference. In parallel, the differences in the V_{max}^{app} values increased from 5.5 fold when using the propeptide-deficient FLEEL to nearly 14-fold with the substrate proPT28. This obviously amplifies the difference between normal and affected cats. On the basis of these data we have formulated the hypothesis that the coagulopathy observed in the affected cats was probably due to a mutation in the hepatic γ-glutamylcarboxylase, leading to an impaired recognition of the pro-sequence in nascent blood coagulation factor precursors. To explain why in vivo the production of the coagulation factors was restored after vitamin K administration, we decided to investigate the possibility of an allosteric interaction between the propeptide-binding site and the KH_2-binding site on normal, non-affected γ-glutamylcarboxylase. If such an interaction could be demonstrated in normal carboxylase, this would at least make plausible that a mutation in the propeptide-binding site may lead to a different vitamin K requirement. Firstly because the limited amounts of Devon Rex liver available did not allow us to address this hypothesis in the same species, and secondly because the question has a general character, not related to species, we decided to perform our subsequent studies in the well-characterized rat and bovine liver systems.

Cofactor or substrate tested	Animal	K_M^{app} (μM)	V_{max}^{app} (pmol/min)
KH_2	normal	25 ± 5.0	77.5 ± 16.9
	affected	17 ± 3.5	14.0 ± 4.2
FLEEL	normal	6700 ± 1200	72.1 ± 13.8
	affected	4000 ± 1050	13.0 ± 4.7
ProPT28	normal	2.9 ± 0.7	3.1 ± 0.5
	affected	35 ± 6	0.23 ± 0.1

Table III. Comparison of apparent kinetic constants of hepatic enzymes from normal and affected cats. *The kinetic constants were calculated by the non-linear least squares technique (59) in which we used the initial reaction rates at five different substrate concentrations ranging from 0.25 to 4 times the K_M^{app} value of the compound tested. The data for KH_2 were tested in the presence of 10 mM FLEEL. All data presented are the means (± S.E.M.) of triplicate experiments in each of either two normal or two affected animals.*

Apparent Michaelis constants for KH_2 using either propeptide-containing or propeptide-lacking substrates.

The K_M^{app} values for KH_2 were studied in ten carboxylating enzyme systems which differed from each other by the nature of the carboxylatable peptide substrates. The substrates used in this experiment are those given in figure 1, and they were added to the reaction mixtures in excess: 10 mM FLEEL, 20 µM d-osteocalcin, 20 µM proPT28, 1 mM proPT28(FA-16), 20 µM proFIXPT28, 20 µM proFIX59 or 20 µM proPTFLEEV. Moreover, in two cases the K_M^{app} values for KH_2 were assessed by a combination of carboxylatable substrate + propeptide (20 µM).

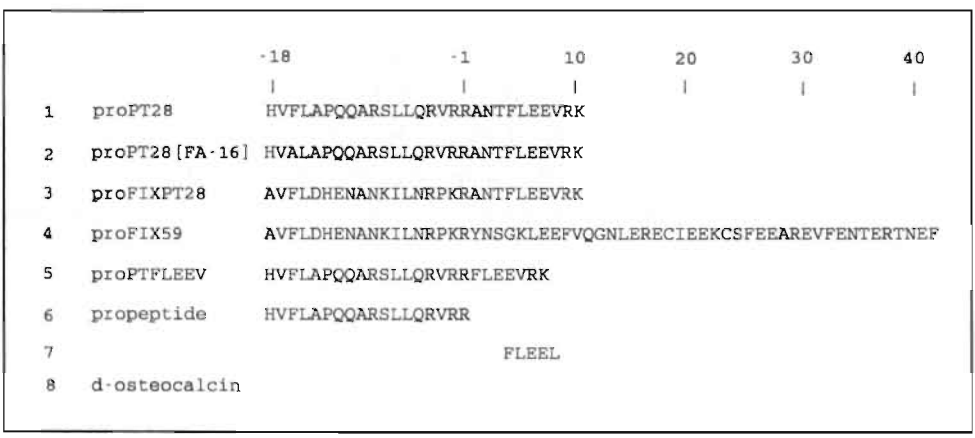

FIG. 1 Primary structure of the peptides used as carboxylatable substrates in the vitamin K-dependent carboxylase reaction. *The numbering from -18 to +40 refers to the amino acid sequence in human preprothrombin. Substrate 1 (proPT28) is a synthetic peptide similar to the amino acid sequence -18 to +10 of human descarboxyprothrombin. Substrate 2 (proPT28(FA-16)) is similar to proPT28 except for the exchange of Phe^{-16} into Ala. Substrate 3 (proFIXPT28) is based on the propeptide (18 amino acids) of factor IX and the first 10 amino acid residues of human descarboxyprothrombin. Substrate 4 (proFIX59) is a 59 residue recombinant peptide containing the propeptide and the complete non-carboxylated Gla-domain of human factor IX. Substrate 5 (proPTFLEEV) is a synthetic peptide composed of the propeptide of human prothrombin and the pentapeptide FLEEV. Substrate 6 (propeptide) is similar to the residues -18 to -1 in human preprothrombin. The sequence of substrate 7 (FLEEL) is found in several coagulation factors (e.g. human factor VII). Substrate 8 (d-osteocalcin) was prepared by thermal decarboxylation of bovine osteocalcin.*

In those cases in which we used propeptide-lacking substrates, the KH_2 concentrations varied from 25 to 400 µM, whereas they ranged from 0.32 to 10 µM if the carboxylatable substrates contained the pro-sequence. It turned out that in the presence of the propeptide-lacking substrates (FLEEL and d-osteocalcin) the K_M^{app} values for KH_2 were rather high (127 and 30 µM, respectively, see table IV, lines 2 and 3). The addition of non-bound propeptide to the various reaction mixtures did not significantly change these data (table IV, lines 4 and 5), which is consistent with earlier observations of Cheung et al. (57). These authors failed to check, however, the effect of substrates in which the propeptide is covalently bound to the carboxylatable Glu-domain. Our data clearly demonstrate, that in reaction mixtures supplemented with propeptide-containing substrates the K_M^{app} for KH_2 values were considerably lower than those obtained in the presence of substrates not covalently bound to the propeptide, and ranged from 0.8 to 3.7 µM (table IV, lines 6-10).

We have concluded, therefore, that the binding of a propeptide-containing substrate to carboxylase results in a decrease of the K_M^{app} for KH_2. The effect is only seen if the propeptide and the carboxylatable sequence are covalently linked via a peptide bond. A second conclusion which may be drawn from these experiments is that, if added in excess (10 times their K_M concentration), both proPT28 and proPT28(FA-16) had the same effect on the K_M^{app} value for vitamin KH_2. This strongly indicates that not the mutation, but the occupation of the substrate-binding place is the factor determining the enzyme's K_M^{app} for KH_2. The apparent interaction between the substrate binding place and the KH_2 binding place was not restricted to the rat, because similar results were obtained in a carboxylating system derived from bovine liver. We also measured the K_M^{app} for CO_2 in the presence of substrates either containing or lacking the pro-sequence, but this constant was independent of the type of substrate used (data not shown).

Apparent kinetic constants for vitamin KH_2 in carboxylase from vitamin K deficient rats.

During vitamin K deficiency the carboxylation process comes to a halt, resulting in the accumulation of precursor proteins in the liver. At least part of these precursor proteins still contain the propeptide, and are tightly bound to γ-glutamylcarboxylase (58). In the in vitro carboxylation reaction this mixture of

immature proteins may serve as a pool of endogenous substrate for carboxylase. Comparison between carboxylase from vitamin K deficient and that from vitamin K sufficient animals thus provided us with an additional tool to test the hypothesis that the K_M^{app} of carboxylase for KH_2 is influenced by the binding of the enzyme with a propeptide-containing substrate. It turned out that in the presence of exogenous substrates lacking the pro-sequence, carboxylase from vitamin K deficient animals had a 10-15 fold lower K_M^{app} for KH_2 than had carboxylase from sufficient rats (table IV, lines 1-3). In the presence of exogenous substrates containing the pro-sequence the values of the K_M^{app} for KH_2 were comparable in both types of carboxylase (table IV, lines 6-10). It seems therefore, that the endogenous precursor proteins bring about the same conformational changes in carboxylase as do the propeptide-containing substrates.

Substrate added	K_M^{app} for KH_2 of carboxylase from	
	K-sufficient rats	K-deficient rats
1. None	-----	8.0 ± 1.50
2. FLEEL	127.0 ± 20.00	11.0 ± 2.40
3. d-osteocalcin	30.0 ± 5.00	3.7 ± 0.95
4. FLEEL + propeptide	154.0 ± 23.00	N.D.
5. d-osteocalcin + propeptide	25.0 ± 4.50	3.2 ± 0.90
6. proPT28	0.8 ± 0.14	1.6 ± 0.25
7. proFIX59	4.2 ± 0.55	2.7 ± 0.80
8. proFIXPT28	0.9 ± 0.16	1.1 ± 0.24
9. proPTFLEEV	1.1 ± 0.20	1.8 ± 0.30
10. proPT28(FA - 16)	1.2 ± 0.25	1.8 ± 0.27

Table IV. Rat carboxylase: K_M^{app} for KH_2 depends on type of substrate used. *The apparent Km values were calculated by the non-linear least squares technique (59) in which we used KH_2 concentrations ranging from 0.32 to 400 μM. Peptide substrates were used in the following concentrations: d-osteocalcin, 20 μM; proPT28, 20 μM; proFIXPT28, 20 μM; proFIX59, 20 μM; proPTFLEEV, 20 μM; FLEEL, 10 mM ; proPT28(FA-16), 1 mM and propeptide, 10 μM. The data (± S.E.M.) are presented as the means of triplicate measurements in each of two either affected or normal rats. N.D. stands for not determined.*

Discussion

The Devon Rex cats which were subject of the investigations reported in this paper were clearly affected by a coagulopathy caused by reduced levels of all vitamin K-dependent blood coagulation factors. The fact that this defect could be totally corrected by pharmacological doses of vitamin K_1 suggested an abnormality on the level of either vitamin K metabolism or vitamin K-dependent post-translational processing of the various coagulation factors. Investigation of the affected cats excluded hepatic disease or fat malassimilation, leaving the ingestion of a coumarin-type rodenticide (which is not uncommon in cats) as the most likely explanation for the observed phenomena. However, this assumption was contradicted by the fact that the high vitamin K requirement remained during long periods of observation of the animals and by the absence of KO in the plasma. Therefore we have analyzed the hepatic enzymes involved in the vitamin K cycle, and found that KO-reductase (the target enzyme for coumarin derivatives) activity in the affected cats was comparable to that in normal ones. This observation definitely excludes the possibility of coumarin ingestion. On the other hand it was shown that the abnormality involved γ-glutamylcarboxylase, and all in vitro data available suggest that this enzyme has undergone a mutation resulting in decreased affinity for the pro-sequence of its natural substrates. This would also explain why no evidence was obtained for elevated levels of the hepatic pool of non-carboxylated precursor proteins complexed to carboxylase. Since descarboxyproteins were neither found in plasma, the fate of the non-carboxylated proteins remains obscure, however.

The question why a mutation in the substrate binding site leads to an increased in vivo requirement for vitamin K in the affected animals may be explained by the in vitro observation that in normal mammalian carboxylase the binding of propeptide-containing substrates induce a high affinity binding site for the active cofactor KH_2. A mutation at the precursor protein binding site of the vitamin K-dependent carboxylase may thus lead to a decreased affinity for KH_2, resulting in an increased dissociation constant for KH_2. Under these conditions the supply of vitamin K may be too low for a full carboxylation of the Gla-containing coagulation factors, which explains why the affected animals are apparently vitamin K deficient at a normal dietary intake of the vitamin. As soon as pharmacological doses of vitamin K are administered, the amount of enzyme/KH_2

complexes is increased so that normal carboxylation of the coagulation factors will occur. It has been shown earlier that non-bound propeptide decreases the K_M^{app} for small Glu-containing substrates, probably by increasing the affinity of the enzyme for peptide substrates (57). Our data strongly suggest that, if the propeptide is covalently linked to the Glu-containing substrates, it also modifies the affinity of carboxylase for its cofactor KH_2.

At this time there is no material available to confirm the above hypothesis. Therefore it is important that more Devon Rex cats are screened for the blood coagulation disorder described here, and identified for further research. This would give a better insight of the extent to which the defect has spread among the breed, and its mode of inheritance. Additionally, the breeding of affected animals may improve the accessibility of the altered carboxylase for experimental analysis.

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CHAPTER 5

Enzymes involved in the recycling of vitamin K

Based on:

- Vermeer, C., Soute, B.A.M., Aalten, M., Knapen, M.H.J., and Thijssen, H.H.W. (1988). Vitamin K reductases in normal and in warfarin-resistant rats. Biochem. Pharmacol. 37, 2876-2878.
- Soute, B.A.M., Groenen-van Dooren, M.M.C.L., Holmgren, A., Lundström, J., and Vermeer, C. (1992). Stimulation of the dithiol-dependent reductases in the vitamin K-cycle by the thioredoxin system: strong synergistic effects with protein disulphide-isomerase. Biochem. J. 281, 255-259.

Introduction

Reduced vitamin K (KH_2) is an obligatory cofactor in the carboxylation reaction, in which it is converted into the epoxide form (KO). Since body pools of vitamin K are extremely low, efficient recycling of the vitamin is an essential physiological requirement. The dietary intake of the vitamin is solely in the quinone form, because of the rapid oxidation by air of an eventually reduced intermediate in foods. Therefore mechanisms for efficient reduction/recycling must be present in the microsomal fraction to meet the needs of the enzyme carboxylase. Although it has never been proven unequivocally, it is generally assumed that the reduction of KO into KH_2 occurs via the quinone form (1). Several enzymes have been identified capable of reducing either vitamin K, KO or both. The presence of at least two NAD(P)H-dependent quinone reductases has been reported in the literature; one found in the cytoplasm, the other in the microsomal fraction (2-4). Fasco and Principe (5) demonstrated the occurrence of an exclusively dithiol dependent activity capable of producing KH_2 both from vitamin K and KO. Whether these reducing steps are exerted by one or two enzymes is still a matter of investigation. It was suggested by Fasco and Lee (6) that the dithiol driven activities are located at separate catalytic sites and that an essential disulfide supplies the reducing equivalents for both reduction steps. Thijssen and Baars (7) recently demonstrated the existence of a microsomal warfarin receptor located at these dithiol-dependent reductases. The in vitro activity of the dithiol-dependent reductases is readily detectable in the presence of synthetic dithiols like dithiothreitol, but the in vivo cofactor is unknown at this time.

As has already been mentioned earlier both dithiol-dependent reductases and not the NAD(P)H-dependent ones (8) are strongly inhibited by coumarin derivatives, which is a clear difference with the dithiol-dependent and NAD(P)H-dependent enzyme systems. In the case of intoxication the dithiol-dependent pathway cannot be used and therefore the second route, via the relatively warfarin-insensitive NAD(P)H reductases, can overcome the warfarin block. A prerequisite in that case is that high amounts of vitamin K are available. Warfarin-binding studies in an experimental animal (rat) model system already revealed that normal microsomes bind warfarin more tightly and in larger amounts than those

from warfarin-resistant rats of a Welsh strain (9,10). In warfarin-resistant rats of a different strain we have focused our attention to the likelihood that both dithiol-dependent reductase activities are exerted by one and the same enzyme.

Dithiol-dependent reductases in normal and in warfarin-resistant rats

The conversion of vitamin K quinone (K) into KH_2 may be accomplished either by a dithiol-dependent reductase or by a NADH-dependent enzyme. The dithiol-dependent reductions of KO and K are extremely sensitive to the action of oral anticoagulants such as warfarin (8). KH_2-dependent carboxylase activity as such can be assessed by reducing vitamin K into KH_2 in a chemical way before it is added to the reaction mixtures. The activity of the two K reductases, on the other hand, is frequently determined in an indirect way, namely by starting the carboxylation reaction with either K + DTT or with K + NADH. A direct measurement of the K reductases by establishing the production of KH_2 is less reliable because the hydroquinone is unstable and is rapidly re-oxidized into its quinone form by traces of oxygen.

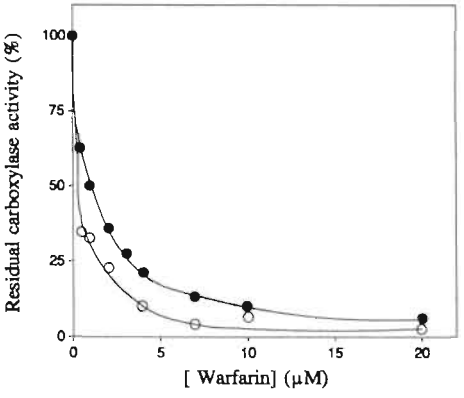

FIG. 1. Warfarin-inhibition of (K + DTT)-dependent carboxylase. *Time course experiments between 0 and 30 min were performed at each warfarin concentration. The initial carboxylation rate was calculated and expressed as a percentage of the non-inhibited reaction. 100% activity corresponds to 2430 dpm/min in susceptible rats (●-●) and to 2970 dpm/min in warfarin resistant rats (O-O).*

Most studies up till now concerning warfarin resistance in rats have been performed with animals of the Welsh resistant strain (HW). It has been demonstrated (10,11) that the resistance in these animals originates from a strongly reduced

inhibitory effect of warfarin on the dithiol-dependent reductase activities (both KO and K reductase). A second warfarin-resistant strain (HS), which had developed in Scotland independent of the Welsh strain, was described by Greaves and Ayres (12). It was demonstrated by Thijssen (13) that in HS rats the in vitro sensitivity of KO reductase for warfarin inhibition was comparable to that in normal rats but that the inhibitory effect was reversible. We have compared the reductase activities in wild-type and in warfarin-resistant HS rats. In our Scottish resistant strain we observed that the dithiol-dependent reductase(s) were inhibited by warfarin to a similar extent as were the wild-type enzymes (figure 1).The in vitro sensitivity for warfarin was measured in the hepatic (K + DTT)-dependent carboxylase assay, and the warfarin-resistant animals were compared with normal Wistar rats. Whereas the wild-type K-reductase bound irreversibly to warfarin, the drug was rapidly washed off from the mutant enzyme strain with tris-buffered saline (figure 2)

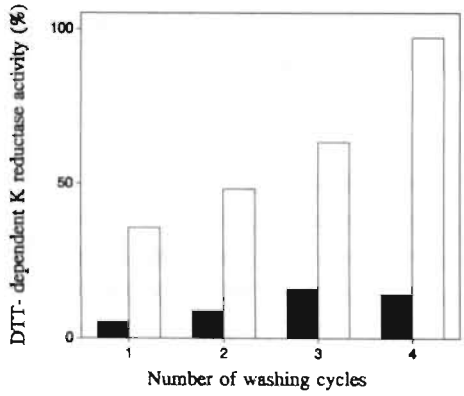

FIG. 2. Effect of washing of microsomes. *Warfarin treated normal (closed bars) and warfarin-resistant (open bars) rats were used for the preparation of microsomes. Step 1 represents the non-washed resolubilized microsomes. Two washing cycles with a low-salt buffer (steps 2 and 3) were followed by a high-salt wash (step 4) and the (K + DTT)-dependent carboxylase activity was measured and expressed as a percentage of that in non-treated animals.*

Like in the Welsh resistant strain, also in the Scottish strain an alteration was found both in KO-reductase (13) as well as in the dithiol-dependent K-reductase. Hence this is the second case in which a mutation leading to warfarin resistance results in an apparent identical mutation in both dithiol-dependent reductases. The probability of simultaneous mutations in two separate genes is extremely low, the chance that simultaneous mutations have taken place in two different strains must be regarded as nil. Therefore we postulate that both dithiol-dependent steps in the vitamin K-cycle are mediated by the same enzyme.

Natural cofactors for the dithiol-dependent reductase

A second point of interest was the origin of the reducing equivalents required for the reduction of KO into KH_2. In an earlier paper from this group it has been demonstrated that in vitro the protein thioredoxin may play a role in the hydrogen transport to KO-reductase (14). We have elaborated on those findings, and postulate a model in which the oxidation of cysteine residues into cystine (mediated by the enzyme protein disulphide isomerase) is coupled to the reduction of KO. In this model two posttranslational models are coupled, and obtain their energy from two successive steps in the oxidation of hydrogen. Additionally it explains why the formation of KO and the formation of Gla are not coupled stoichiometrically: if more cystines than Gla residues are formed (which is probably usual), the recycling of vitamin K must proceed at a higher speed than is required for Gla formation. This provides a rational explanation for the observation that epoxidation and carboxylation are rapidly uncoupled in vitro. Obviously our hypothesis will have to be tested thoroughly. Rather than regarding them as conclusive, we hope that the data obtained thus far will be a starting point for future investigations. For that reason the key paper is reproduced in its complete form.

Conclusions

Both reduction steps in the vitamin K-cycle may be accomplished by dithiol-dependent reductases. Their identical kinetic behaviour towards DTT and inhibition by several compounds (warfarin, lapachol, sulfaquinoxaline) together with the mutation pattern in two independent cases of warfarin resistance strongly supports the idea that both reduction steps are exerted by the same enzyme. In vitro this enzyme may use synthetic dithiols as a reducing cofactor. We have shown that in vitro dithiothreitol may successfully be replaced by a combination of thioredoxin and protein disulphide isomerase. Under these conditions both NAD(P)H and reduced RNAase may serve as reducing cofactors. On the basis of these results it is proposed that the hydrogens which are set free during the formation of disulphide bonds in a posttranslational modification are oxidized via the vitamin K-cycle, and thus provide the energy required for a second posttranslational modification: the conversion of Glu residues into Gla residues.

Stimulation of the dithiol-dependent reductases in the vitamin K-cycle by the thioredoxin system: strong synergistic effects with Protein Disulphide-Isomerase

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Abstract

It has been shown previously that the thioredoxin system (thioredoxin + thioredoxin-reductase + NADPH) may replace dithiothreitol (DTT) as a cofactor for vitamin KO- and K-reductase in salt-washed detergent-solubilized bovine liver microsomes. Here we demonstrate that the system can be improved further by adding protein disulphide-isomerase (PDI) to the components mentioned above. Moreover, NADPH may be replaced by reduced RNAase as a hydrogen donor. In our in vitro system the various protein cofactors were required at concentrations of 2-5 orders of magnitude lower than that of DTT, whereas the maximal reaction rate was about 3-fold higher. PDI stimulated the thioredoxin-driven reaction about 10-fold with an apparent K_M value of 8 μM. These data suggest that in the in vitro system the formation of disulphide bonds is somehow linked to the vitamin K-dependent carboxylation of glutamate residues. In vivo both disulphide formation and vitamin K-dependent carboxylation are post-translational modifications taking place at the luminal side of the endoplasmic reticulum of mammalian secretory cells. The possibility that the reactions are also coupled in vivo is discussed.

Introduction

Most secretory proteins undergo extensive post-translational processing before they are secreted into the extracellular environment. Although more than 100

posttranslational modifications are presently known (15), most of these reactions have been studied in less detail than transcription and translation, the earlier steps in protein biosynthesis. In this paper we focus our attention on two of these post-translational modifications: the conversion of glutamate (Glu) into gammacarboxyglutamate (Gla) residues, and the formation of the disulphide bonds.

The formation of Gla-residues is accomplished during a carboxylation reaction, which is catalyzed by the microsomal enzyme γ-glutamylcarboxylase (hereafter termed: carboxylase). Vitamin K hydroquinone (KH_2) functions as a cofactor in this reaction (16,17). The oxidation of KH_2 into an epoxide (KO) provides the energy required for the carboxylation step. In two successive reduction steps (figure 1) KO is subsequently recycled into KH_2. In vitro the synthetic dithiothreitol (DTT) as well as reduced thioredoxin may serve as a reducing cofactor for KO- and K-reductase (14,18).

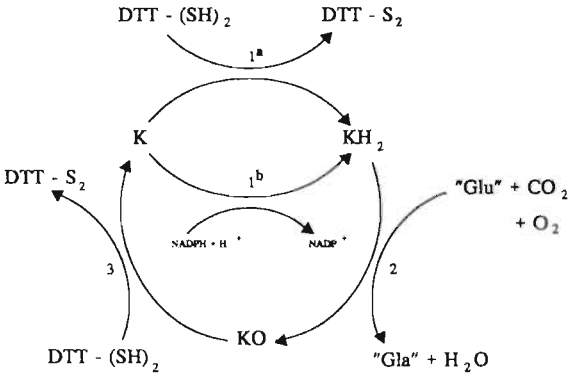

FIG. 1. The vitamin K cycle. Enzymes catalysing the various steps are: 1a, dithiol-dependent vitamin K reductase; 1b, NAD(P)H-dependent K reductase; 2, γ-glutamylcarboxylase; 3, dithiol-dependent KO reductase. 'Glu' stands for peptide- or protein-bound Glu residues.

In its reduced form thioredoxin (12,000 Da) is a powerful protein disulphide-reductase and acts as a hydrogen donor for the enzymatic reduction of ribonucleotides and methionine sulphoxide (19,20). Dihydrolipoic acid (21) and dihydrolipoamide (H.H.W. Thijssen, personal communication) also have reductase cofactor activity. The origin and the nature of the physiological reduction system is still a matter of debate, however (22).

Nascent polypeptide chains entering the lumen of the endoplasmic reticulum contain free SH-groups, which are transformed into native disulphide bonds by

net oxidation and the suggested catalytic action of an enzyme called protein disulphide isomerase (PDI, EC 5.3.4.1, for a review see (21)). PDI is a 57,000 Da acidic protein in the lumen of the endoplasmic reticulum possessing two thioredoxin-like domains with the active site sequence Trp-Cys-Gly-His-Cys. Recently it was demonstrated that the thioredoxin-like domains of oxidized PDI are substrates for NADPH and mammalian thioredoxin-reductase (TR; EC 1.6.4.5), and that PDI has thioredoxin-like activity in the reduction of insulin disulphides (23). PDI and thioredoxin thus share a similar mechanism of action, summarized in equations (1) and (2):

$$PDI\text{-}S_2 + NADPH + H^+ \xrightarrow{TR} PDI\text{-}(SH)_2 + NADP^+ \quad (1)$$

$$PDI\text{-}(SH)_2 + \text{insulin-}S_2 \xrightarrow{\text{spontaneously}} PDI\text{-}S_2 + \text{insulin-}(SH)_2 \quad (2)$$

In equation (1) TR stands for thioredoxin-reductase. PDI-S_2 is also readily reduced by reduced thioredoxin. Hence PDI has a higher redox potential and is a much weaker reductant than is thioredoxin (23). This suggests that PDI is reduced by SH-groups in the nascent proteins according to equation (3):

$$PDI\text{-}S_2 + \text{protein-}(SH)_2 \rightleftharpoons PDI\text{-}(SH)_2 + \text{protein-}S_2 \quad (3)$$

Obviously the PDI-bound sulphydryl groups should be oxidized somehow, but nothing is known about the nature and origin of the oxidizing cofactor(s) involved (24).

The common aspects for the vitamin K-dependent carboxylation and the disulphide bond formation are:

1. Both reactions take place at the luminal side of the rough endoplasmic reticulum (similarity of location).
2. Both reactions occur during the early steps of post-translational modification (similarity in time).
3. Both involve thiol-disulphide exchange reactions, but their redox cofactors have remained obscure despite substantial amounts of research (similarity of problem).

Because of all these similarities we have investigated in an in vitro system whether these two reactions are coupled, so that the disulphide bond formation provides the reducing equivalents required for the reduction of KO and K, i.e. whether the reducing equivalents produced during the formation of the cystine

bonds may be oxidized via the vitamin K cycle. The results of these investigations are presented in this paper.

Materials and methods

Materials. Vitamin K_1 was obtained from Merck (Darmstadt, Germany) and converted to its epoxide form (KO) according to the procedure of Fieser et al. (25). The various forms of vitamin K were added to the reaction mixtures as an ethanol solution. $NaH^{14}CO_3$ (55 Ci/mol) was from Amersham International (Amersham, Bucks., U.K.) and the pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) from Vega Biochemical Co. (Tucson, AZ, U.S.A.). DTT, CHAPS, lipoamide (reduced form), and glutathione (reduced form) were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade or better.

Proteins. The following proteins were prepared from bovine tissues according to previously described methods: PDI (23), thioredoxin (26), TR (27,28). Glutaredoxin was prepared from E.coli (29). E. coli thioredoxin and TR (19) were from Imco (Stockholm, Sweden). Glutathione-reductase (from yeast; EC 1.6.4.2) was purchased from Boehringer Mannheim (Germany) and insulin (from pig pancreas) and RNAase (from bovine pancreas; EC 3.1.27.5), were from Sigma. RNAase was reduced in 100 mg quantities by incubating the enzyme for 30 min in the presence of 200 mM DTT and 8 M urea in buffer C (Tris-buffered saline at pH 8.5) and 37 °C. After removal of the DTT and urea by size exclusion chromatography in buffer C on a PD 10 column (Pharmacia) the samples were flushed with nitrogen gas and kept in sealed tubes at 4 °C until use. Salt-washed, detergent-solubilized microsomes were used as a source of the carboxylase/reductase complex (30). After the extensive washing procedure, the PDI content of these microsomes was determined using the micromethod of insulin reduction (see below) and turned out to be 3.5 µM. The microsomes contained neither thioredoxin nor TR in detectable amounts (by Western blot analysis).

Various assays. Vitamin K-directed carboxylase activity was determined as described earlier (31), but with some modifications to allow for a maximal activity of the PDI/thioredoxin system. Reaction mixtures contained 25 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.4% (w/v) CHAPS, 0.1 mM vitamin K quinone, 5 µCi of $NaH^{14}CO_3$ and 1 mg of microsomal proteins; the

incubation temperature was 20 °C. Reducing cofactors were added as indicated. Initial reaction rates were calculated from time courses obtained after incubations for 0, 5, 10, 20, and 30 min. Each experimental point was determined in duplicate, and blank values (no vitamin K added) were subtracted throughout.

KO-reductase was assayed in 0.25 ml reaction mixtures of a similar composition as described for carboxylase, but the samples were preincubated at 20 °C for 3 min and the reaction was started by adding KO instead of K. Aliquots (50 µl) were taken after incubation for 2.5, 5, and 10 min; during this period the reaction rates were linear. Vitamin K metabolites were extracted from the reaction mixtures and analyzed by HPLC as described by de Boer-van den Berg et al. (32).

Kinetic data were calculated by the non-linear least-squares technique (33), in which we used the initial reaction rates at 6-8 different substrate concentrations, ranging from 0.25 to 4 times the apparent K_M value of the compound tested. The data are presented as the means ± S.E.M. of three independent experiments.

The enzymatic activities of PDI, thioredoxin, TR and glutaredoxin were tested by methods described previously (23,28). Protein concentrations were assessed according to Sedmak & Grossberg (34).

Results

Cofactors for vitamin K-dependent carboxylase. In a first set of experiments we have compared synthetic DTT with seven natural reducing agents for their ability to stimulate the vitamin K-dependent carboxylase reaction in vitro (Table I). A low but distinct activity was found in the presence of NADH and NADPH, but the reaction was insensitive to warfarin up to concentrations as high as 100 µM. In contrast, the reactions stimulated by the other cofactors were all highly sensitive to inhibition by warfarin, with apparent K_i values below 1 µM. This demonstrates once more that the NAD(P)H-dependent reduction of K is accomplished via an alternative pathway (35), which will not be discussed in this paper. From Table I it appears that DTT, thioredoxin and lipoamide are all three capable of stimulating the K-dependent carboxylase reaction, but that thioredoxin differs from the other two cofactors by its low apparent K_M. Therefore we regard thioredoxin as a good candidate for being a natural cofactorfor the reduc-

tases involved in the vitamin K cycle. Maximal activity was only obtained in the presence of the NADPH-dependent enzyme TR, suggesting that in our system oxidized thioredoxin is recycled via this pathway.

Compound added	K_M^{app} (mM)	V_{max}^{app} (relative rate)
1. DTT	0.8 ± 0.2	100 ± 18
2. NADH	1.5 ± 0.3	7 ± 2
3. NADPH	1.1 ± 0.3	8 ± 3
4. Glutathione	n.d.	n.d.
5. Thioredoxin + TR + NADPH	0.022 ± 0.005	32 ± 7
6. Glutaredoxin	n.d.	n.d.
7. PDI	n.d.	n.d.
8. PDI + TR + NADPH	n.d.	10 ± 2
9. Lipoamide	0.6 ± 0.1	21 ± 4

Table I. Reducing agents as cofactors for K-dependent carboxylase. *All measurements were performed under the standard conditions described in Materials and Methods. Both thioredoxin and PDI were tested in the presence of an excess of TR (0.2 μM) and NADPH (4 mM). PDI (up to 24 μM) was also tested in the absence of these compounds. Glutaredoxin (highest concentration: 65 μM) was assayed in the presence of an excess of glutathione (4 mM), glutathione-reductase (10 μg/ml), and NADPH (4 mM). The effect of PDI was checked in the presence of 4 mM NADPH. Oxidation of the protein dithiols was retarded by the presence of 10 μM DTT in all reaction mixtures. Both thioredoxin and TR were from E. coli. N.d. stands for not detectable.*

Additional cofactors for (vitamin K + thioredoxin)-dependent carboxylase. Because the maximal reaction rate of the DTT-stimulated reaction was still 3-fold higher than that of the thioredoxin-catalysed one, we investigated whether the latter reaction could be stimulated further by one of the other cofactors mentioned above. As is shown in Table II only PDI had a measurable effect, and it is notable that the combination of PDI + thioredoxin resulted in a 3-fold higher reaction rate than was obtained with DTT. The apparent K_M (8 μM) for PDI was about 12-fold higher than the concentration of endogenous PDI (0.7 μM, see Materials and Methods) in our reaction mixtures, which explains the stimulatory role of added PDI. We have also compared the characteristics of a

system containing thioredoxin and TR from E. coli with a system in which all cofactors were of bovine origin.

Compound added	K_M^{app} (mM)	V_{max}^{app} (relative rate)
1. None	---	32 ± 5
2. DTT	0.8 ± 0.3	100 ± 24
3. Glutathione	---	29 ± 4
4. Glutaredoxin	---	30 ± 3
5. Lipoamide	n.d.	36 ± 7
6. PDI	0.008 ± 0.002	335 ± 66

Table II. Stimulation of (thioredoxin + K)-dependent carboxylase. *All reaction mixtures contained thioredoxin (0.2 mM), TR (0.2 μM) and NADPH (4 mM). Further details are as described in the legend to Table I.*

As can be seen from Table III, the apparent K_M values for thioredoxin and PDI were similar in both systems, whereas in the homologous system that for TR was slightly (4-fold) higher than in the heterologous system. This may be due to the rather high sensitivity of mammalian thioredoxin/TR systems to irreversible inactivation by molecular oxygen (36), or to the fact that bovine TR has a broader substrate specificity than the E. coli enzyme (19).

Reaction component	K_M^{app} (μM) in system with Trx/TR from	
	E.coli	calf thymus
Thioredoxin	20 ± 6	21 ± 5
Thioredoxin reductase	0.019 ± 0.006	0.081 ± 0.012
PDI	8.1 ± 1.8	7.9 ± 2.4

Table III. Apparent K_M for cofactors of vitamin K-dependent carboxylase *All measurements were performed under standard conditions. Trx stands for thioredoxin. If added in excess, the concentration of the various reaction components was 10 times the K_M value. Further details are as described in the legend to Table I.*

In all experiments mentioned thus far NADPH was used as a hydrogen donor for the PDI-containing systems; the physiological reductant of PDI is probably not NADPH but more likely a reduced protein. Therefore we have measured the

kinetic properties of the (PDI + thioredoxin)-driven carboxylase reaction in the presence of either NADPH or reduced RNAase (Table IV).

System used	$^{14}CO_2$ incorporated (dpm)
DTT	5,419 ± 1,877
Trx/TR + NADPH	2,864 ± 724
Trx/TR + PDI + NADPH	16,320 ± 4,518
Trx/TR + reduced RNAase	92 ± 25
Trx/TR + PDI + reduced RNAase	12,941 ± 2,932

Table IV. Reduced RNAase as a cofactor for K-dependent carboxylase. *All incubations were performed for 30 min under standard conditions, and the results are expressed as dpm incorporated per mg of microsomal proteins. Each experimental point was determined in triplicate, and the values are means ± S.E.M. Trx stands for thioredoxin. The various reaction components were used in the following concentrations: DTT, 4 mM; E. coli thioredoxin, 0.2 mM; E. coli TR, 0.2 μM; PDI, 24 μM; NADPH, 4 mM; reduced RNAase, 0.2 mM. Further details are as described in the legends to Tables I and II.*

No differences between the two systems were observed, except that the RNAase-directed reaction could be blocked completely by 100 μM warfarin, whereas in the presence of NADPH the inhibition by warfarin was only 92%. The residual activity probably originated from the warfarin-insensitive NADPH-dependent K reductase mentioned earlier in this paper.

Cofactors for KO-reductase. The reductases of the vitamin K cycle may be tested directly by extraction of the various K metabolites from the reaction mixtures, followed by HPLC analysis. At rate-limiting concentrations of the various cofactors this method was not sufficiently accurate to yield reproducible kinetic data. Therefore we have measured the effect of the various cofactors for KO reductase under non-limiting conditions only. The results are summarized in Table V, and demonstrate the same tendency as was found for K-dependent carboxylase: PDI alone is not a cofactor for the enzyme, the cofactor activity of (thioredoxin + TR) is 30-40% that of DTT, and the combination of thioredoxin, thioredoxin-reductase and PDI may stimulate the reaction to a level which is several fold higher than that reached with DTT alone. Like in the case of K-dependent carboxylase, the data obtained with thioredoxin and TR from E. coli were slightly better than those obtained with the all-bovine system.

Cofactor(s)	Source of Trx/TR	Rate of KO reduction (pmol K.mg^{-1}.min^{-1})
DTT	---	25.6 ± 3.2
PDI	---	0
Trx	E. coli	13.6 ± 2.9
Trx	bovine thymus	9.7 ± 2.7
Trx + PDI	E. coli	57.1 ± 14.8
Trx + PDI	bovine thymus	49.8 ± 11.5

Table V. Cofactor activity of various reductants for KO reductase. *All measurements were performed under standard conditions; the concentrations of the various components (if added) were 10 times the K_M^{app} values found for the K-dependent carboxylation reaction. The results are expressed as pmol K formed per mg of microsomal proteins per min. Each experimental point was determined in triplicate, and the values are means ± S.E.M. Trx stands for thioredoxin. Further details are as in the legend to Table I.*

Discussion

Despite considerable efforts neither KO-reductase nor vitamin K reductase have yet been purified to homogeneity. Therefore we have used the rather crude system of salt-washed detergent-solubilized microsomes for all experiments described in this paper. It was shown that under these experimental conditions the combination of reduced RNAase, PDI and thioredoxin/TR is superior to DTT in providing the reducing equivalents required in the vitamin K cycle. This is demonstrated by the fact that the apparent K_M values for these compounds are between 2 and 5 orders of magnitude lower than that for DTT, whereas the maximal reaction rate is about 3 fold higher. These conclusions hold true for homologous systems in which all proteins are of bovine origin, but also for systems in which thioredoxin and thioredoxin-reductase are from E. coli. Advantages of the latter proteins are that they are commercially available, and that they are less sensitive to inactivation by thiol oxidation with molecular oxygen (19). Our data clearly demonstrate that in an in vitro system the process of disulphide bond formation may be coupled to the reductive steps in the vitamin K cycle. All reaction components required for this coupling are natural compounds, the occurrence of which in the endoplasmic reticulum has been demon-

strated (37-39). However, the way in which this coupling is brought about remains uncertain.

The fact that reduced thioredoxin is the only reaction component displaying cofactor activity for KO and K reductase seems to support the reaction pathway shown in figure 2A. This is consistent with results from Gardill and Suttie (40), who demonstrated that E.coli thioredoxin may be used as an affinity ligand for the partial purification of KO-reductase. On the other hand it is likely that PDI is involved in disulphide bond formation in the way shown in figure 2B, and it is tempting to speculate about the possibility that both processes are directly coupled (i.e. X = PDI and Y = thioredoxin). However, such a sequence of events is not in line with the respective redox potentials of the proteins involved, thioredoxin being a much stronger reductant than is PDI (23,41). This was previously also concluded from experiments in which mixtures containing equimolar concentrations of reduced thioredoxin and oxidized PDI were incubated, and in which an equilibrium was reached after 90% of the thioredoxin and 10% of the PDI were in the oxidized state (36). It should be realized, however, that in the optimal carboxylating system the apparent K_M for PDI is about 3-fold lower than that for thioredoxin (Table III), and that the concentration of PDI in the lumen of the endoplasmic reticulum is very high (21). Under these conditions, and provided that most of the PDI occurs in the reduced state, it cannot be excluded that sufficient amounts of thioredoxin are reduced to allow for its involvement in the vitamin K cycle. The likelihood of such a mechanism being operational in vivo therefore depends on the ratio between the luminal concentrations of PDI and thioredoxin in the endoplasmic reticulum. But although with the aid of immunohistochemical techniques the occurrence of thioredoxin in the lumen of the endoplasmic reticulum has been demonstrated unequivocally (39), its local concentration is not known at this time.

An alternative explanation for our data is that in the in vitro system thioredoxin (a powerful protein disulphide reductase) is required to reduce critical disulphides on the carboxylase, which have artificially formed during the microsome preparation and which inactivate the enzyme. Additionally, both thioredoxin and PDI may be active as reductants supplying electrons during the reduction of KO. This explanation implies that in vivo PDI-$(SH)_2$ may even function as the only cofactor for KO-reductase. It is well known that the synthesis of Gla (carboxylase activity) and the formation of KO (epoxidase activity) only proceed at equi-

molar rates at saturating concentrations of CO_2 and carboxylatable substrate (42). Most probably these conditions are not met in vivo, and it is an intriguing to speculate why the recycling of vitamin K may be uncoupled from the carboxylase activity, and may exceed by far the rate of Gla formation. A putative relation between disul-phide bond formation and the reductive steps in the vitamin K cycle readily explains the necessity of such an uncoupling, however; in vivo the number of disulphide bonds formed during protein synthesis will probably exceed that of Gla-residues; hence the oxidation rate of KH_2 must be higher than the protein carboxylation rate.

An alternative explanation for the putative involvement of PDI in the vitamin K cycle would be that the enzyme has a structural role. In our model all proteins involved in the carboxylation reaction (PDI, TR, thioredoxin and the enzymes of the vitamin K cycle) must be aligned in a complex protein/ phospholipid aggregate, which is disrupted by the preparation procedure for the microsomes and by the detergent used in the assay. It is known that PDI is the β-subunit of prolyl hydroxylase, where it serves as a structural component (43), and it might have a similar role in the carboxylase/reductase complex. This hypothesis requires further confirmation, however. Experimental systems should be developed, in which it is possible to demonstrate whether the model proposed in figure 2 occurs in vivo. If the coupling between disulphide bond formation and vitamin K recycling proves to be a physiological route, an explanation will also have to be found for the mechanism of disulphide bond formation during periods of oral anticoagulant treatment.

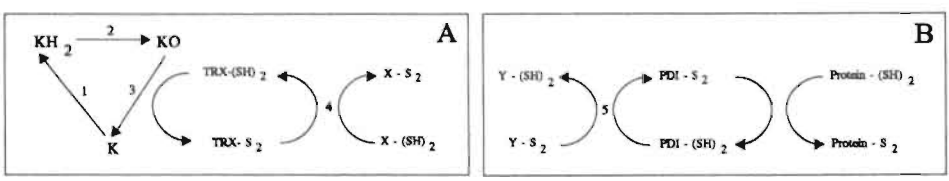

FIG. 2. Oxidoreductases involved in the reduction of KO and K (A) and possible redox carriers for the oxidation of protein-bound cysteine SH groups (B). *Both reactions 4 and 5 may occur spontaneously, but are catalyzed by TR. X and Y stand for hypothetical redox cofactors, not yet identified. The possibility that the recycling of PDI is directly coupled to that of thioredoxin and vitamin K is discussed in the text.*

The conclusions which can be drawn thus far are that for in vitro studies the PDI/thioredoxin system is an improvement over the commonly used synthetic dithiols like DTT. Maximal activity is only obtained if both proteins (PDI as well as thioredoxin/TR) are present in the reaction mixture, but the precise mechanism by which they participate in the reduction of KO and K is presently uncertain. The PDI/thioredoxin system is a good candidate to be the physiological 'cofactor' providing the reducing equivalents required in the vitamin K cycle, but whether these dithiol proteins are involved in the vitamin K cycle in vivo remains to be proven.

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CHAPTER 6

Substrates for vitamin K-dependent carboxylase

Based on:

- Soute, B.A.M., Budé, R., and Vermeer, C. (1991). Vitamin K-dependent carboxylation of poly-L-glutamate. Biochim. Biophys. Acta 1073, 434-436.
- Soute, B.A.M., Balland, A., Faure, T., de la Salle, H., and Vermeer, C. (1989). In vitro carboxylation of a blood coagulation factor IX precursor produced by recombinant-DNA technology. Thromb. Haemostas. 61, 238-242.
- Wu, S.-M., Soute, B.A.M., Vermeer, C., and Stafford, D.W. (1990). In vitro gammacarboxylation of a 59-residue recombinant peptide including the propeptide and the gammacarboxyglutamic acid domain of coagulation factor IX: effect of mutations near the propeptide cleavage site. J. Biol. Chem. 265, 13124-13129.

Introduction

Since the discovery of the enzyme γ-glutamylcarboxylase extensive investigations have been carried out to understand substrate recognition requirements and to develop good substrates for the in vitro assay of the enzyme. In the first carboxylating enzyme systems the pool of endogenous precursor proteins, accumulated during in vivo warfarin treatment or vitamin K-deficiency, served as a substrate for carboxylase (1). However, because of the poor possibilities to quantitate the endogenous substrate and the number of carboxylation events, these systems were less useful for kinetic studies. The development of synthetic exogenous peptides gave new impulses to the study of the enzymology of the system (2,3). The first generation of artificial substrates invariably consisted of short peptides homologous to the N-termini of the Gla-containing plasma proteins. Examples are the pentapeptide Phe-Leu-Glu-Glu-Val, corresponding to residues 5 to 9 of the bovine prothrombin precursor, and Phe-Leu-Glu-Glu-Leu, corresponding to residues 5 to 9 in the human FVII precursor (2,4). They have been of great value in elucidating molecular details of the carboxylase reaction and have facilitated efforts to partially purify the enzyme. However, their high K_M^{app} (2-8 mM), their low efficiency of carboxylation (5) and their inability to carboxylate adjacent glutamic acid residues made it less probable that they were comparable with the endogenous substrates (6). The reason for their suitability as substrates in the carboxylation reaction is probably the fact that they can be used in very high concentrations (5-10 mM). In the late 1980's substrates were developed which contain the carboxylase recognition (or "pro") sequence (7,8). Because of their high affinity for carboxylase these so-called second generation substrates have played a crucial role in the purification of the enzyme.

Vitamin K-dependent carboxylation of Poly-L-Glutamate

The "vitamin K-dependent" plasma proteins contain a number of Gla-Gla sequences which have formed the base for the first synthetic substrates. Peptides with Glu-Glu sequences are in general better substrates than those containing only one Glu-residue and also the residue preceding the Glu-Glu sequence seems to be relevant for substrate activity (9). We have investigated whether

sequence homology with Gla-containing coagulation factors is a prerequisite for proper carboxylation. Therefore three poly-L-glutamate preparations of varying chain length were used as substrates for bovine liver γ-glutamylcarboxylase. The quality of these substrates (as measured by their apparent kinetic constants) was compared to that of TPT (the tripeptide Boc-Glu-Glu-Val) and FLEEL (the pentapeptide Phe-Leu-Glu-Glu-Leu) two other well known substrates for vitamin K-dependent carboxylase and to that of free glutamic acid and N-t-Boc-Glu-αBE. All experiments were performed both in the presence and in the absence of $(NH_4)_2SO_4$. The effect of the latter component was checked because it has been reported to stimulate the carboxylation of various synthetic substrates (10). As is shown in table I all three poly-Glu preparations could serve as a substrate for carboxylase, and the amount of CO_2 incorporated increased with increasing molecular weight of the substrate.

Substrate	Concentration (mM)	$^{14}CO_2$ incorporated (pmol)	
		$+(NH_4)_2SO_4$	$-(NH_4)_2SO_4$
Glutamic acid	50	8.1	---
N-t-Boc-Glu-αBE	25	22.5	6.7
TPT	1	384.9	77.0
FLEEL	1	449.1	80.2
Poly-Glu_{5-10}	1	122.8	14.9
Poly-Glu_{10-50}	1	214.8	45.9
Poly-Glu_{50-100}	1	793.7	206.3

Table I. Carboxylation of various substrates. *All reaction mixtures were incubated for 30 min at 20 °C under standard conditions.*

The stimulation by $(NH_4)_2SO_4$ was 4-9 fold, which is comparable to that with the pentapeptide FLEEL. As is shown in table II, the apparent K_M^{app} values were closely similar for all three poly-Glu preparations. On the other hand, the maximal reaction rates increased at increasing peptide chain length in such a way that the ratio between V_{max}^{app} and molecular weight was more or less constant. An explanation for this phenomenon might be that carboxylation only takes place at isolated, widely separated positions of the poly-Glu chain, and that the longitudinal movement of carboxylase along the polypeptide chain is much faster than the association/dissociation of the enzyme and its substrate.

Presently we have no arguments to support this hypothesis, however. From these experiments we have concluded that the presence of a specific aminoacid sequence for a Glu-containing peptide to be used as a substrate for vitamin K-dependent carboxylase is not required. It seems as if the only restriction is that both the α-carboxyl and the amino group form part of a peptide bond. As may be calculated all poly-Glu substrates were carboxylated with a very low efficiency: less than one Gla-residue was formed per 2000 Glu-residues per h.

Substrate	Apparent kinetic constants for poly-Glu carboxylation	
	K_M^{app} (mM)	V_{max}^{app}(pmol min^{-1})
Poly-Glu_{5-10}	1.5	1.66
Poly-Glu_{10-50}	1.8	3.24
Poly-Glu_{50-100}	1.1	8.08

Table II. Apparent kinetic constants for poly-Glu carboxylation in the presence of $(NH_4)_2SO_4$. *Standard reaction mixtures contained varying amounts of either poly-Glu_{5-10}, poly-Glu_{10-50} or poly-Glu_{50-100}. Initial carboxylation rates were determined after incubation of the various mixtures for 0, 5, 10, 20 and 30 minutes at 20 °C. The V_{max}^{app} is expressed as pmoles $^{14}CO_2$ incorporated per min.*

Vitamin K-dependent carboxylation of descarboxy-coagulation factors

Another group of substrates lacking the prosequence is formed by the "natural substrates". Anticoagulant treatment of cattle and humans results in the excretion of descarboxy forms of the vitamin K-dependent coagulation factors. In contrast to earlier expectations several authors reported these descarboxy proteins either to have no substrate activity at all, or be very poor substrates (11,12). K_M^{app} values have been found in the range of those for the peptides mentioned above, i.e. far beyond normal plasma levels. Therefore descarboxy proteins are not likely to function as substrates for carboxylase in vivo. From the cDNA sequences of vitamin K-dependent plasma proteins it was predicted that the primary gene

products of these proteins contain a propeptide (13). In recent years this propeptide has shown to function as a recognition site for the enzyme γ-glutamylcarboxylase (14,15). The presence of this γ-carboxylation recognition site was first detected in the propeptide of FIX (16), and with site-specific mutagenesis the significance of the propeptide for carboxylation has been shown (16,17.18). Based upon this knowledge various peptides containing the propeptide have been constructed (7,8), which all were characterized by K_M^{app} values of at least three orders of magnitude lower than those for the peptides not containing a prosequence. In a collaborative project with Transgene (Strasbourg) recombinant descarboxy FIX was constructed, containing the propeptide necessary for γ-carboxylation. It was shown that these large protein substrates could be carboxylated efficiently (K_M^{app} = 0.12 μM) in vitro and that when analysed by PAGE in SDS, the carboxylated product was similar to normal plasma FIX. FIX procoagulant activity was not found and could neither be expected because:

a) the strongly reducing conditions during the carboxylation reaction will rapidly destroy all disulphide bonds in the precursor protein and
b) the propeptide, which remains attached to the carboxylated product, has been reported to prevent normal procoagulant activity (17).

If the production of biologically active FIX via expression in eukaryotic cell lines turns out not to be successful, the in vitro carboxylation method might be an alternative approach to produce biologically active FIX from incompletely carboxylated recombinant material. In that case it should be checked whether by the replacement of DTT by the PDI/thioredoxin system (19) the disulphide bonds will remain intact. Also the propeptide will have to be removed, e.g. by the recently purified microsomal liver endopeptidase (20).

A recent paper of our group on this subject is reproduced at the end of this chapter.

In vitro γ-carboxylation of a 59-residue recombinant peptide including the propeptide and the γ-carboxyglutamic acid domain of coagulation factor IX

Since it has become apparent that a major part of the signal, necessary for carboxylase to recognize the substrate, resides in the propeptide of the vitamin K-dependent coagulation factors, the importance of the propeptide in carboxylation has been documented by the use of synthetic peptides. However, until three years ago the best peptide substrate reported was proPT28 that was carboxylated in vitro to only 45% of the theoretical value. The short synthetic peptides used failed to include the complete Gla domain (array of glutamic acid residues that are normally carboxylated).

There is evidence indicating that sequences other than the propeptide are important in enzyme recognition. Decarboxylated bone Gla protein (21) and fragment 13-29 of prothrombin (22) were found to be good in vitro substrates for carboxylase. The role of residues other than the propeptide in recognition by the carboxylase is still not resolved, and there may be other, as yet unrecognized, signals that are necessary for processes like translocation of the substrate across the carboxylase enzyme or release of the substrate from the enzyme. Answers to these questions may require the use of still longer peptides. Therefore the production in Escherichia coli of a fusion protein from which a 59-residue peptide has been isolated has been started by Wu and Stafford. The peptide included the propeptide and all of the glutamic acid residues of factor IX which are normally carboxylated. In addition peptides with mutations identical to $FIX_{San\ Dimas}$ (arginine to glutamine at -4) or $FIX_{Cambridge}$ (arginine to serine at -1) as well as a peptide with both mutations in the propeptide sequence have been prepared and the effect of the mutations on in vitro carboxylation has been examined. The 59-amino acid peptide is an efficient substrate for in vitro γ-carboxylation (see table I). Its K_M^{app} (0.55 μM) is several thousand-fold lower than that of the commonly used substrate FLEEL and about 5 times lower than proPT28 or proFIX28 (7,8). The difference with the latter two substrates indicates that besides the propeptide sequence, part of the binding site for the carboxylase resides in the Gla-domain itself. This is consistent with the suggestion of Price et al. (23) that a sequence similarity (Gla-X-X-X-Gla-X-Cys at the positions +17 to +23) together with an essential sulfhydryl group in the carboxylase which forms a

disulphide bond with the substrate during carboxylation, play an additional role in the substrate selection by carboxylase.

Substrate	V_{max}^{app} (cpm/min)	K_M^{app} (μM)	V_{max}^{app}/K_M^{app} (cpm/min/μM)
FIXGla	1015	0.55	1845
FIXQ-4	1455	0.87	1672
FIXS-1	1403	0.70	2004
FIXQ/S	1504	0.74	2032

Table I. Enzyme kinetic studies of the FIXGla peptide and three mutant forms. *Three kinetic parameters K_M^{app}, V_{max}^{app} and V_{max}^{app}/K_M^{app} were determined by Lineweaver-Burk and Eadie-Hoffstee plots. FIXGla stands for a 59-residue peptide including the propeptide and the complete FIX Gla domain in its non-carboxylated form; FIXQ-4 is the mutant form in which arginine is changed into glutamine at -4; FIXS-1 is the mutant form in which arginine is changed into serine at -1; FIXQS has both mutations.*

In addition, FIXGla is the first peptide substrate that is carboxylated in vitro to more than one Gla/molecule (6-11 gammacarboxyglutamic acids/molecule). These high levels of carboxylation were obtained after prolonged incubation (48 h at 10 °C). The extent of carboxylation was calculated from the specific activity of the radioactive CO_2 and the concentration of the peptide. Enzyme kinetic studies revealed no significant difference in V_{max}^{app}/K_m^{app} values between normal and mutant substrates. Maximum CO_2 incorporation was achieved with the double mutant. Several mutations have been described in which the cleavage of the propeptide from factor IX is hampered. Two of these mutants, factor $IX_{San\ Dimas}$ (24) and factor $IX_{Cambridge}$ (25), have been reported to be undercarboxylated to the same extent (7 Gla/molecule). From our study, we propose that the highly conserved residues arginine -1 and arginine -4 are not important for γ-carboxylation but function as the recognition site for the propeptidase. The presence of both arginine residues appears to be critical for the cleavage of propeptide by the enzyme propeptidase as both factor $IX_{San\ Dimas}$ and factor $IX_{Cambridge}$ retain their propeptide, which probably explains their dysfunction. Bone matrix protein, which contains a propeptide-like sequence with a single arginine corresponding to the -1 position of other vitamin K-dependent coagulation proteins, is not

cleaved, and the propeptide-like domain is retained in the mature protein. Recently Kawabata & Davie (20) have purified a microsomal endopeptidase the activity of which for proteolytic cleavage was dependent on arginine residues located at the -1 and -4 position. This finding supports the hypothesis that both arginine residues are only important for the cleavage of the propeptide and not for carboxylation.

From these data we conclude that 1) FIXGla and the mutants described above are excellent substrates for studying the mechanism of gamma-carboxylase, 2) Although arginines at positions -4 and -1 are highly conserved in the propeptide sequence of all the vitamin K-dependent proteins, neither is critical for γ-carboxylation.

Conclusions

During the last decade our knowledge about the substrates in the vitamin K-dependent carboxylation reaction has been substantially increased. A number of peptide substrates similar to the Gla-containing sequences in some coagulation factors have been synthesized, except that Gla had been replaced by Glu. K_M^{app} values of these substrates were all in the millimolar range which meant that they were poorly recognized by the enzyme. The presence of a γ-carboxylation recognition site seemed to be required for efficient in vitro carboxylation. The construction of the propeptide and peptide substrates containing the propeptide demonstrated that a carboxylation recognition site indeed was critical for efficient carboxylation. Not only the understanding of the mechanism of γ-glutamylcarboxylation has been facilitated with the propeptide but finally Wu et al. (26) and Berkner et al. (27) have succeeded in the purification of carboxylase making use of the tight binding of enzyme and propeptide.

In vitro carboxylation of a blood coagulation factor IX precursor produced by recombinant DNA technology

Berry A.M. Soute, Alain Balland, Thérèse Faure, Henri de la Salle, Cees Vermeer

Abstract

Blood coagulation factor IX (Christmas factor) is a plasma protein which is required for normal haemostasis. A functional deficiency of factor IX results in haemophilia B, a bleeding disorder which is generally treated by infusions of factor IX concentrates prepared form pooled human plasma. The use of human blood products is connected with the risk of transmitting viral agents responsible for diseases such as hepatitis B and AIDS. Recombinant DNA techniques may provide in the means to produce the required proteins without exposing the patients to these risks and at lower costs. One of the problems which has to be overcome before recombinant factor IX can be used for therapeutical purposes is related to the vitamin K-dependent carboxylation of its 12 NH_2-terminal glutamate residues. In cell cultures this carboxylation, which is required to render the protein its procoagulant activity, is far from complete, especially at high expression levels. In this paper we describe the in vitro carboxylation of non and/or partly carboxylated recombinant factor IX produced by transformed Chinese hamster ovary cells. The identity of the newly formed Gla residues was verified and it could be demonstrated that all carboxyl groups had been incorporated into the recombinant factor IX.

Introduction

The clinical use of proteins obtained by recombinant DNA technology may be hampered due to the inability of certain hosts to correctly process molecules normally subjected to post translational modifications. Some of these problems

have been solved by using eukaryotic cell lines as host. Recombinant factor IX produced by baby hamster kidney cells (28), Chinese hamster ovary (CHO) cells (29) and human hepatoma cells (30) is partially active, indicating at least some correct post translational processing. After cleavage of the 28 amino acid signal peptide, factor IX undergoes post translational modifications including glycosylation, hydroxylation at aspartic acid residue 64 and the vitamin K-dependent γ-carboxylation of the first 12 glutamate residues of the mature protein (13,31,32). This latter modification is thought to be directed by an 18 amino acid N-terminal prosequence which is cleaved off before factor IX appears in the plasma (15-17,33). It is generally accepted that the γ-carboxylation is the limiting factor in the expression of high levels of active recombinant factor IX. It has been documented that at high expression levels, recombinant factor IX secreted by CHO cells has a very low specific activity due to inefficient γ-carboxylation (29).
In principle two procedures may lead to an improved carboxylation of the recombinant factor IX: a search for a cell line expressing high levels of the enzymes of the vitamin K cycle (carboxylase, KO reductase and K reductase) or, alternatively, the in vitro carboxylation of the insufficiently carboxylated proteins. We have investigated the second possibility using a partly purified carboxylating enzyme system from cow liver and recombinant factor IX produced by Chinese hamster ovary (CHO) cells.

Materials and methods

Chemicals. Vitamin K_1 was obtained from Hoffmann-La Roche (Switzerland) and chemically reduced as described by de Metz et al. (34). Dithiothreitol and CHAPS were from Sigma (USA) and $NaH^{14}CO_3$ (40 Ci/mol) from Amersham International (UK). Ultrogel AcA 34 was purchased from LKB (Sweden), heparin agarose (A4R) from IBF (France), and CNBr-activated Sepharose 4B from Pharmacia (Sweden). Polyclonal, immunopurified antibodies against the various human coagulation factors as well as against the proteins C an Z were kindly supplied by Dr. R. Bertina (Leiden, The Netherlands). Atomlight (New England Nuclear, FRG) was used as a scintillation liquid for radioactivity measurements. All other chemicals were of the highest purity commercially available.

Factor IX assays. Factor IX antigen was monitored using an enzyme-linked immuno-sorbent assay (Stago, France). Factor IX procoagulant activity was determined with a cephalin-kaolin one-stage assay in the presence of deficient plasma (35).

Cell culture conditions. A CHO transformed cell line (622.4) producing recombinant factor IX is routinely grown in selective medium consisting of α2000 (Gibco, UK), supplemented with 10% dialysed foetal calf serum, glucose, glutamine, hypoxanthine, thymidine, xanthine and mycophenolic acid (36). The production of recombinant factor IX was performed in multichamber trays (Nunc, Denmark) in non-selective medium, consisting of α2000 supplemented with 10% inactivated foetal calf serum and vitamin K_1 (50 μg/ml). Factor IX secreted under these cell conditions had a procoagulant activity of 10-15% from that of normal pooled plasma.

Partial purification of recombinant factor IX. Supernatant culture medium of the 622.4 cells was adjusted to pH 6.0 with Mes-Tris buffer and loaded onto a heparin agarose column (2 x 20 cm). The proteins were eluted at 20 ml/h with a linear gradient from 0.1 to 1.5 M NaCl in 20 mM Mes-Tris buffer, pH 6.0 and 1 mM benzamidine. Fractions containing factor IX antigen were pooled and purified further by chromatography on an Ultrogel AcA 34 column (1.5 x 100 cm) in buffer A (0.15 M NaCl, 20 mM Tris/HCl, pH 7.5). The fractions containing factor IX antigen were pooled and concentrated with an Amicon diaflo system using a PM 30 membrane filter. The average yield of factor IX antigen ranged from 55 to 65% of the crude cell supernatant and the purification was about 50-fold. The proportion of biologically active molecules was about 13%. Preparations thus obtained, contained 100 μg/ml of recombinant factor IX with a purity of 1% and they were used for all experiments described in this paper.

Carboxylase. Vitamin K-dependent carboxylase was prepared from normal cow liver and partly purified as described by Soute et al. (5). Standard reaction mixtures (0.125 ml) for in vitro carboxylation contained 0.25 mg of carboxylase, 2 μg of recombinant factor IX, 0.15 M NaCl, 1 M $(NH_4)_2SO_4$, 20 mM Tris/HCl, pH 7.5, 8 mM $MnCl_2$, 5 μCi $NaH^{14}CO_3$, 4 mM dithiothreitol, 0.05% (w/v) CHAPS, 0.4 mg/ml phosphatidylcholine, and 0.4 mM vitamin K hydroquinone. Phosphatidylcholine was added as mixed micelles with cholate in a 1:1 (w/w) ratio as described by de Metz et al. (37). The reaction mixtures were incubated at 25 °C for the indicated periods and the reaction was stopped by adding 1 ml of

5% (w/v) trichloroacetic acid. After gentle boiling for 1 min the samples were counted in Atomlight. In some cases the carboxylation reaction was not terminated by adding trichloroacetic acid but by cooling the samples in ice, removal of non-bound $^{14}CO_2$ by gentle bubbling with $^{12}CO_2$ gas and dialysis against cold buffer A.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out in 10% slab gels in the presence of 0.1% sodium dodecyl sulphate using a Bio Rad Mini Protean II dual slab gel system. If [^{14}C]r-factor IX was analyzed, reaction mixtures containing approximately 10,000 dpm of labelled protein were adsorbed onto Sepharose-bound anti-factor IX, eluted with 2 M KCNS and extensively dialysed. The samples were supplemented with 7.5 µg of non-labelled plasma-factor IX, concentrated to 100 µl and 40 µl aliquots were applied onto the gel. The gels were either stained with 0.25% Coomassie Brilliant Blue and destained with 30% methanol/10% acetic acid in distilled water or blotted electrophoretically on nitrocellulose paper (Bio Rad Trans-Blot). Proteins were visualized on the nitrocellulose paper by staining with 0.1% amidoblack and factor IX-related material was specifically stained with the Western blot technique using horse radish peroxidase-labelled goat anti rabbit as a second antibody. The presence of radiolabel was detected by slicing the nitrocellulose paper into 0.25 cm strips which were counted as such in a liquid scintillation counter.

Other techniques. Protein-bound Gla-residues were determined after alkaline hydrolysis of the samples with the HPLC technique described by Kuwada and Katayama (38). The correct incorporation of $^{14}CO_2$ into Glu-containing substrates was checked by comparing the amount of radiolabel after acid and after alkaline hydrolysis. Since Gla is acid-labile, a carboxyl group is rapidly lost under acidic conditions. This carboxyl group may be either $^{14}CO_2$ or $^{12}CO_2$. Because no stereospecificity has been observed for the decarboxylation of Gla into Glu, a theoretical loss of 50% of the label is expected, whereas Gla remains stable under alkaline conditions. The number of Gla-residues present in several recombinant factor IX preparations was quantified using free Gla as a standard. Assuming that all Gla-residues originated from the recombinant factor IX, the mean Gla-content of the latter follows from the quotient of the concentrations of protein-bound Gla and recombinant factor IX antigen.
Protein concentrations were measured according to Sedmak and Grossberg (39).

Results

A stable transformed Chinese hamster ovary (CHO) cell line constitutively expressing human factor IX has been established as was described elsewhere (36). The sequencing of the purified protein showed that two recombinant species are secreted by CHO cells. One is correctly processed and possesses the N-terminal sequence expected for the mature protein. The other fraction corresponds to the precursor form with an 18 amino acid extension at the NH_2 terminus. The majority of recombinant proteins thus obtained present a deficiency in gamma-carboxyglutamic acid content as was tested by direct Gla analysis (see below).
Purification of the recombinant factor IX secreted into the culture medium can be performed using a protocol derived from conventional methods of plasma fractionation (36). In this purification scheme the first step involves barium citrate precipitation. During this process only molecules which are sufficiently carboxylated are expected to be linked to barium ions. Such a treatment eliminates incomplete or non-carboxylated recombinant molecules which would be potential substrates for the carboxylase. We therefore performed a limited purification procedure (excluding the barium precipitation step) based on affinity chromatography on heparin agarose previously developed to fractionate natural coagulation factors (40). The recombinant factor IX molecules present in the crude cell supernatant are totally adsorbed on heparin agarose and eluted in high salt conditions with a yield ranging from 65 to 75%. After a second purification step (size exclusion chromatography on an AcA 34 column), the preparation was analyzed by polyacrylamide gel electrophoresis in SDS, followed by immunoblotting on nitrocellulose paper. As is shown in figure 1 the migration rate of the majority of the antifactor IX-reaction material was slightly slower than that of factor IX from human plasma. This difference is too large, however, to be explained by an impaired cleavage of the leader sequence alone. Possibly the high molecular weight material represents a form of factor IX which has been excessively glycosylated. As stated above, the best results in our carboxylation assays were obtained with recombinant factor IX preparations of limited purity (about 1%) and this material was used for all experiments described below. However, the vast excess of contaminating proteins in these preparations did not allow us to detect the Gla-residues. In more purified recombinant factor IX preparations (10-20% purity) the Gla-content was invariably found to be 3 re-

sidues per molecule or less. Therefore, we assume that the majority of the recombinant factor IX used in this paper also contained less than 3 Gla residues per molecule.

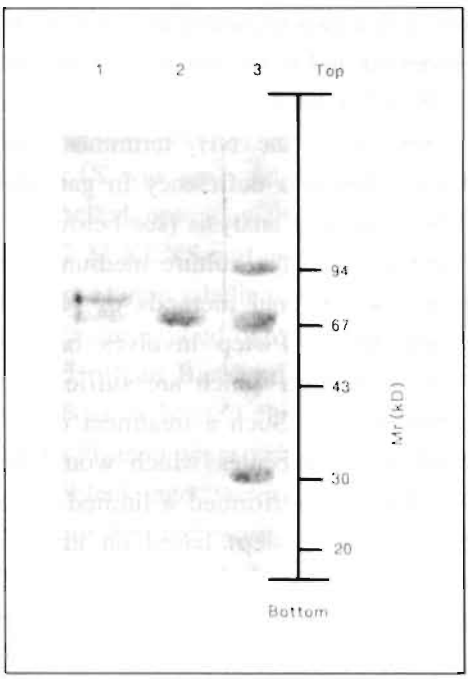

FIG. 1. Analysis of recombinant factor IX. *Partly purified recombinant factor IX was analyzed on 10% polyacrylamide gels in 0.1% sodium dodecyl sulphate (lane 1) and compared with normal plasma factor IX (lane 2) and marker proteins (lane 3). After electrophoresis the gels were blotted on nitrocellulose paper and stained either with the immunoblot technique (lanes 1 and 2) or with amidoblack (lane 3).*

The recombinant factor IX was used as a substrate for vitamin K-dependent carboxylase and a time-course of its in vitro carboxylation is shown in figure 2A. After a short lag-phase the reaction rate was constant for more than 1 h. From the linear parts of the curves (15-75 min) obtained at various substrate concentrations we calculated the initial carboxylation rate, which was then plotted in a Lineweaver-Burk diagram (figure 2B). This resulted in a straight line and from the interception with the abscissa the K_M^{app} for recombinant factor IX could be calculated to be 0.12 μM, which is lower than that of any other exogenous substrate presently known. To make sure that the protein-bound $^{14}CO_2$ had been incorporated into the recombinant factor IX, three control experiments were performed. Firstly is was verified that the incorporated label could be identified as Gla. After completion of the in vitro carboxylation, aliquots from the reaction

mixtures were submitted to either alkaline or acid hydrolysis as described in Materials and Methods. The acid-induced loss of $^{14}CO_2$ was calculated for all recombinant factor IX concentrations shown in figure 2A and amounted to 56.3 ± 4.1%.

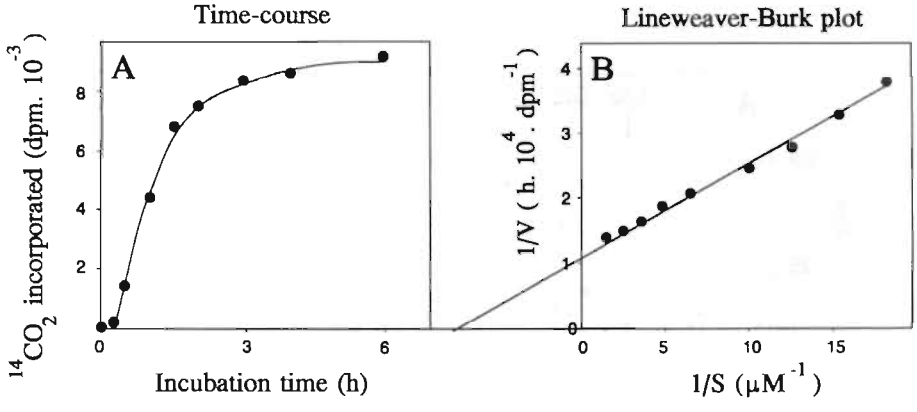

FIG. 2. In vitro carboxylation of recombinant factor IX.

A: Standard reaction mixtures containing 20 µg of recombinant factor IX were incubated for different periods at 25 °C.

B: Standard reaction mixtures containing varying amounts of recombinant factor IX were incubated and the initial reaction rate (V) was calculated from the slope of the time course between 15 and 75 min. V^{-1} was then plotted against the reciprocal substrate concentration (S^{-1}).

After hydrolysis the samples were also analyzed by HPLC and it turned out that after alkaline hydrolysis the ^{14}C-label was exclusively found at the position of Gla, whereas after acid hydrolysis the label eluted at the position of Glu (figure 3). Moreover, in the latter case the total amount of label recovered was about 50% from that after alkaline hydrolysis. From these results we concluded that indeed Gla-residues had been formed during the carboxylation reaction.

A second control experiment was the immuno-adsorption of [^{14}C]r-factor IX to immobilized antibodies. Polyclonal, immuno-purified antibodies against normal human prothrombin, factor IX, factor X, factor VII, protein C and protein Z were linked to CNBr-activated Sepharose (1 mg of protein per g Sepharose) and it was verified that they were monospecific for each of the corresponding plas-

ma proteins. The affinity of [^{14}C]r-factor IX (4,100 dpm per reaction mixture) for each of these antibodies was measured by incubating the labelled product with the Sepharose beads. The mixtures were subsequently centrifuged (5 min at 1,500 x g) and the supernatants and washed pellets were counted (Table I). In all tubes a low amount of label was found to be associated with the Sepharose.

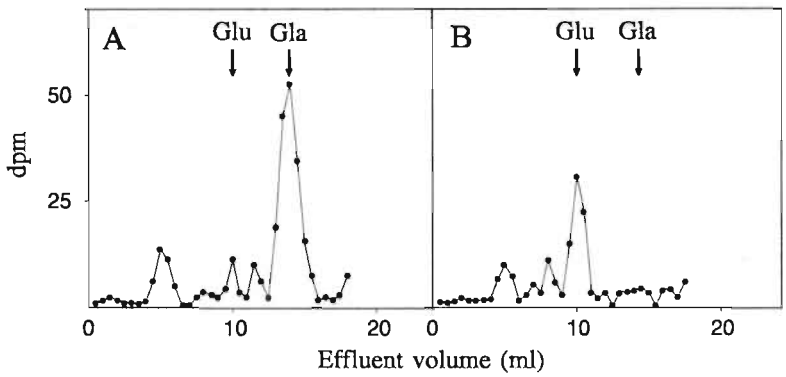

FIG. 3. Identification of ^{14}C-labelled reaction product. *Reaction mixtures containing 10,000 dpm of carboxylated recombinant factor IX were extracted with Sepharose-bound antibodies against factor IX (see Table I) and the adsorbed proteins were eluted from the solid phase with 2 M KCNS. After dialysis the samples were subjected to alkaline (fig. 3A) and acid (fig. 3B) hydrolysis and aliquots containing 580 (A) and 320 (B) dpm were fractionated by HPLC in the way described for Gla analysis. Fractions containing 0.5 ml were collected and counted in a liquid scintillation counter. The positions of Glu and Gla (arrows) were determined in a separate run using the free amino acids.*

This might be the result of non-specific adsorption of some precipitation (denaturation) of the material. The antibodies against factor IX, however, bound more than 75% of the labelled proteins, thus indicating that the product of the carboxylation reaction consists of recombinant factor IX. Finally, the radiolabeled product was analyzed on polyacrylamide gels (figure 4). Since the samples had been supplemented with an excess of plasma factor IX (see Materials and Methods), the immunoblot shows the presence of normal factor IX, whereas the label indicates the position of in vitro carboxylated material. Obviously the two bands coincide.

From these data we concluded that the recombinant factor IX had indeed been carboxylated, and we have tried to estimate the number of Gla residues formed per molecule of recombinant factor IX. For these calculations it was assumed that the concentration of non-labelled atmospheric CO_2 in the reaction mixtures was similar to that dissolved in distilled water and the various buffers (approximately 2 mM).

Immobilized antibody against	Carboxylated product (dpm)	
	bound	non bound
Prothrombin	326	3,532
Factor X	487	3,299
Factor IX	3,321	856
Factor VII	28	4,025
Protein C	394	3,778
Protein Z	260	3,605

Table I. Affinity of carboxylated recombinant factor IX to Sepharose bound antibodies. *A carboxylating reaction mixture (2.5 ml) containing 40 µg of recombinant factor IX was prepared and incubated for 3 h at 25 °C. After incubation the mixture was degassed by gentle bubbling with CO_2 gas and dialysed against 10 l of buffer A overnight. It was checked that all free $^{14}CO_2$ had been removed from the sample by taking an aliquot and showing that all radiolabel was precipitable with trichloroacetic acid. The mixture was then subsampled in 0.1 ml portions (containing 4,100 dpm each), which were supplemented with Sepharose-bound antibodies (100 µl of slurry) and 800 µl of buffer A and rotated end over end at 4 °C. After 16 h the tubes were centrifuged at 1,500 x g and the supernatants were counted directly. The pellets were washed four times with 1 ml of buffer A before counting. The data given are the means of duplicate measurements and it was checked that in all cased the Sepharose-bound antibodies had been added in excess.*

Because standard reaction mixtures contain 1 mM of $^{14}CO_2$, the specific activity of the label is reduced to about 15 Ci/mol. When using 2 µg (36×10^{-6} µmole) of recombinant factor IX, a maximal level of incorporation of 10^4 dpm (3×10^{-4} µmole) could be reached. From these data it follows that under optimal conditions about 8 moles of CO_2 were incorporated per mole of recombinant factor IX.

Obviously this figure is only a rough estimate, the height of which varies with the estimated level of atmospheric CO_2 present in the reaction mixtures. Nevertheless these calculations clearly show that at least several Gla residues are formed per molecule of recombinant factor IX.

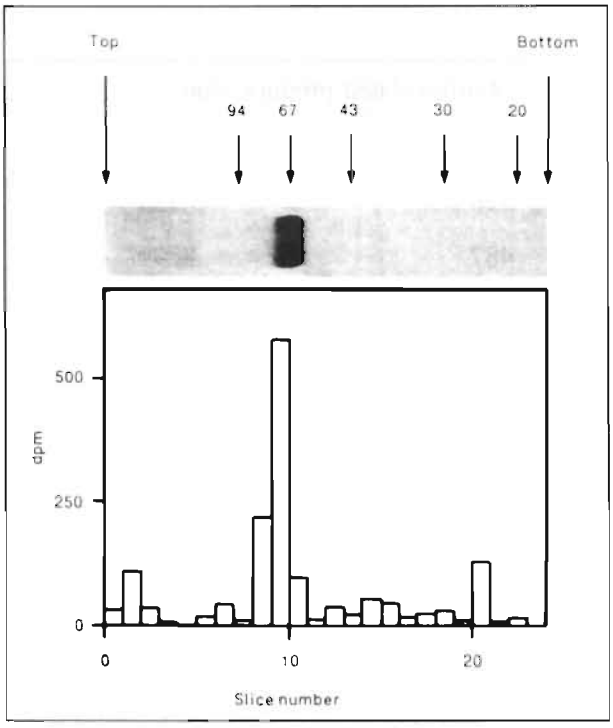

FIG. 4. Analysis of [^{14}C]recombinant factor IX. *The carboxylated substrate was analyzed as described in the legend to Fig. 1. the nitrocellulose paper containing the labelled material was stained with the immunoblot technique and after the photograph was made, the nitrocellulose was sliced into 2.5 mm strips which were counted in a liquid scintillation counter. The position of the peak was compared with that of a set of marker proteins (arrows).*

Discussion

Factor IX undergoes several post translational modifications and an N-terminal processing prior to secretion into the plasma as a mature protein. The factor IX precursor, recently discovered by analysis of two inactive forms (factor IX Oxford 3, factor IX Cambridge) isolated from haemophilic patients, carries an N-terminal extension of 18 amino acid residues (15,17). In the case of factor IX Cambridge, the natural mutation of the arginine residue at position -1 has been shown to impair the γ-carboxylation (15). The other vitamin K-dependent proteins are also synthesized as precursors and although the exact size of the pro-

sequence has not been determined for each one, the comparison between published cDNA sequences (41-45) shows clear homology in their respective propeptide regions.

These observations suggest that this aminoterminal extension is important for the post translational carboxylation and could act as the recognition site for carboxylase. Furthermore, several experiments manipulating the cDNA sequence by either removing the prosequence or altering particular aminoacids within it have been performed using both factor IX and protein C (16,33). In all cases studied, alteration of the prosequence resulted in a decrease or the complete abolition of the carboxylation reaction.

We describe elsewhere (36) the purification and the characterisation of the recombinant factor IX used in this study, and demonstrate that at least two recombinant species are secreted by CHO cells: one corresponding to the mature protein, the other being the precursor. The CHO cells thus present an enzymatic deficiency which lead to the incomplete processing after Arg^{-1}. We can take advantage of the presence of this precursor form synthesized in a mammalian expression system as it should be a good substrate for the carboxylase. Indeed, by analogy with our previous results showing that non-carboxylated plasma prothrombin was not a substrate for the vitamin K-dependent carboxylase (22), we can speculate that partially carboxylated or non-carboxylated mature recombinant factor IX would be equally disappointing. On the other hand, the precursor form of human factor IX is probably the ideal target for carboxylase. Attempts to purify the recombinant factor IX before it was added to an in vitro carboxylating enzyme system invariably lead to an increased ratio between factor IX procoagulant activity and factor IX antigen. In parallel also the ability of the preparation to serve as a substrate for carboxylase decreased considerably. Explanations for this phenomenon may be that either the various purification steps tend to select for normally carboxylated recombinant factor IX, or the leader sequence is prone to proteolytic digestion, so that it is cleaved off during the purification procedure.

In this study we have used therefore a partly purified recombinant factor IX preparation and we have tested if it could be carboxylated in vitro. We have shown that the incorporated $^{14}CO_2$ is recovered in a protein which reacts with monospecific antibodies against plasma factor IX (Table I) and which is found exclusively at the position of factor IX after Western blot analysis (Fig. 4).

Moreover the radiolabel is found in the HPLC peak corresponding to Gla residues. The present work shows that the post translational formation of Gla residues in recombinant factor IX may be increased in vitro by using partly purified bovine liver carboxylase. It is probable that other recombinant proteins (e.g. factor X, protein C) can also be processed in this way. At this stage of our investigation an increase of procoagulant activity is not really expected for two reasons. Firstly, the strongly reducing conditions, required for an efficient carboxylation reaction will rapidly destroy the disulphide bonds in the precursor protein. The recent discovery that thioredoxin from E. coli may replace dithiothreitol as a cofactor for the vitamin K reducing enzymes of bovine origin shows that a system may be developed in which the disulphide bonds in protein substrates for carboxylase remain intact (46), but the activity of this system will have to be increased. In this respect it would be interesting to investigate if the results could be improved by using thioredoxin from cow liver, thus creating an all bovine enzyme system. Secondly, the propeptide still present on the molecule probably interferes with the adequate activation of the zymogen. For example Bentley et al. (17) have shown that factor IX Oxford 3, an inactive form of factor IX carrying an 18 aminoacid N-terminal extension nevertheless possesses the twelve normal Gla residues. In the event of producing a fully γ-carboxylated recombinant factor IX with attached prosequence, a putative "propeptide peptidase" would have to be isolated and used to dissociate the fully carboxylated factor IX from its propeptide. Certain transformed cell lines have been described which produce recombinant factor IX lacking the propeptide and it will be interesting to see whether these proteins can be carboxylated in vitro in the presence of a synthetically prepared and hence not covalently bound propeptide.

Acknowledgements

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CHAPTER 7

Summary and conclusions

Summary and conclusions

During maturation, most secretory proteins undergo extensive post-translational processing before they are secreted into the extracellular environment. One of these post-translational modifications is the conversion of glutamate (Glu) into γ-carboxyglutamate (Gla) residues. The formation of Gla-residues is accomplished during a carboxylation reaction, which is catalyzed by the microsomal enzyme γ-glutamylcarboxylase, which is predominantly found in the membranes of the rough endoplasmic reticulum. Vitamin K hydroquinone (KH_2) functions as an essential cofactor in this reaction. The oxidation of KH_2 into vitamin K 2,3-epoxide (KO) provides the energy required for γ-carboxylation. In two successive reduction steps KO is subsequently recycled into KH_2. In this way vitamin K may be re-used several thousand times before it is excreted. At least one, but probably several, reductases are involved in the recycling process of vitamin K. This sequence of events is generally known as the 'vitamin K cycle'. In Chapter 1 of this thesis a review is presented of our current knowledge concerning the γ-glutamylcarboxylation reaction and the role of vitamin K in this reaction.

Gla residues have only been demonstrated in a limited number of proteins, and in these proteins they are found at well defined positions. The first protein demonstrated to contain Gla was prothrombin, one of the blood coagulation factors produced by the liver. Hence the first enzyme systems in which vitamin K-dependent carboxylase could be tested in vitro were prepared from liver homogenates. Later on it became clear that carboxylase is present in tissues differing from each other as much as kidney, spleen, testis, lung, vessel wall and bone. At least six Gla-containing proteins are involved in the blood coagulation process and the presence of Gla in these proteins is a prerequisite for their biological activity. Hence a sufficient supply of vitamin K is of vital importance for normal haemostasis. Besides vitamin K deficiency the intake of 4-hydroxycoumarin derivatives may result in incompletely carboxylated Gla-proteins entering the circulation in an undercarboxylated form. During coumarin treatment the vitamin K cycle is interrupted by a blockade of the enzyme KO-reductase, leading to increased levels of circulating KO, and to a rapid exhaustion of the KH_2 store. This results in an accumulation of non-carboxylated precursor

proteins in the endoplasmic reticulum and, if the blockade is continued, in the cellular secretion of maturated Gla-proteins in a non- or undercarboxylated form (descarboxyproteins). Whether or not descarboxyproteins are secreted depends on the dose and the duration of coumarin intake, as well as on the species. In cattle and in humans descarboxyproteins are secreted into the bloodstream from where they may be purified with conventional methods. In the early years of carboxylation it was thought that descarboxyproteins would be the target substrates for the enzyme γ-glutamylcarboxylase. Hence the effect of carboxylation was supposed to be detectable from an increase of the enzymatic activity of descarboxyproteins. However, it is now known that by the absence of a recognition-site descarboxyproteins fail to act as good substrates and therefore this approach turned out not to be successful. The development of some sensitive coagulation assays was the positive outcome of this period. Coagulation kinetics initially worked out by Hemker et al. could be proven with these reagents. In Chapter 2 the preparation and sensitivity of these reagents is described. Secondly we report an improved method for the separation and detection of descarboxy and normal coagulation factors by HPLC.

In Chapter 3 the optimizing of an in vitro system for measuring vitamin K dependent carboxylation is described. In the past years the purification of carboxylase has been the main goal of many investigators, since the lack of purified preparations has been a major limitation in understanding the mechanism of carboxylation. Whereas most investigators use rat liver as a starting material for preparing microsomes, in our lab the bovine system has always been the principal source for carboxylase. In our attempts to purify γ-glutamylcarboxylase from bovine liver two methods have been developed leading to partially purified preparations, one of which is bound to a solid-phase, the other one is soluble. Although none of these methods resulted in a purified enzyme, the partially purified carboxylase preparations have been helpful in defining optimal conditions for carboxylase activity. Remarkably this activity was enhanced by a number of organic solvents and by high concentrations of $(NH_4)_2SO_4$.

In Chapter 4 we have focussed our attention on the cofactor of carboxylase: vitamin K. During the formation of Gla-residues KH_2 is converted into K epoxide (KO). Under normal in vitro conditions dithiol-dependent reductases will catalyse the recycling of vitamin K. However, during warfarin treatment KO reductase is inhibited resulting in a retardation of the carboxylation reaction.

Placental carboxylase differed from the liver systems because only vitamin K hydroquinone and not vitamin K quinone could be used as a coenzyme for the carboxylation reaction. Obviously, the dithiol-dependent K reductase was absent in these preparations. In the second part of Chapter 4 attention is focused on the two different forms of vitamin K found in nature: vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone). Both are characterized by a functional naphtoquinone ring and an aliphatic side chain. Phylloquinone is mainly found in green plants whereas the menaquinones are produced by the intestinal flora. The extent to which each of the K vitamins contributes to the carboxylation of blood coagulation factors is still a matter of investigation. Phylloquinone and various menaquinones were compared for their ability to serve as a cofactor for the hepatic vitamin K-dependent carboxylase. It was found that the cofactor activity of the menaquinones varied with the length of the aliphatic side chain and that it showed an optimum at MK-3. Under in vitro conditions both vitamin K_1 and the K_2-vitamins were rapidly metabolized into a mixture of the quinone, the hydroquinone and the epoxide form. The fact that at equilibrium the levels of these three metabolites were independent of the starting material shows that the vitamin K cycle is operational for vitamin K_1 as well as for K_2. Finally we describe in this chapter the occurrence of an allosteric interaction between the vitamin K binding site and the propeptide binding site on carboxylase. By this mechanism the affinity for KH_2 of the enzyme/substrate complex is about 20-fold higher than that of carboxylase alone.

In Chapter 5 we have investigated some aspects of the reductases involved in the recycling of vitamin K. Both reduction steps in the vitamin K-cycle may be accomplished by dithiol-dependent reductases. Whether this activity is exerted by one or different enzymes is still a matter of investigation. However, the mutation pattern found in two independent cases of warfarin resistance strongly supports the idea that only one reductase is responsible for the recycling of vitamin K. In vitro the synthetic dithiothreitol (DTT) as well as reduced thioredoxin may serve as reducing cofactor for KO- and K-reductase. It was shown that in vitro the formation of disulphide bonds may be linked to the vitamin K-dependent carboxylation of glutamate residues. In vivo both disulphide formation and vitamin K-dependent carboxylation are post-translational modifications taking place at the luminal side of the endoplasmic reticulum. It is possible that also in vivo both reactions are coupled, which would explain the source of

reducing equivalents necessary for carboxylation and the source of oxidating equivalents necessary for the formation of a disulphide bond. A model in which this hypothesis is worked out is presented at the end of this chapter.
In Chapter 6 data concerning substrates in the vitamin K-dependent carboxylase reaction are described. During the last 15 years two types of substrates have been developed: those containing and those lacking a pro-sequence of the natural Gla-proteins. Examples of the first type are FLEEL and FLEEV. All substrates of this type are characterized by high K_M^{app} values. The fact that poly-Glu preparations are carboxylated at a comparable rate as are the pentapeptide substrates demonstrated that a specific aminoacid sequence is not an essential requirement for carboxylation. In vitro it has been shown that the carboxylation of Glu-containing substrates is substantially enhanced when the substrates are extended with the propeptide. The improvement turned out to be the result of a decrease of the K_M^{app}. Propeptide-containing substrates in the in vitro carboxylase reaction are, for example, pro-PT28 and recombinant factor IX. The in vitro carboxylation of the latter substrate, produced by transformed Chinese hamster ovary cells, is described and the identity of the newly formed Gla residues is verified. Finally a 59-amino acid peptide containing the propeptide sequence and Gla-domain (residues -18 to 41) of human factor IX (FIXGla), is demonstrated to be an efficient substrate for in vitro gamma-carboxylation. Its K_M^{app} (0.55 μM) is several thousand-fold lower than that for the commonly used substrate FLEEL and about 5 times lower than that for proPT28 or proFIX28. It is the first peptide substrate that is carboxylated in vitro to more than one Gla/molecule (6-11 Gla/molecule). In addition, peptides with mutations similar to those in $FIX_{San\ Dimas}$ or $FIX_{Cambridge}$ as well as a peptide with both mutations in the propeptide sequence have been prepared. Enzyme kinetic studies revealed no significant difference in V_{max}^{app}/K_M^{app} values between normal and mutant substrates, indicating that arginines at positions -4 and -1, although highly conserved in the propeptide sequence of all the vitamin K-dependent proteins, is not critical for γ-carboxylation.

The purification of γ-glutamylcarboxylase has turned out to be more laborious and complex than anyone had imagined. At this time purified, homogenous carboxylase is available in at least three leading research groups, which means that we are experiencing a turning point in science: behind us is a period in

which carboxylase research was performed exclusively in non-purified systems. By the availability of purified carboxylase this research area has finally come to maturation. In front of us are scientific challenges such as the elucidation of the detailed mechanism of the carboxylation reaction, the mechanism of termination of Gla-formation as well as possible interactions between carboxylation and other posttranslational modifications. We hope and expect that the Maastricht group will continue to actively participate in this fascinating field of science.

Samenvatting en conclusies

De meeste secretie-eiwitten ondergaan een aantal belangrijke post-translationele modificaties tijdens hun synthese voordat ze door de cel uitgescheiden worden. Een van deze veranderingen is de omzetting van glutaminezuurresiduen (Glu) in γ-carboxyglutaminezuurresiduen (Gla). De omzetting van Glu in Gla vindt plaats tijdens een carboxyleringsreactie die wordt gekatalyseerd door het microsomale enzym γ-glutamylcarboxylase, dat voornamelijk in de membranen van het ruw endoplasmatisch reticulum wordt gevonden. De gereduceerde vorm van vitamine K (KH_2) is een belangrijke cofactor bij deze reactie. De energie die nodig is voor carboxylering wordt geleverd door de oxydatie van KH_2 tot vitamine K epoxide (KO). In twee opeenvolgende reductiestappen wordt KO vervolgens weer omgezet in KH_2. Op deze manier kan vitamine K duizenden malen opnieuw worden gebruikt voordat het wordt uitgescheiden. Tenminste één maar waarschijnlijk meerdere reductases zijn betrokken bij de recycling van vitamine K. Dit hele proces staat bekend als de "vitamine K cyclus". In hoofdstuk 1 van dit proefschrift wordt een overzicht gegeven van onze hedendaagse kennis over de carboxylerings reactie en de rol die vitamine K daarin speelt.
Gla residuen zijn tot nu toe slechts aangetoond in een beperkt aantal eiwitten waar zij op specifieke plaatsen worden gevonden. Prothrombine, één van de stollingseiwitten die door de lever worden geproduceerd, was het eerste eiwit waarin Gla werd aangetoond. Daarom werden lever homogenaten als uitgangsmateriaal gebruikt voor de ontwikkeling van de eerste enzymsystemen waarin vitamine K afhankelijke carboxylering kon worden gemeten. Het werd later duidelijk dat carboxylase in veel weefsels gevonden wordt zoals o.a. in de nier, milt, testes, long, vaatwand en bot. Tenminste zes Gla bevattende eiwitten zijn betrokken bij het bloedstollingsproces, waarbij de aanwezigheid van Gla een belangrijke voorwaarde is voor hun biologische activiteit. Daarom is voldoende aanvoer van vitamine K van essentieel belang voor een normaal bloedstollingsproces. Behalve een tekort aan vitamine K kan ook de inname van 4-hydroxycoumarine derivaten leiden tot de uitscheiding van niet volledig gecarboxyleerde Gla eiwitten. Tijdens coumarine behandeling wordt het enzym KO-reductase geblokkeerd waardoor de vitamine K cyclus wordt onderbroken. Dit heeft tot gevolg dat het circulerend KO niveau zal stijgen en de KH_2 voorraad snel zal

afnemen. Dit zal leiden tot een ophoping van niet gecarboxyleerde eiwitten in het endoplasmatisch reticulum en als de blokkade wordt voortgezet uiteindelijk tot de uitscheiding van niet of gedeeltelijk gecarboxyleerde eiwitten (descarboxyeiwitten). Of deze descarboxyeiwitten al dan niet worden uitgescheiden hangt af van de dosis en de duur van de coumarine behandeling evenals van de behandelde species. Bij de koe en de mens worden descarboxyeiwitten in het bloed uitgescheiden waaruit zij gezuiverd kunnen worden met gebruikelijke zuiveringstechnieken. In de beginjaren van carboxylering werd aangenomen dat deze descarboxyeiwitten als substraat konden dienen voor het enzym γ-glutamylcarboxylase en dat het carboxyleringsproces gevolgd kon worden door meting van de toename van de biologische activiteit van de descarboxyeiwitten. We weten nu echter dat door de afwezigheid van een herkenningsplaats descarboxyeiwitten geen goede substraten zijn en dat daardoor de destijds gevolgde procedure niet succesvol kon zijn. De ontwikkeling van reagentia waarmee gevoelige stollingsbepalingen werden opgezet kan beschouwd worden als een nuttige zijlijn uit deze periode. Met behulp van deze reagentia kon de door Hemker et al. gepostuleerde stollingskinetiek bewezen worden. In hoofdstuk 2 wordt de bereiding en de gevoeligheid van deze reagentia beschreven evenals een verbeterde methode voor de scheiding en detectie van descarboxy- en normale stollingsfactoren met behulp van HPLC.

In hoofdstuk 3 wordt de optimalisering van de vitamine K afhankelijke carboxylering in vitro beschreven. Omdat gezuiverd carboxylase een absolute noodzaak is om het carboxyleringsmechanisme te kunnen begrijpen, is de zuivering van carboxylase in de afgelopen jaren het voornaamste doel geweest van veel onderzoekers. Hoewel rattelever meestal gebruikt werd als uitgangsmateriaal voor de bereiding van microsomen is dit in ons lab altijd lever van de koe geweest. Onze pogingen om γ-glutamylcarboxylase te zuiveren hebben geleid tot de ontwikkeling van twee methoden voor de bereiding van gedeeltelijk gezuiverde preparaten. Het eerste preparaat is gebonden aan een vaste drager, het tweede in oplossing. Hoewel geen van beide methoden een gezuiverd enzym hebben opgeleverd, speelden beide preparaten wel een belangrijke rol bij de bepaling van de optimale condities voor carboxylering en bij de ontdekking van een aantal essentiële kenmerken, welke o.a. geleid hebben tot de uiteindelijke zuivering van het enzym. Opmerkelijke bevindingen waren dat de carboxylaseactiviteit gestimuleerd werd door een aantal organische oplossingen en door hoge

concentraties $(NH_4)_2SO_4$.

In hoofdstuk 4 wordt de aandacht gericht op de cofactor voor carboxylase, vitamine K. Tijdens de vorming van Gla residuen wordt KH_2 omgezet in K epoxide (KO). In vitro wordt onder normale condities de recycling van vitamine K gekatalyseerd door dithiol-afhankelijke reductases. Na toediening van warfarine echter wordt het KO-reductase geremd, wat een onderbreking van de K cyclus en daardoor een onvolledige carboxyleringsreaktie tot gevolg heeft. Bij carboxylase uit placenta werd gevonden dat alleen vitamine K hydroquinon en niet vitamine K quinon als cofactor gebruikt kon worden in de carboxyleringsreaktie, in tegenstelling tot levercarboxylase. Waarschijnlijk komt dit door de afwezigheid van het dithiol-afhankelijke K-reductase in deze preparaten. In het tweede gedeelte van hoofdstuk 4 worden twee verschillende vormen van vitamine K bestudeerd die in de natuur gevonden worden: vitamine K_1 (phylloquinon) en vitamine K_2 (menaquinonen). Beide vormen worden gekenmerkt door een functionele naftaquinon ring en een alifatische zijketen. Phylloquinon wordt voornamelijk gevonden in groene planten, terwijl menaquinonen worden geproduceerd door o.a. de bacteriën in de darm. De mate waarin beide vitamines K een rol als cofactor spelen bij de carboxylering van de bloedstollingsfaktoren wordt nog steeds onderzocht. De cofactoractiviteit van phylloquinon en een aantal menaquinonen werd vergeleken in levercarboxylase. De cofaktoractiviteit van de menaquinonen bleek afhankelijk van de lengte van de alifatische zijketen en was optimaal bij menaquinon-3. In vitro werden zowel vitamine K_1 als vitamine K_2 snel omgezet in een mengsel van de quinon, de hydroquinon en de epoxide vorm. Het feit dat in evenwichtstoestand de hoeveelheid van deze drie metabolieten onafhankelijk was van het uitgangsvorm bewijst dat de vitamine K cyclus zowel met vitamine K_1 als met vitamine K_2 werkt. Tenslotte wordt in dit hoofdstuk een allosterische interactie tussen de vitamine K bindingsplaats en de propeptide bindingsplaats bij carboxylase beschreven. Hierbij werd gevonden dat de affiniteit van KH_2 voor het enzym/substraatcomplex ongeveer 20 maal groter was dan voor carboxylase alleen.

In hoofdstuk 5 worden enkele aspecten onderzocht van de reductases die betrokken zijn bij de recycling van vitamine K. Beide reductie-stappen in de vitamine K cyclus kunnen uitgevoerd worden door dithiol-afhankelijke reductases. Onderzocht wordt nog altijd of deze activiteit gekatalyseerd wordt door één of meerdere enzymen. Mutaties, gevonden in twee verschillende gevallen van warfarine

resistentie, ondersteunen echter sterk de theorie dat slechts één reductase zorgt voor recycling van vitamine K. In vitro kunnen zowel het synthetische dithiothreitol (DTT) als gereduceerd thioredoxine optreden als reducerende cofactor voor het KO- en K-reductase. In vitro werd aangetoond dat de vorming van disulfide bruggen gekoppeld kan worden aan de vitamine K afhankelijke carboxylering van glutaminezuur residuen. In vivo zijn zowel de disulfide vorming als de vitamine K afhankelijke carboxylering posttranslationele modificaties die plaatsvinden aan de luminale zijde van het endoplasmatisch reticulum. Het is mogelijk dat ook in vivo beide reacties gekoppeld zijn, waarmee dan ook een verklaring gevonden zou zijn voor de herkomst van de reducerende equivalenten die nodig zijn bij carboxylering en voor de herkomst van de oxiderende equivalenten die nodig zijn voor de vorming van een disulfide band. Aan het eind van dit hoofdstuk wordt een model gepresenteerd waarin deze hypothese uitgewerkt is.

In hoofdstuk 6 worden enkele gegevens beschreven van substraten in de vitamine K afhankelijke carboxylerings reactie. In de afgelopen 15 jaar zijn er twee soorten substraten ontwikkeld: substraten met en zonder de prosequentie zoals die gevonden wordt in de natuurlijke Gla-eiwitten. Voorbeelden van het eerste type zijn FLEEL en FLEEV. Alle substraten van dit type worden gekenmerkt door hoge K_M^{app}-waarden. Het feit dat poly-Glu preparaten dezelfde K_M^{app}-waarden hebben als de pentapeptides, laat zien dat een specifieke aminozuurvolgorde geen essentiële voorwaarde is voor carboxylering. In vitro werd aangetoond dat Glu-bevattende substraten betere substraten zijn wanneer zij een propeptidesequentie bevatten. Deze verbetering wordt veroorzaakt door een verlaging van de K_M^{app}-waarde. Voorbeelden van substraten voor de in vitro reactie die een propeptidesequentie bevatten zijn bijvoorbeeld pro-PT28 en recombinant factor IX. De in vitro carboxylering van het laatstgenoemde substraat, geproduceerd door een CHO cellijn, wordt beschreven evenals de identificatie van het gevormde produkt. Tenslotte word aangetoond dat een peptide, bestaande uit 59 aminozuurresiduen met daarin de propeptide volgorde en het Gla-domein (residuen -18 tot 41) van humaan factor IX (FIXGla), een goed substraat is voor carboxylase in vitro. De K_M^{app} (0.55 µM) is enige duizenden malen lager dan die van proPT28 en die van proFIX28. Het is het eerste peptidesubstraat waar in vitro meer dan één Glu per molecule werd gecarboxyleerd (6-11 Gla's per molecule). Tevens werden peptides gemaakt met mutaties gelijk aan die in

$FIX_{San\ Dimas}$ en $FIX_{Cambridge}$, en een peptide met beide mutaties in de prosequentie. Kinetische studies toonden geen wezenlijke verschillen in V_{max}^{app} en K_M^{app} tussen normale en gemuteerde substraten, waarmee wordt aangetoond dat arginines op de posities -4 en -1 niet noodzakelijk zijn voor γ-carboxylering.

De zuivering van γ-glutamylcarboxylase is bewerkelijker en complexer gebleken dan men zich in eerste instantie voor kon stellen. Op dit ogenblik is gezuiverd carboxylase beschikbaar in tenminste drie leidende onderzoeksgroepen, hetgeen betekent dat we nu op een keerpunt staan in het carboxylaseonderzoek: achter ons ligt een periode waarin dit onderzoek uitsluitend werd uitgevoerd in ongezuiverde systemen. Door de beschikbaarheid van gezuiverd carboxylase is dit onderzoeksgebied eindelijk naar volwassenheid gegroeid. Voor ons liggen wetenschappelijke uitdagingen, zoals de opheldering van het gedetailleerde mechanisme van de carboxyleringsreaktie, het mechanisme voor het stoppen van de Gla-vorming nadat in een eiwit een strikt gedefinieerd aantal Gla-residuen is gevormd, en ook de mogelijke interacties tussen carboxylase en andere posttranslationele modificaties. We hopen en verwachten dat de Maastrichtse groep actief zal blijven deelnemen aan dit fascinerende onderzoeksgebied.

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Curriculum vitae

Berry Soute werd geboren op 2 oktober 1948 in Batavia. Na het behalen van het diploma HBS-A te Eindhoven en het diploma HBO-B Biochemie te Amsterdam kwam hij in dienst bij het Centraal Laboratorium voor de Bloedtransfusiedienst in Amsterdam waar hij twee jaar werkzaam was op de afdeling Bereiding Stollingsfactoren (Dr.H.G.J. Brummelhuis). In juli 1975 trad hij in dienst als analist bij de vakgroep Biochemie van de Rijksuniversiteit Limburg, deelproject vitamine K onder leiding van Dr. Cees Vermeer. In die tijd werd de grondslag gelegd voor het onderzoek beschreven in dit proefschrift. In juni 1985 werd hij Limburgs Wielerkampioen der Veteranen en in februari 1986 schaatste hij de volledige Elfstedentocht. In 1990 begon hij een aanvullende studie aan de Hogeschool Heerlen ter verkrijging van het HLO-diploma Chemie. In juli 1991 werd dit diploma behaald waarna hij toelating kreeg voor dit promotieonderzoek.

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