Hepatic and non-hepatic vitamin K-dependent reactions

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Hepatic and non-hepatic vitamin K-dependent reactions

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PROEFSCHRIFT

ter verkrijging van de graad van doctor in de geneeskunde
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus Prof. Dr. F.I.M. Bonke,
volgens besluit van het College van Dekanen,
in het openbaar te verdedigen in de aula van de universiteit
op vrijdag 13 maart 1987, des namiddags te vier uur

door

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geboren te Amsterdam

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'Heel het leven bestaat uit iets achterlaten
en een sprong voorwaarts maken naar iets nieuws'

Paul Pruijser

aan mijn ouders
aan Jurjen
voor alle gemiste uren

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C H A P T E R 1

VITAMIN K AND VITAMIN K ANTAGONISTS: HISTORICAL BACKGROUND

Vitamin K was discovered by Henrik Dam in 1935, when he studied the effects of a cholesterol-free diet on chickens (1). He observed that the animals had developed hemorrhages and that their blood exhibited a prolonged coagulation time. These hemostatic defects turned out to be related to the deficiency of a fat soluble vitamin, which was called Koagulations-vitamin (coagulation vitamin) or shortly vitamin K. The bleeding symptoms provoked by vitamin K-deficiency were similar to those described for a hemorrhagic disease in cattle (2). These hemorrhages were found to be caused by feeding the animals improperly cured sweet clover hay. The active compound in the clover was identified as 3,3 methylene-bis (4-hydroxycoumarin) (3), which is present in a chemically bound inactive form in the fresh plants, but which may be liberated during the curing of the clover. In its free form the 4-hydroxycoumarin derivative acts as an antagonist of vitamin K. In the following years numerous coumarin-derivatives have been synthesized and used for the treatment of patients to antagonize undesired blood coagulation during thrombogenic episodes. Some of them are also used in the form of rodenticides. The structures of vitamin K and some frequently used coumarin-derivatives are shown in fig. 1.

DISCOVERY OF 4-CARBOXYGLUTAMIC ACID

After the discovery that vitamin K was involved in blood coagulation, various hypotheses about the action of vitamin K were postulated (4). A

vitamin K₁ or phylloquinone

4-hydroxycoumarin derivatives:

	R ₁	R ₂
acenocoumarol	CH ₂ COCH ₃	NO ₂
phenprocoumon	CH ₂ CH ₃	H
warfarin	CH ₂ COCH ₃	H

Fig. 1.

Structures of vitamin K₁ and some 4-hydroxycoumarin derivatives

real step forward was the postulation of Hemker et al. (5) that blood from patients receiving oral anticoagulant therapy contains a mixture of normal and abnormal prothrombin. The abnormal prothrombin was called "PIVKA" (protein induced by vitamin K antagonists) and it was regarded as a precursor of prothrombin. This hypothesis was strengthened by the demonstration of a protein antigenically similar to prothrombin but lacking biological activity in the plasma of patients receiving coumarin-therapy (6,7). The abnormal prothrombin seemed to be very similar to the normal one but appeared to lack the ability to bind calcium ions (8,9). In 1974, it was reported independently by the groups of Stenflo (10), Nelsetuen (11), and Magnusson (12), that the Ca²⁺-binding sites in normal prothrombin were formed by an abnormal amino-acid, the 4-carboxyglutamic acid or γ -carboxyglutamic acid (Gla, for structure see fig. 2). Since this modified amino-acid is absent in PIVKA, it was concluded that vitamin K is involved in the posttranslational carboxylation of glutamic acid residues in a prothrombin precursor protein. Gla-residues were also demonstrated in the coagulation factors VII, IX and X (13) and more recently in protein C (14), and protein S (15), which are also involved in blood coagulation and protein Z (16), which function is unknown. Presently we know that vitamin

Fig. 2.

Structural formulae of glutamic acid (Glu) and 4-carboxyglutamic acid (Gla)

K-dependent carboxylation is not uniquely restricted to proteins involved in blood coagulation. The formation of Gla-residues is a modification, which is undergone by numerous other proteins, synthesized in various tissues (17,18).

SYNTHESIS OF VITAMIN K-DEPENDENT PROTEINS

All vitamin K-dependent proteins discovered up to now belong to the so-called secretory-proteins. These proteins are excreted into the extracellular fluid or to the outer cell membrane after their maturation has been completed. The mechanism by which they are synthesized has been clarified by Blobel et al. (19) and Meijer et al. (20,21), while the groups of MacGillivray and Davie have studied in particular the biosynthesis of prothrombin (22,23,24). The basic concept in their biosynthesis is the presence of a hydrophobic leader sequence, consisting of a signal presegment (18-34 residues) and a basic pro-segment (5-9 residues) at the N-terminal side of the growing peptide chain. Once it has been synthesized and emerged from the ribosome the signal sequence will be recognized and bound by the signal recognition particle (SRP) in the cytoplasm, causing a stop of any further polypeptide chain elongation. A second protein involved

in this process is located on the rough endoplasmic reticulum membrane. This so-called "docking protein" is able to recognize the SRP-ribosome complex and to mediate its binding to the endoplasmic reticulum, resulting in a reinitiation of protein synthesis. The hydrophobic signal sequence rapidly penetrates into the phospholipid membrane, thus facilitating the transport of the growing protein through the membrane to the luminal side of the endoplasmic tubuli. Here are located the enzymes required for posttranslational modifications, such as glycosylation, hydroxylation and vitamin K-dependent carboxylation. Obviously not all secretory proteins undergo all possible modifications and the mechanism, responsible for the selection of carboxylatable Glu-residues is still a matter of research.

PARTIAL PURIFICATION OF VITAMIN K-DEPENDENT CARBOXYLASE

Since the discovery of vitamin K-dependent carboxylase activity many attempts have been undertaken to purify the enzyme. The enzyme acts as a typical integral membrane protein. Relatively high concentrations of detergent (Triton X-100, CHAPS, Lubrol) are required and even after solubilization the enzyme remains associated with phospholipids and contaminating proteins. Further desintegration of these structures inevitably leads to an almost complete loss of carboxylase activity (4).

Canfield et al. (25) and Wallin and Suttie (26) developed procedures for the specific extraction of a number of rat liver microsomal membrane proteins, using various detergents and chaotropic agents. By this procedure the specific enzymatic activity could be increased about 100-fold, but this increase was mainly due to the elimination of an inhibitor of the vitamin K dependent carboxylation rather than to the removal of a substantial amount of contaminating proteins.

Another attempt was done by Olson et al. (27), who tried to purify on a heparin affinity column the carboxylase-bound prothrombin precursors in a liver homogenate from warfarin treated rats. The enzyme/substrate complexes could be bound and subsequently eluted with a recovery of about 10%. The total purification was 100-fold but it is not sure whether this is due to the removal of an inhibitor as reported above or to the elimination of contaminating proteins.

A slightly different approach was followed by De Metz et al. (28) who first determined the identity of the accumulated substrate for carboxylase in the livers of warfarin-treated cows. It turned out that about 60% of the carboxylatable material consists of factor X precursors whereas only 25% could be identified as prothrombin precursors. Consequently purified antibodies against normal bovine factor X were covalently linked to Sepharose and used for the extraction of enzyme/substrate complexes from detergent treated microsomes. Because vitamin K-dependent carboxylase is firmly bound to its endogenous substrate, the binding of factor X-precursor to the insolubilized antibodies resulted in the specific extraction of enzyme/substrate complexes. By this method, part of the carboxylase activity could be bound to the Sepharose beads. After washing away the non bound material, a preparation resulted which was designated as solid-phase carboxylase and resulted in a 50-100 fold purification of the enzyme. A schematic representation of solid-phase carboxylase is given in fig. 3.

Fig. 3.
Schematic representation of solid-phase carboxylase

Solid-phase carboxylase is able to incorporate $^{14}\text{CO}_2$ into endogenous substrates as well as in F L E E L. Later on Olson et al. (27) prepared in a similar way a solid-phase carboxylase from rat liver using Sepharose-bound antibodies against rat prothrombin. Conventional methods for the breakage of the antigen-antibody interaction failed to resolubilize the carboxylase enzyme, because the carboxylation activity was irreversibly lost (29).

Recently Girardot (30) has claimed a 400 fold purification of the carboxylation enzyme from the hepatic microsomal fraction from warfarin treated rats. The vitamin K dependent enzyme was solubilized with the detergent (CHAPS) and further purified by low-pressure chromatography on ion exchange and size exclusion columns. The final enzyme preparation still contained an endogenous substrate for carboxylase but showed no reductase activity (see Vitamin K cycle) anymore. Its molecular weight was determined to be lower than 350000 D. This is the smallest size obtained for the active enzyme complex thus far. Unfortunately this purification procedure has not yet been confirmed by other laboratories.

VITAMIN K DEPENDENT CARBOXYLATION IN VITRO

In 1973, Suttie demonstrated an accumulation of coagulation factor precursors in rat liver induced by warfarin (31). In 1975, Esmon et al. found that these precursors could be carboxylated in a cell-free system, provided that the reaction mixtures were supplemented with vitamin K and $\text{NaH}^{14}\text{CO}_3$ (32). Under these conditions, $^{14}\text{CO}_2$ was incorporated into the endogenous substrate proteins which could subsequently be separated from the bulk of bicarbonate by trichloroacetic acid precipitation. So, both the vitamin K-dependent carboxylase enzyme complex and the corresponding endogenous substrate had been found in the same microsomal fraction. Since then, the vitamin K-dependent reaction has been studied most extensively in liver microsomal preparations from vitamin K-deficient or warfarin-treated rats (33,34) and from warfarin-treated cows (35). Its main characteristics are summarized in table 1.

Although the use of the carboxylase preparations mentioned above had several advantages (e.g. the correct substrate is complexed to carboxylase in such a way that it is readily carboxylated upon adding vitamin K) the

Table 1

Properties of vitamin K-dependent carboxylase

Absolute requirements:

- vitamin K quinone + reducing agent (NADH, DTT) or vitamin K hydroquinone
- O_2
- CO_2
- carboxylatable substrate

Stimulating agents:

- DTT
- pyridoxalphosphate or Mn^{2+}
- detergents
- antichaotropic agents
- ketones or DMSO

Inhibiting agents

- chloro-K
 - 2,3,5,6-tetrachloro-4-pyridinol
 - coumarin-like anticoagulants
 - sulphhydryl reagents
 - chaotropic agents
 - CN^-
-

Abbreviations: DTT, dithiothreitol; DMSO, dimethylsulphoxide; chloro-K, 2-chloro-3-pyridyl-, 1,3-naphthoquinone.

drawbacks are also obvious. During attempts to purify carboxylase, for instance, the substrate may be lost, which inevitably will lead to the loss of enzyme activity. A second drawback is the fact that kinetic studies may require a substrate present in varying and well-defined amounts. Therefore also exogenous substrates have been developed. In the first place synthetic substrates have been made (36,37), which all resemble a short Gla-containing amino acid sequence in one of the coagulation factors, but in which Gla has been replaced by Glu. An example of these substrates is Phe-Leu-Glu-Glu-Leu (F L E E L) which shows sequence homology with bovine factor VII and protein C (38). When offered to the carboxylating system, these synthetic pentapeptides were all readily carboxylated, although their K_m values were found to be rather high (2-10 mM). Another synthetic substrate, a tripeptide linked to menadione, has been reported to have a K_m of about 50 μM (39). In higher concentrations this substrate and its analogues appeared to be potent inhibitors of the carboxylation. Another type of exogenous substrates is formed by the proteolytic split products

derived from PLVKA's. One of them is fragment Su, derived from descarboxy-prothrombin and consisting of the amino acid residues 13-29 (40). Its K_m value is about 2 μM . Although the bound endogenous proteins are suggested to be carboxylated nearly completely (41) the pentapeptides are thought to be carboxylated for less than 1%. Moreover, only the first Gla-residue has been found to be carboxylated (42,43). The presence of the earlier mentioned leader sequence may be essential for the binding and hence for the efficient carboxylation of the proteins. This idea is supported by the fact that intracellular prothrombin precursors appear to contain a basic prosegment (44). A competitive inhibitor with regard to peptide substrates has been prepared by Gaudry et al. (45). This pentapeptide Phe-Leu-1-threo-4-methyl Glu-Glu-Leu exhibited a K_i of about 65 μM in the carboxylation of F L E E L. Another competitive inhibitor, the peptide Boc-4-methylene Glu-Glu-Val, with a K_i of about 50 μM was synthesized by the group of Azerad (46).

In the carboxylation reaction the K_m values for CO_2 has been estimated to be 0.2-0.4 mM (47) while the K_m values for O_2 ranged between 10-15 μM in intact microsomes (48) and 45 μM in detergent solubilized microsomes (49). The K_m value for KH_2 is not known, in most studies 50-500 μM KH_2 is used and this quantity does not seem to be rate limiting.

VITAMIN K CYCLE

In the liver three metabolites of vitamin K have been identified: vitamin K quinone (K), vitamin K hydroquinone (KH_2) and vitamin K 2,3 epoxide (KO). Calculations based on the daily requirement for vitamin K relative to the formation of Gla, as measured by its excretion, show that each molecule of the vitamin must be recycled several thousand times before it is metabolized further and excreted (50). Therefore it has been proposed by several groups (50,4) that the 3 forms of vitamin K are interconverted into each other in a cyclic way as is depicted in fig. 4. In step 1, KH_2 , the active cofactor in carboxylation, is converted in KO by the vitamin K epoxidase (51). This enzyme may be linked to or identical with the carboxylase. This hypothesis is strengthened by the observation that increasing the concentration of a peptide substrate, which results in an increase in the number of carboxylation events, also leads to an increase

Fig. 4.

Schematic representation of the vitamin K cycle; for abbreviations see text

in epoxidase activity (52). It is generally believed now that the oxidation of KH_2 provides the energy required for the carboxylation of glutamic acid residues (34). In step 2, KO is reduced to K by vitamin KO reductase. In vitro the reducing equivalents required for this reaction are provided by dithiothreitol (DTT) but its physiological counterpart has not yet been discovered. From recent data (53,54,55) it has been suggested that a free sulphhydryl group on the KO reductase attacks the epoxide ring in KO, resulting in a thioether adduct. The next step would be the formation of a 3-hydroxy-vitamin K-enolate, including the reformation of the disulfide form of the enzyme. The quinone form, K, would be subsequently formed by elimination of H_2O from the 3-hydroxy-K intermediate. This intermediate has been demonstrated to be present in microsomes from warfarin-resistant rats (56). Step 2 is extremely sensitive to coumarin derivatives (57,58). The close structural similarity between the postulated enolate form of hydroxyvitamin K and 4-hydroxy-coumarins might be an explanation for the coumarin induced inhibition (54,56,59). In step 3, vitamin K can be reduced

to KH_2 by several possible enzymes, one driven by DTT and several by NADH. The NADH-dependent DT-diaphorase was purified from rat liver by Wallin et al. (60). It was found that DT-diaphorase, although in majority cytosolic, is also present in microsomes. DT-diaphorase can be inhibited by relatively high concentrations of warfarin (50). More recently it was shown that only 45% of the NAD(P)H dependent K reductase activity in Triton X-100 solubilized red liver microsomes could be neutralized by antibodies against pure DT-diaphorase. The remaining NADH-dependent K reductase activity appeared to be warfarin-insensitive. It was assumed that the unidentified NADH-dependent dehydrogenase(s) are responsible for the enhanced coagulation factor synthesis, triggered by high concentrations of vitamin K in the presence of coumarin drugs (62,63,64). The existence of a warfarin-insensitive NADH stimulated K reductase was also found by Roncaglioni et al. (65). A DTT-dependent K reductase was identified by Fasco and Principe (66). This enzyme was reported to be very sensitive to warfarin (58). From the present data it is not clear whether both the DTT-dependent KO and K reductase activities are catalyzed by the same protein or by two different ones (67,68,69).

POSTULATED MECHANISMS OF THE VITAMIN K-DEPENDENT CARBOXYLATION REACTION

When Glu residues are converted into Gla-residues, a proton from the fourth C-atom in Glu must be abstracted and replaced by a carbondioxide group. Originally, two possibilities for vitamin K action in this process were proposed: 1) as a CO_2 -carrier and 2) as a cofactor utilized to labilize the abstractable hydrogen at the 4-position (4,70). Friedman et al. (71) have shown that the cleavage of the 4-carbonyl-hydrogen bond is KH_2^- and O_2 -dependent, but independent on CO_2 concentrations. From these observations it has been concluded that vitamin K is involved in the hydrogen abstraction.

Dubois et al. (72) and Decottignies-Le Marechal et al. (73) demonstrated that the pro S-hydrogen at the 4-position of the Glu-residue is eliminated prior to carboxylation. These data afford a direct proof for a Glu-site specificity. However, these data do not establish whether a radical-mediated or a ion-mediated mechanism is involved in the carboxylation process. McTigue et al. (74) have recently shown a KH_2^- and

O_2 -dependent 3H incorporation from tritiated water. This result would favour the hypothesis of an ion-mediated mechanism. The role of vitamin K would be the abstraction of the 4-carbonyl-hydrogen as a proton, leaving a carbanion which can react with CO_2 to form 4-carboxyglutamic acid.

Some other theories include the formation of radicals via KH_2 . Friedman et al. (71) and Larson et al. (75) assumed the formation of a 4-carbonyl-radical and a vitamin K radical, while the 4-carbonyl hydrogen is released, followed by the addition of the carbonyl group. De Metz et al. (76) proposed a concerted reaction mechanism via which the proton abstraction, the CO_2 addition and the KO formation all occur by a single electron shift after the reaction components have been properly aligned on carboxylase.

Current ideas of the carboxylation mechanism stress its relationship to the K epoxidase activity. It is not clear how epoxide formation and 4-hydrogen abstraction are coupled but it could be by an oxygenated intermediate of KH_2 in the form of $KHOOH$ (70). Until now, there is no direct proof for the formation of such an intermediate, however.

Obviously, the investigation concerning the carboxylation mechanism are hampered by the fact that the enzyme system has not yet been completely purified.

NON-HEPATIC VITAMIN K-DEPENDENT CARBOXYLASE

It has been assumed for many years that vitamin K-dependent carboxylase was only involved in the synthesis of the coagulation factors II, VII, IX and X in the liver. However, Vitamin K was demonstrated to be present not only in liver, but in many other tissues as well (77,78). The only known function of vitamin K is its cofactor role in a carboxylation reaction. It may not be surprising, therefore, that vitamin K-dependent carboxylase activity can also be demonstrated in various non-hepatic tissues. In 1976 Hauschka et al. (79) demonstrated vitamin K-dependent carboxylase activity in kidney microsomes. The activity appeared to be localized in the tubular cells (80). Microsomes from embryonic chicken bone also showed vitamin K-dependent carboxylase activity towards synthetic substrates and an endogenous bone-gla-protein precursor (81). The synthesis of the latter appeared to be inhibited by warfarin (82). Bone carboxylase is thought to be present in the osteoblast (83). Vitamin K-dependent carboxylase activity

was also detected in microsomal preparations from lung (84,85,86), spleen (85,86), pancreas (85,87) and testis (85) from normal and warfarin-treated animals, and from arteries, cartilage, thymus, thyroid and uterus from a calf, treated with high doses of warfarin. In all tissues mentioned, Vermeer et al. (85) observed an increase in the amount of endogenous substrate after warfarin treatment. Buchtal et al. (87) reported the presence of vitamin K dependent carboxylase in microsomal preparations from several rat and murine tumors, i.e. lymphoma, mammary gland, and melanoma. Recently, vitamin K-dependent carboxylase activity has been reported in rabbit V₂ carcinoma (88). Moreover, the enzymatic activity was also demonstrated in fibroblasts (89).

IDENTIFICATION OF GLA-CONTAINING PROTEINS

Except for the coagulation factors II, VII, IX and X, blood plasma contains three more circulating Gla-containing proteins, the proteins C, S and Z. Protein C, synthesized in the liver, is a glycoprotein, composed of 2 chains, the molecular weight of which is 41000 D and 21000 D respectively (14). It contains 11 Gla-residues on the NH₂-terminal side in the light chain (90). Like the coagulation factors, protein C occurs in plasma in a zymogen form and can be activated into a serine protease by proteolysis with thrombin (91). The function of the activated protein C seems to be the inactivation of activated factor V and hence it is involved in one of the control mechanisms which limit the coagulation process whenever it has started (92). The activity of activated protein C is enhanced by the presence of protein S. The latter is a glycoprotein consisting of a single polypeptide chain with a molecular weight of about 65000 D and containing 9 Gla-residues (93). Protein S has been reported to be synthesized by endothelial cells (94). Protein Z is also a single-chain glycoprotein with a molecular weight of approximately 50000 D, containing 13 Gla residues per molecule, but its function and its place of synthesis are not yet clear (90). A considerable sequence homology has been found between all the Gla-containing proteins occurring in blood plasma (90). Although in vitro they rapidly bind to insoluble barium and calcium salts, in vivo they exclusively occur dissolved in plasma. In the presence of calcium ions,

these proteins have a high affinity towards negatively charged phospholipid surfaces, the binding to which highly stimulated their enzymatic activity (95).

As early as in 1976, Hauschka et al. (79) reported the presence of a membrane-bound Gla-containing protein in the kidney. The protein was not further characterized but it may be similar or related to the Gla-containing protein found in calcified renal stones (96). This protein, which is extremely acidic, has a molecular weight of 17000 D and contains about 7 Gla-residues per molecule. It does not seem to be related with any of the plasma-Gla-proteins (82). From human urine a Gla-containing protein has been isolated (97). This protein has been reported to prevent crystal growth of calcium oxalate.

Osteocalcin is a Gla-containing protein found in bone (98). It is among the six most abundant proteins in man and it comprises over 25% of the non-collagenous proteins in bone. Human-osteocalcin contains 49 aminoacid residues, among which are three Gla residues (99). The exact function of osteocalcin is not known, but it may play a role in bone development. When coumarin derivatives are administered to women during the first trimester of pregnancy the so-called "fetal warfarin syndrome" (chondrodysplasia punctata) can occur (100). The syndrome is characterised by bone abnormalities in the developing fetus. These are caused by the excessive and irregular precipitation of calcium phosphate in the rapidly growing parts of the bone (101). In the calcified areas the growth is stopped in an irreversible way. In vitro studies have demonstrated that osteocalcin has a very high affinity for hydroxylapatite. In vitro, low concentrations of osteocalcin are able to efficiently stop the growth of calcium phosphate crystals from supersaturated solutions (98,102). A second Gla-containing protein, which is different from osteocalcin, has also been isolated (103). It is called matrix-Gla-protein, its molecular weight is about 15000 D and it contains 5-6 Gla-residues per molecule.

In the calcified matrix of atherosclerotic human aorta a Gla-containing protein has been reported to be present (104,105). This protein was originally called atherocalcin and reported to have a molecular weight of about 80000 D (104). Later on, it turned out that the reported molecular weight should be ascribed to a protein complex also containing albumin and osteocalcin (105). A non-complexed Gla-containing protein, dissimilar to

osteocalcin, was isolated from the calcified atherosclerotic plaques. Its molecular weight was estimated to be 6000-10000 D (105,106).

Other Gla-containing proteins have been found in dentin (107), in calcified skin noduli (108) and in corals (109). The biological function of these proteins is not clear yet, but they may function in the regulation or reduction of the deposition of insoluble calcium salts.

The Gla-containing protein, found in the chorio-allantoic membrane of chicken eggs is supposed to be involved in the Ca^{2+} transport from the egg shell to the developing embryo (110).

Gla-containing proteins have also been discovered in the venom of various snakes (111,112). These proteins are also capable of binding to phospholipid micelles and in this way they can activate the blood coagulation process.

In some warfarin-sensitive tumor cells a vitamin K-dependent procoagulant protein has been demonstrated. This protein is a cysteine proteinase with factor X activating properties (113).

VITAMIN K-DEPENDENT CARBOXYLASE IN HUMAN TISSUES

In microsomes from human liver (114) and placenta (114,115) vitamin K dependent activity was demonstrated. No substantial differences were found between the vitamin K-dependent carboxylase systems in bovine and human liver microsomes with regard to the K_m values for F L E E L and KH_2 and the warfarin concentration which inhibits 50% of the activity in the K+DTT stimulated carboxylation reaction. In the microsomal preparations from human livers, however, a rather high amount of endogenous substrate was found as compared to the bovine preparations. The reason for it is not clear yet. Recently, Wallin et al. (116) also demonstrated K- and KO-reductase activities in microsomal preparations from human liver. The amount of microsomal DT-diaphorase was found to be substantially lower than in rat hepatic microsomes.

INTRODUCTION TO THIS THESIS

Vitamin K is involved in a posttranslational carboxylation reaction, in which specific glutamic acid residues are converted to 4-carboxyglutamic acid (Gla) residues. This reaction is commonly reported to occur in liver. The importance of the Gla-residues is clearly demonstrated by their role in blood coagulation. The formation of Gla-residues and hence the synthesis of biologically active coagulation factors is inhibited by administration of 4-hydroxycoumarin drugs, which are often used in anticoagulant therapy.

It is presently known that vitamin K-dependent carboxylation also occurs in various non-hepatic tissues. Until now, the biological significance of these non-hepatic vitamin K-dependent systems is hardly known. Therefore, it was felt necessary to start investigations concerning a) the properties of the non-hepatic vitamin K-dependent enzymes, b) the characteristics of the proteins which are carboxylated by these non-hepatic systems, and c) the effects of the administration of 4-hydroxycoumarin drugs on the non-hepatic systems. Further insight in these aspects may be of relevance for the treatment of patients with 4-hydroxycoumarin drugs.

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C H A P T E R 2

VITAMIN K-DEPENDENT CARBOXYLASE IN SKIN

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SUMMARY

Vitamin K-dependent carboxylase is demonstrated in skin microsomes from man, rat, rabbit and mouse. This enzyme converts a number of distinct protein-bound glutamic acid residues into 4-carboxyglutamic acid residues, which strongly interact with Ca^{2+} ions. The enzymatic activity (expressed per mg protein) in skin is about 20% from that in liver. Vitamin K-dependent carboxylase is present in both epidermic and dermic tissue. It is demonstrated that warfarin treatment in mice results in an accumulation of non-carboxylated precursor proteins in both dermic and epidermic microsomes. Most probably this effect of warfarin is not restricted to mice, but occurs also in the skin of patients under oral anticoagulant therapy.

A possible relation between vitamin K-dependent skin carboxylase and the 4-carboxyglutamic acid-containing protein in calcified nodules from patients with scleroderma and dermatomyositis is discussed.

INTRODUCTION

Vitamin K is involved in a posttranslational modification of proteins (1,2). This modification consists of the carboxylation of a number of distinct glutamic acid residues into 4-carboxyglutamic acid residues (Gla). The presence of Gla has initially been discovered in blood coagulation factors, which are synthesized in the liver (3,4). The Gla-rich regions are essential for the proper functioning of these coagulation factors. Coumarin derivatives, such as warfarin, inhibit the formation of Gla-residues and therefore they are used as anticoagulants (5).

For a long period it has been thought, that the vitamin K-dependent carboxylation was restricted to the liver. During the last decade, however, similar enzyme systems have been detected in various other tissues such as kidney, spleen, testis, lung and vessel wall (6,7). In vivo, the administration of low doses of warfarin affects both the hepatic as well as the non-hepatic carboxylase systems (8). It is to be expected that all these vitamin K-dependent enzymes are involved in the production of Gla-containing proteins, but in most cases their precise function and even their place of action are unknown. Gla-containing proteins have been demonstrated in for instance bone (9), renal stones (10), calcified atheromatous plaques (11), calcium containing material extruded from the skin of scleroderma-patients and in the calcified skin and subcutaneous plaques from patients with dermatomyositis (12). Whereas it has been suggested that the bone Gla-protein is synthesized by the osteoblasts (13), the origin of the Gla-containing proteins in the calcified plaques occurring in the skin diseases mentioned above has remained obscure until now. It was of interest therefore, to examine skin tissue for the presence of vitamin K-dependent carboxylase. The results of our investigations are presented in this paper.

MATERIAL AND METHODS

Materials

Vitamin K₁ (konakion^R) was obtained from Hoffmann-La Roche (Basel, Switzerland). Vitamin K hydroquinone was prepared as described earlier

(13). 3-([3-cholamidopropyl])dimethyl-ammonio)-1-propane sulfonate (CHAPS), dithiothreitol (DTT) and warfarin were from Sigma (St. Louis, U.S.A.) and the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu (F L E E L) was from Vega Biochemicals (Tucson, U.S.A.) $\text{NaH}^{14}\text{CO}_3$ (40-60 Ci/mol) was purchased from Amersham (Amersham U.K.) and Atomlight from New England Nuclear (Dreieich, FRG). Dispase protease neutrale from *Bacillus polymixa* grade II was obtained from Boehringer Mannheim (Mannheim, FRG). All other chemicals were from Merck (Darmstadt, FRG). Athymic, hairless mice, strain NMRI, nu/nu were obtained from TNO (Zeist, The Netherlands).

Methods

Skin tissue was obtained from 12-16 weeks old hairless, athymic mice, newborn Lewis rats and newborn Hollander rabbits, shortly after the animals were sacrificed under ether anaesthesia. Skin tissue from man without known skin diseases was obtained from the Departments of Pathology in hospitals at Maastricht and Amsterdam. All pieces of skin tissue were obtained less than 3 hours after death and they were immediately frozen at -80°C . For some experiments, livers, kidneys, lungs and testes were excised from athymic mice shortly after their sacrifice. For some experiments these mice were injected intraperitoneally with warfarin dissolved in 150 mM NaCl, pH 7.4, in a dose of 10 mg per kg body weight, 18 hours before sacrifice.

Epidermal tissue was separated from the underlaying connective tissue by dispase treatment. Therefore we slightly modified the method described by Kitano et al. (15). Skin tissue was cut into pieces of about 2 x 4 cm and incubated at room temperature with 150 ml 0.5% dispase (w/v), 0.15 NaCl pH 7.4, during 4-6 hours. After this incubation the skin could be divided into epidermis and dermis with a small spatula. To control the separation, pieces of tissue were fixed in phosphate buffered formalin and embedded in paraffin. 4 μm tissue sections were stained with hematoxylin or azan and examined with a light microscope.

The microsomal fractions of the tissues were prepared in a similar way as described earlier (6). After three washing cycles the microsomes were suspended in buffer A (0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 1 mM EDTA, 20% (v/v) ethyleneglycol) to a concentration of 3-10 mg protein per ml and stored at -80°C .

The carboxylase activity was measured by incubating 0.25 ml reaction mixtures at 25°C in buffer A, containing 0.4 mg microsomal protein, 0.1 mg

CHAPS, 10 mM F L E E L, 8 mM DTT, 0.2 mM vitamin K hydroquinone, 0.01 mCi $\text{NaH}^{14}\text{CO}_3$ and 1 M $(\text{NH}_4)_2\text{SO}_4$. The reaction was stopped and non-bound $^{14}\text{CO}_2$ was removed by adding trichloroacetic acid (5%) and degassing the solution for 2 min at elevated temperatures. The samples were supplemented with 10 ml Atomlight and counted in a Beckman scintillation counter. Since under the incubation conditions all reaction components are present in excess, the initial reaction rates are proportional to the respective amounts of carboxylase. To measure the amount of endogenous substrate no F L E E L was added to the reaction mixture. In all experiments parallel incubations were performed in the absence of vitamin K hydroquinone and DTT; these blank values were subtracted. It was ascertained that dispase had no effect on the carboxylase-activity measurements.

Protein concentration was determined according to Sedmak and Grossberg (16).

Gla residues were determined by alkaline hydrolysis of homogenized tissue and subsequent HPLC analysis on a Nucleosil 5SB column (Chrompack) as described by Kuwada and Katayama (17).

RESULTS

The detection of vitamin K-dependent carboxylase activity

Skin tissue was obtained from man, hairless mice and from newborn rats and rabbits. After tissue homogenization the microsomal fractions were prepared. In all these species vitamin K-dependent carboxylase activity could be demonstrated (table I). The rather low amounts measured in the rabbit and rat preparations may be caused by a less efficient homogenization of the tissues. All experiments described below were performed with hairless mice as their skin is most readily homogenized.

To compare the amounts of vitamin K-dependent carboxylase activity in skin and other tissues we also prepared the microsomal fractions of liver, kidney, spleen and testes from the same animals. In all microsomal preparations carboxylation proceeded in a linear way for at least 30 min. The skin carboxylase activity is comparable with that in other non-hepatic tissues, but much lower than the liver carboxylase activity (table II). The

Table I

Vitamin K-dependent carboxylase activity in skin from various species

species	carboxylase activity (dpm/min per mg protein)
man	500
rabbit	105
rat	200
mouse	590

Microsomes were prepared from about 10 g of skin tissue, and carboxylase activity was measured as described in Materials and Methods. Blank values without added KH_2 and DTT (15-30 dpm/min per mg protein) were subtracted. The data are the mean of duplicate measurements.

Table II

Vitamin K-dependent carboxylase activity in various tissues from athymic mice

tissue	$^{14}\text{CO}_2$ incorporated (dpm)		relative carboxylase activity (%)
	+ KH_2	- KH_2 -DTT	
skin	6470	245	100
liver	36200	200	580
kidney	3810	340	56
lung	12010	220	190
spleen	2720	370	38
testis	5250	290	80

The vitamin K-dependent carboxylase activities are given as the amount of $^{14}\text{CO}_2$ incorporated in 25 min in the presence and absence of vitamin KH_2 and DTT. All reaction mixtures contained 0.4 mg of microsomal protein. In the last column the activities are expressed as a percentage of the skin carboxylase activity per mg microsomal protein.

Fig. 1 Skin tissue of athymic mice

A: Skin before disperse treatment, stained with hematoxylin

B: Epidermis, stained with hematoxylin

C: Dermis, stained with azan

Bar = 0.1 nm

ratio between the enzyme activities in liver, kidney, spleen, lung and testes microsomes from these mice is in reasonable agreement with results earlier found for rats (7).

The localization of skin carboxylase

In earlier experiments we demonstrated a considerable vitamin K-dependent carboxylase activity in bovine vessel wall. It cannot be excluded that this vascular enzyme accounts, at least partly, for the carboxylase activity demonstrated in microsomes derived from several whole tissue homogenates. Experiments were undertaken to investigate whether the skin carboxylase activity could be attributed to the vascular vitamin K-dependent carboxylase system. As the epidermis does not contain any blood vessels, skin was separated into epidermic and dermic tissue by dispase treatment. It was ascertained by histological techniques that the epidermic cells did not contain any detectable amount of dermic tissue and vice versa (fig. 1). Carboxylase activity was detected in both microsomal preparations. The carboxylation rate was constant for about 30 min as is shown in fig. 2.

Effects of warfarin treatment in vivo

In liver, warfarin treatment causes the accumulation of non-carboxylated precursors of blood coagulation factors. A part of these precursors remain firmly bound to the microsomal carboxylase-complex and can be carboxylated in vitro (endogenous substrate). We investigated whether a similar effect of warfarin would occur in skin tissue. Athymic mice were injected intraperitoneally with a dose of 10 mg warfarin per kg body weight. After 24 hours the mice were sacrificed, the skin was removed and the livers and kidneys were excised. Microsomes were prepared and the amounts of endogenous substrates were measured. In all preparations an increase of non-carboxylated precursor proteins was demonstrated after warfarin treatment (table III). This increase was comparable in skin and kidney, whereas in liver a higher level of endogenous substrate was detected. This is in agreement with earlier results obtained from bovine and rat experiments in which invariably a higher accumulation of endogenous

Fig. 2 Time course of vitamin K-dependent carboxylation

The carboxylation was performed as described in Materials and Methods. Blank values without added vitamin KH_2 and DTT (200-450 dpm per incubation) were subtracted.

- dermis microsomes
- epidermis microsomes

substrate was found in liver than in other tissues (6,7,8). The sum of the quantities of endogenous substrate found in the microsomes from isolated dermic and epidermic tissue was slightly lower than the amount of endogenous substrate found in total skin microsomes. This was due to the disperse treatment. Nevertheless, an increase of non-carboxylated precursor proteins was demonstrated after warfarin treatment in epidermis and dermis as well. To determine if $[^{14}\text{C}]$ Gla was formed in the precursor proteins, incubated samples of microsomes from warfarin treated mice were heated at 110°C in 6 N HCl for 24h. This treatment resulted in a loss of 59% of the bound ^{14}C in total skin microsomes, 47% in dermic microsomes, 56% in

Table III

Amounts of accumulated endogenous substrate after warfarin treatment

tissue	amounts of endogenous substrate (dpm/60 min per mg protein)	
	normal	after warfarin treatment
liver	4970	14460
kidney	770	1540
skin	1260	2560
dermis	970	2000
epidermis	800	2230

Warfarin was given intraperitoneally in a dose of 10 mg/kg body weight to 16 athymic mice. Microsomes were prepared from the pooled tissues of normal and warfarin treated mice respectively and endogenous substrate was measured as described in Materials and Methods. Blank values (250-500 dpm/60 min per mg protein) were subtracted. The data are the mean of triplicate measurements.

epidermic microsomes and 54% in liver microsomes. The theoretical loss of ^{14}C from [^{14}C] Gla labeled in one of the carboxyl groups would be 50%. From these results we concluded that under our experimental conditions Gla-residues had been formed by skin microsomes.

The presence of vitamin K-reductase

In bovine liver and non-hepatic microsomes, vitamin K-dependent carboxylase has been shown to be closely linked to another enzyme, vitamin K reductase. The latter enzyme is responsible for the reduction of vitamin K quinone to vitamin K hydroquinone, which is an essential cofactor in the carboxylation reaction. To detect the presence of vitamin K reductase in dermis and epidermis, in vitro carboxylation studies were performed with vitamin K quinone as a cofactor instead of vitamin K hydroquinone. The carboxylation rates were about 50% of the vitamin K hydroquinone stimulated reaction for dermis, epidermis as well as for total skin microsomes. This

is comparable with previous results found in bovine liver microsomes. In addition, the in vitro inhibition by warfarin of vitamin K quinone reductase was measured. The carboxylation was carried out with vitamin K quinone as a cofactor and at various warfarin concentrations. In all microsomal preparations, a warfarin concentration of 0.002 mM inhibited the carboxylation rate for 50%. From these results we concluded that vitamin K reductase is present in both dermic and epidermic microsomes.

DISCUSSION

It has been well established, that the vitamin K-dependent carboxylation reaction is not restricted to the liver, but that it occurs in many non-hepatic tissues as well (6,7). In a previous study we have also demonstrated that the vitamin K-dependent carboxylases from bovine liver, kidney, lung and testis may be regarded as a group of iso-enzymes, which mainly differ in their substrate specificity (18). In the present paper we report the discovery of vitamin K-dependent carboxylase in skin and, like in other tissues, the enzyme is located in the microsomal fraction of tissue homogenates. It remains to be investigated whether skin carboxylase is identical with one of the previously discovered carboxylases or if it is another distinct member of the group of vitamin K-dependent carboxylases.

The Gla-residues which are formed in the carboxylation reaction are known to bind strongly and selectively to Ca^{2+} . The Gla-containing proteins discovered up till now may be classified as follows (2):

- a) Gla-proteins in blood plasma (e.g. various coagulation factors), which are all able to bind to phospholipid surfaces via the complexed Ca^{2+} ;
- b) Gla-proteins found in depositions of insoluble calcium salts such as bone, dentin, renal stones and atheromatous plaques;
- c) membrane-bound Gla-proteins, examples of which are found in the kidney and in the chorioallantoic membrane of chicken eggs and
- d) proteins which do not fit in one of the three former groups, such as a snail neurotoxin (18) and the Gla-protein in spermatozoa.

Whereas most of the Gla-proteins found in plasma are involved in the process of blood coagulation, the function of the other proteins is less clear at this moment.

In an attempt to estimate the physiological importance of skin carboxylase, we have tried to identify its putative product. Obviously, this should be a Gla-containing protein and we have tried to find such a protein in KOH extracts of hair and nail, in sweat and in normal human and animal skin homogenates. In none of these products Gla could be detected. However, skin homogenates, like liver homogenates, contain too many proteins to detect Gla without further purification. On the other hand a Gla-containing protein has been reported to occur in the pathological depositions of calcified material in the skin of patients suffering from scleroderma and dermatomyositis (12). Obviously the latter protein belongs to the category mentioned under b) (see above), but whether it is indeed produced by skin carboxylase is not sure at this moment.

Speculating about the function of the skin Gla-protein we would like to mention two possibilities. In the first place the protein might play a role in the calcium metabolism in skin. Indications in favour of this hypothesis were obtained from clinical studies in which patients with dermatomyositis and massive depositions of subcutaneous calcific nodules were treated with low doses of warfarin. This treatment induced a profound and rapid decrease of the calcified lesions (20). As warfarin inhibits the formation of Gla, these data suggest a possible role for the skin Gla-protein in the regulation of the calcification process.

Alternatively, also a pathological effect of warfarin on skin has been described: especially during the early stages of oral anticoagulant treatment skin necrosis may occur (21). It has been proposed that the latter phenomenon may be the result of an increased thrombogenic activity in the capillaries induced by an protein C deficiency (22). Protein C is a Gla-containing plasma protein, produced by the liver and does have anticoagulant activity (23). Without doubts its synthesis is inhibited by warfarin. It is remarkable however, that the disturbed balance between protein C and the vitamin K-dependent coagulation factors has only been reported to cause ischemic necrosis of capillaries in skin and not in other tissues. Therefore another protein may also be involved in the occurrence of this type of thrombosis. Such a protein might be the putative skin Gla-protein which is produced by skin carboxylase. In this paper we have shown that coumarin derivatives directly inhibit skin carboxylase and thus the carboxylation of the skin Gla-protein. This might result in a decreased biological activity

of the latter, which may play an additional role in the observed skin necrosis.

Obviously further research is wanted to elucidate the function of the skin Gla-protein and thus the physiological importance of the vitamin K-dependent skin carboxylase.

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C H A P T E R 3

THE IN VIVO EFFECTS OF ACENOCOUMAROL, PHENPROCOUMON AND WARFARIN ON VITAMIN K EPOXIDE REDUCTASE AND VITAMIN K-DEPENDENT CARBOXYLASE IN VARIOUS TISSUES OF THE RAT

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SUMMARY

In rats the in vivo effects of a chronic low dose treatment (± 60 $\mu\text{g}/\text{rat}/\text{day}$) with different coumarins (acenocoumarol, phenprocoumon and warfarin) on hepatic and non-hepatic vitamin K-dependent enzyme systems were compared. The plasma concentrations of the three coumarins differed largely but these differences were not reflected in the microsomal coumarin contents. The non-hepatic microsomes contained less than 20% of the coumarins found in liver microsomes. No substantial differences were observed between the following effects of the three anticoagulant treatments:

- 1) The blood coagulation factor activities were about 10% of normal.
- 2) The hepatic microsomal vitamin K epoxide reductase activity was diminished to about 35% of control values. The vitamin K epoxide reductase activities present in kidney, lung, spleen, testis and brain microsomes were less influenced by the coumarin treatments; activities ranged between 45 and 65% of normal.
- 3) In liver microsomes a 15-fold accumulation of non-carboxylated precursor proteins was found; in the non-hepatic microsomes this effect was less pronounced but still present.
- 4) The hepatic vitamin K-dependent carboxylase activity was enhanced but the corresponding non-hepatic enzyme activities were slightly or not affected.

In addition, the effects of a chronic low dose warfarin treatment were compared with those after an acute high dose of the drug.

INTRODUCTION

Vitamin K is involved in the conversion of peptide-bound glutamic acid residues into 4-carboxyglutamic acid residues. This reaction is catalyzed by vitamin K-dependent carboxylase, located at the luminal side of the endoplasmic reticulum. In this carboxylation, the active cofactor vitamin K hydroquinone, is oxidized to vitamin K 2,3 epoxide, which is reduced in a second step to vitamin K quinone and to vitamin K hydroquinone by one or more specific reductases (1-3). It is generally believed now that vitamin K antagonists such as warfarin inhibit the vitamin K epoxide reductase. The result of this inhibition is that the supply of vitamin K hydroquinone is exhausted, which decreases the rate of the carboxylation reaction. In vivo this sequence of events results in the appearance of descarboxy coagulation factors in blood and in an increase of non-carboxylated precursor proteins in the liver.

For many years it has been thought that vitamin K-dependent carboxylation exclusively occurred in the liver to form the biologically active coagulation factors II, VII, IX and X. Administration of warfarin and other coumarin drugs has been used frequently for the control of blood coagula-

tion during thrombogenic episodes. In the last 10 years, it became evident that vitamin K-dependent carboxylation is a more common process, also occurring in other tissues such as kidney, lung, spleen, bone and testis (4,5). Obviously, all these enzyme systems will produce 4-carboxyglutamic acid-containing proteins, but only few of them have been isolated and characterized. Since the only known function of 4-carboxyglutamic acid residues is a selective interaction with Ca^{2+} ions, it may be expected that these proteins are involved in Ca^{2+} metabolism, Ca^{2+} transport or Ca^{2+} phospholipid interactions (6). In an earlier study concerning rat liver, lung, spleen and testis carboxylase we have reported that the *in vivo* administration of a low dose of warfarin affected both the hepatic and non-hepatic vitamin K dependent systems. This was demonstrated by the accumulation of non-carboxylated precursor proteins in the various tissues (7).

	R_1	R_2
ACENOCOUMAROL	CH_2COCH_3	NO_2
PHENPROCUMON	CH_2CH_3	H
WARFARIN	CH_2COCH_3	H

Fig. 1.

Structural formulas of acenocoumarol, phenprocoumon and warfarin.

Recently Thijssen et al. showed the presence of vitamin K epoxide reductase activity in rat kidney and testis tissue (8). Whereas single doses of warfarin (up to 1 mg/kg) hardly affected the non-hepatic vitamin K epoxide reductase activity, a more pronounced effect was observed after chronic warfarin administration. In liver, the enzymatic activity appeared to be more decreased than in kidney and testis. Differences in the tissue distribution of warfarin were postulated to be responsible for the observed selectivity.

Since in Europe, instead of warfarin two other vitamin K antagonists, acenocoumarol and phenprocoumon, are generally used for oral anticoagulant therapy in man, it seemed interesting to compare the effects of the administration of the three coumarin drugs (for structures see fig. 1) on the vitamin K-dependent systems in various tissues. The results of these investigations are presented in this paper.

MATERIALS AND METHODS

Materials

Phenprocoumon (Marcoumar^R) and water-solubilized vitamin K (Konakion^R) were obtained from Hoffman-La Roche (Switzerland) and vitamin K hydroquinone was prepared as described earlier [9]. Pure vitamin K was obtained from Merck (F.R.G.) and vitamin K 2,3 epoxide was prepared from it according to the method of Tishler et al. (10). Warfarin, dithiothreitol, tocopherolacetate and 3-([3-cholamidopropyl]dimethyl-ammonio)-1-propane sulfonate (CHAPS) were purchased from Sigma (U.S.A.) and acenocoumarol (Sintrom^R) from Ciba Geigy (Switzerland). The synthetic substrate Phe-Leu-Glu-Glu-Leu (FLEEL) was obtained from Vega Biochemical Co (U.S.A.), $\text{NaH}^{14}\text{CO}_3$ (50-60 Ci/mol) from Amersham (U.K.) and Atomlight from New England Nuclear (F.R.G.) A Lichrosorb 5 RP18 column (150 x 4.6 mm) was obtained from Chrompack (The Netherlands). All other chemicals were obtained from Merck (F.R.G.).

Methods

Male Lewis rats, 10-12 weeks old, were used throughout the experiments. Warfarin, acenocoumarol or phenprocoumon was dissolved in 20 mM phosphate buffer pH 9 to a final concentration of 6.5 $\mu\text{mol/l}$. The rats received these

solutions as their sole source of drinking water, the daily intake was approximately 30 ml ($\pm$ 60 μ g coumarin). Control rats received only phosphate buffer. In some experiments a warfarin solution was administered intraperitoneally in a dose of 20 mg/kg body weight. Before sacrificing the rats under ether anaesthesia, 4.5 ml blood was taken from the aorta and mixed immediately with 0.5 ml 0.1 M trisodiumcitrate. Coagulation factor activities were determined with specific reagents according to van Dam-Mieras et al. (11).

Various tissues i.e. liver, kidney, spleen, testis, lung, brain, heart and femoral muscle were excised, rinsed with buffer A (0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 1 mM EDTA, 20% ethyleneglycol) and immediately frozen at -80°C until further use. After thawing, the livers, kidneys etc. of one group of at least five experimental rats were pooled and microsomes were prepared in a similar way as described before (12). After three washing cycles the microsomes were suspended in buffer A containing 1 M NaCl, to a concentration of 10-30 mg protein/ml and stored at -80°C . Protein concentrations were determined according to Sedmak and Grossberg (13).

The concentration of warfarin, acenocoumarol and phenprocoumon was determined in plasma, tissue homogenates and microsomes using an HPLC assay, as described previously (14). Calculations were made from the peak area ratios 4-hydroxycoumarin/internal standard and a calibration curve.

The vitamin K dependent incorporation of $^{14}\text{CO}_2$ was measured by incubating 1 mg microsomal protein in 0.25 ml reaction mixture in buffer A, supplemented with 8 mM dithiothreitol, 0.2 mM vitamin K hydroquinone, 4 mM F L E E L, 0.2% CHAPS (w/v), 0.01 mCi $\text{NaH}^{14}\text{CO}_3$ and 1 M $(\text{NH}_4)_2\text{SO}_4$. Incubation was performed in a waterbath at 25°C under occasional shaking. The reaction was stopped and non-bound $^{14}\text{CO}_2$ was removed by adding 1 ml trichloroacetic acid (5%) followed by degassing the reaction mixture for 2 min at elevated temperatures. The samples were supplemented with 10 ml Atomlight and counted in a Beckman scintillation counter. Under the conditions mentioned above, the reaction rates were linear for at least 20 min for all tissues examined. The $^{14}\text{CO}_2$ incorporation in each sample was measured after 5, 10 and 20 minutes incubation and the carboxylase activity is expressed as dpm incorporated $^{14}\text{CO}_2$ per min per mg protein. To measure the amount of non-carboxylated precursor proteins (endogenous substrate),

F L E E L and $(\text{NH}_4)_2\text{SO}_4$ were omitted from the reaction mixture, the incubation time was prolonged to 60 min and 2.5-4 mg microsomal proteins were used per incubation. The amount of the endogenous precursor proteins is expressed as dpm incorporated $^{14}\text{CO}_2$ per h per mg protein. In all experiments, parallel incubations were performed in the absence of vitamin K hydroquinone and dithiothreitol. These blank values were subtracted.

To determine vitamin K epoxide reductase activity, 1 mg of microsomal protein was mixed with 0.02 M Tris-HCl, pH 7.4, 0.15 M KCl to a final volume of 0.5 ml. After 3-5 min preincubation in a shaking waterbath at 30 °C, dithiothreitol was added to a final concentration of 5 mM and the mixture was incubated for another 3 min before adding 0.05 μmol vitamin K epoxide as substrate. Vitamin K epoxide was dissolved in propanol-2 (10 μmol vitamin K epoxide/ml). Liver microsomes were incubated for 20 min; microsomes from other tissues for 45 min. It was ascertained that the reaction rates were linear for at least 1 h. The reaction was stopped by mixing 0.4 ml of the reaction mixture with 4 ml of propanol-2/ H_2O (3:1, v/v). To prevent the formation of association products of vitamin K epoxide or vitamin K hydroquinone and dithiothreitol due to co-extracted dithiothreitol, we modified the extraction procedure after terminating the reaction as follows: 0.1 ml of a AgNO_3 solution (0.1 M) was added immediately and the resulting mixture was briefly shaken on a whirlmix. Tocopherol acetate was added (12.5 μg in 50 μl propanol-2) as an internal standard and the mixture was extracted with 2 ml hexane by shaking it for 5 min. The hexane layer was washed with 4 ml of a 0.15 M NaCl solution, and evaporated to dryness at 35°C under a stream of nitrogen. The residue was redissolved immediately in 0.1 ml of propanol-2.

Analysis was performed by HPLC and UV adsorption at 250 nm. Separation was achieved on a Lichrosorb 5RPL8 column using acetonitrile:dichloromethane:water (100:10:1, v/v/v) as the eluent with a flow rate of 2 ml/min. Retention times were: K-epoxide, 4 min; internal standard, 5,5 min; K quinone, 6,0 min.

The amount of vitamin K quinone formed was determined by comparing the peak heights of vitamin K quinone and the internal standard with a calibration graph. The vitamin K epoxide reductase activity is expressed as the amount of vitamin K quinone formed per min and per mg microsomal

protein. Blank incubations without vitamin K epoxide added, were carried out for each tissue. In none of those samples vitamin K quinone could be detected.

RESULTS

Blood coagulation factor activities.

Male Lewis rats received a low dose of either acenocoumarol, phenprocoumon or warfarin via their drinking water. The daily intake was about 60 μ g per rat. After one week the coagulation factor activities as measured in their plasma-samples were all decreased to levels which are therapeutically relevant in man (table I).

Table I

Coagulation factor activities in plasma of coumarin-treated rats

Treatment	Coagulation factor activities (% of normal values)				
	II	V	VII	IX	X
Control	100	98	92	95	92
Chronic dose treatment with:					
acenocoumarol	10	94	7	16	8
phenprocoumon	11	87	10	23	14
warfarin	14	90	9	15	10
Acute high dose of warfarin	15	95	2	2	3

The coagulation factor activities were determined according to Van Dam-Mieras et al. (11) in the pooled plasma-samples of each group of 5 rats. The values are expressed as a percentage of a reference value obtained from a plasma-pool of 20 normal Lewis rats.

Table II

The amounts of acenocoumarol (A), phenprocoumon (P) and warfarin (W) present in plasma and in the microsomal fractions of various tissues

tissue	amount of coumarin (ng/ml and ng/mg)			
	chronic low dose treatment			acute high dose
	A	P	W	W
plasma	37+10	110+35	425+13	12670+340
liver	30	26	27	18
kidney	6	2	3	2
testis	6	5	3	4
spleen	3	2	3	2
lung	2	2	2	3

The amount of coumarin was determined using an HPLC method as described in Materials and Methods. Values are expressed as ng coumarin/ml plasma + SD and ng coumarin/mg microsomal protein. The microsomal fractions were prepared from pooled tissues of 5 rats.

No remarkable differences were found between the three coumarin-treatments. In addition, one group of male Lewis rats received a single high dose of warfarin (+ 6 mg/rat, intraperitoneally) and these rats were sacrificed 20 hours later. Except for prothrombin the coagulation factor activities in the plasma-samples of these rats were lower than in the chronic experiments.

Amounts of coumarin present in plasma and in the microsomal fractions of various organs.

The amounts of coumarins were determined in all plasma-samples and in the microsomal fractions of liver, kidney, lung, spleen, testis, heart, brain and muscle-tissue varied considerably. As is shown in table II, the plasma coumarin concentrations were different. The amount of acenocoumarol for instance is much lower than the amount of phenprocoumon, which on its

turn is only about 25% of the warfarin concentration. These dissimilarities are due to differences in the pharmacokinetics of the particular drugs (15,16,17). In spite of the different plasma-concentrations no substantial differences were observed in the microsomal contents of the three coumarins (table II). In kidney, lung, spleen and testis microsomes, the coumarin contents were about equal, but at least five fold lower than in liver microsomes. An acute high dose of warfarin resulted in a lower warfarin concentration in liver microsomes than the chronic warfarin treatment did, while no differences were observed in the non-hepatic microsomal fractions. The coumarin concentrations in brain, muscle and heart microsomes were too low to quantify although low amounts of all three drugs could be demonstrated in the tissue homogenates.

The effects of different coumarin treatments on vitamin K epoxide reductase activities

The reduction of vitamin K epoxide to vitamin K quinone in liver has been shown to be inhibited in vivo by warfarin. Recently, this inhibition has also been demonstrated in rat kidney and testis microsomes. We have compared the effects of acenocoumarol, phenprocoumon and warfarin-treatments with respect to the inhibition of vitamin K epoxide reductase activities in liver, kidney, testis, lung, spleen, brain and muscle microsomes. In all microsomal preparations from normal rats vitamin K epoxide reductase activity could be demonstrated, although the values measured for heart and muscle microsomes were very low (table III). Therefore it was not possible to determine accurately any inhibition by coumarins in heart and muscle. Comparing the effects of the various drugs we must conclude that in all tissues phenprocoumon appeared to be slightly less inhibitory than acenocoumarol and warfarin (table IV). By each drug the non-hepatic vitamin K epoxide reductases were all inhibited to a similar extent, while the hepatic enzyme was found to be clearly more reduced. The acute high dose of warfarin resulted in 10-20% more inhibition than the chronic low-dose warfarin treatment did. Again, the liver reductase appeared to be more affected than the non-hepatic enzymes.

Table III

Vitamin K epoxide reductase activities in tissues of normal Lewis rats

tissue	vitamin K epoxide reductase activity (pmol vitamin K/min per mg protein)
liver	28
kidney	15
testis	16
lung	7
spleen	5
brain	6
heart	<0.5
muscle	<0.5

Vitamin K-epoxide reductase activities were measured in microsomal fractions of various tissues of non-treated rats. The data are the mean of duplicate measurements.

The effects of various coumarin treatments on the amounts of non-carboxylated precursor proteins

It has been demonstrated before, that as a result of warfarin treatment non-carboxylated precursor proteins (the so-called endogenous substrate for carboxylase) accumulate in both hepatic and non-hepatic tissues. The purpose of this experiment was to investigate possible differences in the amounts of these endogenous substrates after different coumarin treatments. As is shown in table V a chronic low dose treatment of either acenocoumarol, phenprocoumon or warfarin resulted in similar levels of endogenous substrate. In liver, the increase in endogenous substrate is at least tenfold, in the other tissues this increase is less pronounced. In liver the chronic low dose coumarin treatment resulted in a larger amount of endogenous substrate than the acute high dose of warfarin did.

Table IV

Effects of acenocoumarol (A), phenprocoumon (P) and warfarin (W) treatment on vitamin K-epoxide reductase activity in various tissues

tissue	vitamin K-epoxide reductase activity (% of non-treated values)			
	7 days low-dose treatment			single high dose
	A	P	W	W
liver	30	36	33	23
kidney	46	66	52	34
testis	44	62	47	29
lung	47	62	46	36
spleen	45	59	52	32
brain	46	66	51	38

Vitamin K epoxide reductase activities were measured in the microsomal fractions of various tissues of coumarin-treated rats. The data are the mean of duplicate measurements and are expressed as percentage of the corresponding activities in non-treated rats.

The effects of various coumarin treatments on vitamin K-dependent carboxylase activities

The vitamin K-dependent carboxylase activity was determined in the microsomal fractions of the various tissues. From the data shown in table VI, it will be clear that vitamin K-dependent carboxylase activity can be demonstrated in all tissues examined, even in brain, heart and muscle, although the activities in the latter tissues are less than 10% that in normal liver microsomes. The data obtained in these experiments are in reasonable agreement with the findings of Buchtal and Bell (5). After the chronic low dose coumarin treatments the vitamin K-dependent carboxylase activity in liver was increased. This effect has been shown before in hypothyrombinemic rats (18,19). The increase in carboxylase activity is, however, less pronounced or absent in the non-hepatic tissues. No significant differences in vitamin K-dependent carboxylase activities were

Table V

Effects of acenocoumarol (A), phenprocoumon (P) and warfarin (W) treatment on the amounts of non-carboxylated precursor proteins

tissue	amount of non-carboxylated precursor proteins				
	normal	chronic low dose treatment			single high dose
	(dpm/mg protein)	(% of normal)			(% of normal)
		A	P	W	W
liver	1250	1760	1640	1400	800
kidney	480	230	215	270	150
testis	200	130	145	215	140
lung	375	150	190	170	190
spleen	350	120	170	150	200
brain	50	200	250	220	270
heart	45	170	200	240	270
muscle	65	150	170	200	200

The amounts of non-carboxylated precursor proteins were measured in the microsomal fractions of various tissues. Values are expressed as dpm $^{14}\text{CO}_2$ incorporated per mg microsomal protein. Blank values, ranging between 10 and 30 dpm per mg microsomal protein were subtracted. The amounts of endogenous substrate found after coumarin treatments are presented as percentages of the content in the corresponding tissues of non-treated rats. The data are the mean of triplicate measurements.

found between acenocoumarol, phenprocoumon or warfarin treatment. The administration of an acute high dose of warfarin resulted in equal or slightly increased vitamin K-dependent carboxylase activities as compared to normal values. The enhanced vitamin K dependent carboxylase activity might either be due to stabilisation of the enzyme by its endogenous substrate during isolation (18) or by changes in the quantity (de novo synthesis) or quality (enhanced enzymatic activity) of the enzyme, induced during the hypocoagulability state of the animals.

Table VI

Effects of acenocoumarol (A), phenprocoumon (P) and warfarin (W) treatments on vitamin K dependent carboxylase activities in various tissues

tissue	vitamin K-dependent carboxylase activity				
	normal (dpm/min per mg protein)	chronic low dose treatment (% of normal)			single high dose (% of normal)
		A	P	W	W
liver	3600	240	310	280	150
kidney	680	125	120	135	110
testis	340	125	150	200	190
lung	920	110	110	100	125
spleen	460	105	110	140	160
brain	220	120	160	145	190
heart	100	140	200	180	225
muscle	100	160	120	140	160

Vitamin K-dependent carboxylase activities were measured in the microsomal fractions of various tissues. Values are expressed as dpm $^{14}\text{CO}_2$ incorporated per min per mg microsomal protein. Blank values, ranging between 5 and 15 dpm per min per mg microsomal protein were subtracted. The activities found after coumarin treatments are presented as a percentage of the corresponding activities in normal rats. The data are the mean of triplicate measurements.

DISCUSSION

In this study we confirmed that both vitamin K-epoxide reductase and vitamin K-dependent carboxylase are present in liver as well as in many non-hepatic tissues like kidney, lung, spleen, testis and brain. Even in heart and muscle microsomes these enzymes were demonstrated although the measured activities were low. The concentrations of vitamin K epoxide reductase in liver which were found in our experiments are in good agreement with the results of Wallin et al. (21), somewhat higher than

those reported by Hildebrandt et al. (22) and Thijssen et al. and somewhat lower than the values found by Fasco et al. (23). The reductase activities measured in kidney and testis microsomes were reasonably higher than previously reported (8). These discrepancies may be explained by differences in the rat strains used and in the methods of preparing the microsomes and testing the activities.

No significant differences were found between the effects of chronic treatments with either acenocoumarol, phenprocoumon or warfarin: the clotting factor activities were all reduced to the same extent, the vitamin K epoxide reductase activities had been comparably decreased and no differences were found in the accumulation of non-carboxylated precursor proteins. From these results it is suggested that the three coumarins act in a similar way. Although the plasma-concentrations of acenocoumarol, phenprocoumon or warfarin differed largely, these differences were not reflected in the microsomal coumarin contents. In hepatic microsomes, the coumarin content per mg microsomal protein was at least five fold higher than in non-hepatic fractions. This might be explained by the presence of more vitamin K-dependent systems per mg microsomal protein. However, a correlation between vitamin K epoxide reductase activity and microsomal coumarin concentration was not observed. The effects of the coumarins are obviously more pronounced in liver than in non-hepatic tissues. The hepatic vitamin K epoxide reductase activities for instance appeared to be clearly more reduced than the non-hepatic enzymes. These findings support the data from recent pharmacokinetic experiments with S-warfarin supplied by osmotic minipumps for 3 days (8). Furthermore, a largely increased amount of non-carboxylated hepatic precursor proteins was measured, while this increase was considerable lower for the non-hepatic precursor proteins. No correlation was found between the amount of the endogenous substrate and the percentage of inhibition of the vitamin K epoxide reductase. However, the possibility that non-carboxylated precursor proteins from non-hepatic origin are more easily excreted than the hepatic ones cannot be ruled out.

In an earlier study we reported a greater increase of endogenous substrate for carboxylase in lung, spleen and testis microsomes after 7 days of low dose warfarin treatment. Apart from individual rat differences, this apparent discrepancy between the data may be explained by the fact

that we now use a slightly different and more sensitive test. In former experiments we used to subtract as blank values the amount of $^{14}\text{CO}_2$ incorporated in incubation mixtures in the absence of added vitamin K hydroquinone. However, there is still a small amount of endogenous vitamin K quinone present in microsomes (unpublished data). By adding dithiothreitol to the incubation mixture the quinone is reduced in vitro to hydroquinone and used as a cofactor for carboxylation. Incubations in the absence of both vitamin K hydroquinone and dithiothreitol result in lower amounts of incorporated $^{14}\text{CO}_2$ than the formerly used blank incubations. Therefore a higher amount of non-carboxylated precursors is detected now in the microsomal fraction of normal animals.

The acute high dose of warfarin resulted in warfarin contents in the non-hepatic microsomal fractions similar to those elicited by the chronic low dose treatment. On the contrary, in liver microsomes, the coumarin content was $\pm$ 60% of the amount after chronic treatment. Vitamin K epoxide reductase activities were 10-20% lower than after the chronic warfarin treatment. In the liver the latter treatment resulted in the accumulation of about twice as much endogenous substrate than after the acute high dose of warfarin. This may be explained by the fact that the time elapsed between the acute blockade of the vitamin K-cycle (inhibition of vitamin K quinone reductase and vitamin K epoxide reductase) and sacrifice of the rats is too short to reach a steady state between synthesis and excretion of the precursor-proteins in liver.

In some studies a vitamin K deficient diet is preferably used instead of an acute dose of warfarin because the diet is thought to result in a higher vitamin K-dependent carboxylase enzymatic activity in liver microsomes. As a vitamin K-deficient diet is rather expensive, a prolonged treatment of rats with a low dose of warfarin in the drinking water might be a good alternative.

Our results may have relevance for the application of coumarin anti-coagulant therapy in man. It might be expected that hardly any differences will be observed in the effects on vitamin K-dependent enzymes after either acenocoumarol, phenprocoumon or warfarin treatment. In addition it might be expected that the non-hepatic vitamin K-dependent enzymes will be also inhibited, although to a lesser extent than the hepatic ones. To confirm

these results in human vitamin K-dependent systems, investigations are now in current progress in our institute.

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C H A P T E R 4

DECARBOXYLATED BONE GLA-PROTEIN AS A SUBSTRATE FOR HEPATIC VITAMIN K-DEPENDENT CARBOXYLASE

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SUMMARY

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Chemicals

Vitamin K_1 (Konakion^R) was obtained from Hoffmann-La Roche and vitamin K hydroquinone was prepared as in (10). Dithiothreitol (DTT), benzamidine, aprotinin and 3-([3-cholamidopropyl]dimethylammoniol)-1-propane sulfonate (CHAPS) were from Sigma and the pentapeptide Phe-Leu-Glu-Glu-Leu(F L E E L) from Vega Fox. $\text{NaH}^{14}\text{CO}_3$ (40-60 Ci/mol) was purchased from Amersham and Sephadex G-25 from Pharmacia. All other chemicals were obtained from Merck.

Preparation of decarboxylated Bone Gla-Protein.

Fresh bovine femurs and tibias were obtained from the Maastricht Slaughter House, cut into slices and demarrowed. The bones were carefully cleaned, heated at 120 °C overnight and ground to powder mechanically. Powder (100 g) was extracted by stirring overnight with 200 ml of buffer A (1 M EDTA, 0.2 M KCl, 0.1 M benzamidine, pH 8.0). Insoluble material was removed by filtration and the solution was desalted on a Sephadex G-25 column (60x5 cm) in water. The protein peak was collected and the pH was adjusted to 2.5 by adding 1 M HCl. The preparation was subsequently lyophilized and decarboxylated by heating at 110 °C for 5h under vacuum (11). The resulting material was dissolved in buffer B (0.1 M KCl, 0.02 M Tris-HCl, pH 7.4) to a final concentration of 5 mg/ml. Since after the decarboxylation step the proteins remain acidic, it is important that the pH of the solution is checked at this stage. The preparation thus obtained is referred to as crude decarboxylated Bone Gla-Protein (d-B.G.P.).

Various Assays

Vitamin K-dependent carboxylase was prepared from normal cow tissues (3) and carboxylase activity was measured by the incorporation of $^{14}\text{CO}_2$ into exogenous substrates in the presence of vitamin K hydroquinone (10). High performance liquid chromatography (HPLC) was carried out using a Beckmann-112 system and a Mono Q column (Pharmacia) for anion exchange chromatography and a Spherogel-TSK 2000 SW column (Beckmann, 300 x 7.5 mm) as a molecular sieve. Protein concentrations were determined according to Lowry (12) and the N-terminal aminoacid was identified as described by Weiner et al. (13). Gla and Glu residues were determined by alkaline hydrolysis of the protein and subsequent HPLC on a Nucleosil 5SB column (Chrompack) according to the method of Kuwada and Katayama (14).

RESULTS

During the preparation of Bone Gla-protein (B.G.P.) it turned out that the bone powder could be extracted a number of times and that after each extraction about 70 mg of protein was present in the extract. Five subsequent extracts obtained from one portion of 100 g bone powder were separately processed for the production of d-B.G.P. The Gla/Glu ratio in

Fig. 1. Fractionation of d-B.G.P.

A: one mg of crude d-G.B.P. was adsorbed to a Mono Q ion exchange column and eluted with a linear salt gradient from 0.05 to 0.5 M NaCl in 20 mM Tris-HCl, pH 7.4. The eluate was recorded at 280 nm and the start of the tracing represents the start of the gradient. The various peaks were collected and tested with the carboxylase assay. All d-B.G.P. activity was recovered in the peak eluting after 22.76 min.

B: the eluted d-B.G.P. was analyzed on a TSK-2000 molecular sieve and all activity was recovered in the peak eluting after 11.48 min. Trasylol eluted after 11.56 min.

these extracts was 0.03 before and 0 after decarboxylation. The decarboxylated preparations were used as substrates for carboxylase and in all cases the incorporation of $^{14}\text{CO}_2$ amounted about 120000 dpm per mg of substrate after an incubation of 3h at 25 °C under standard conditions. Since no differences were found between the various preparations they were pooled and used for the purification of d-B.G.P.

The crude d-B.G.P. was purified by HPLC on an ion exchange column (fig. 1A). When analyzed on a size exclusion column the preparation thus obtained eluted in a single peak (fig. 1B) shortly before trasylol (aprotinin), and its apparent molecular weight was about 6800; the NH_2 -terminal amino-acid was identified as tyrosine. Since similar characteristics have been reported for bovine osteocalcin (15,16) it is highly probable that d-B.G.P. is similar to decarboxylated osteocalcin. The optimal reaction conditions for the carboxylation of d-B.G.P. were compared with those for the carboxylation of F L E E L and it turned out that they were similar for both substrates. Therefore the following conditions were used in all experiments described below: 0.25 ml reaction mixtures containing 4 mg of microsomal proteins, 0.4 M KCl, 0.05 M Tris-HCl, pH 7.4, 0.4% CHAPS, 12% ethylene glycol, 16 mM DTT, 0.1 mM vitamin K hydroquinone and 0.01 mCi $\text{NaH}^{14}\text{CO}_3$ were incubated at 25 °C. Under these conditions the carboxylation of both substrates was markedly enhanced by the addition of $(\text{NH}_4)_2\text{SO}_4$. Unless stated otherwise we therefore added $(\text{NH}_4)_2\text{SO}_4$ to our reaction mixtures to a final concentration of 1 M.

When we compared the kinetic constants of the two substrates we found the K_M of F L E E L to be 4.8 mM and that of d-B.G.P. 0.025 mM. The respective maximal reaction rates ($V_{\max}$) were 14375 and 4825 dpm/min. It was checked that the presence of $(\text{NH}_4)_2\text{SO}_4$ did not affect the K_M of the two substrates but only increased the $V_{\max}$. The fact that the $V_{\max}$ of F L E E L is higher than that of d-B.P.G. probably results from its low molecular weight, which will be accompanied by a high diffusion rate.

Time courses of the carboxylation of the two substrates (both, in the presence and absence of $(\text{NH}_4)_2\text{SO}_4$) are shown in fig. 2 and it is clear that $(\text{NH}_4)_2\text{SO}_4$ not only increases the initial carboxylation rate 4-5-fold, but that also at the end point of the reaction (after 4 h) the amounts of incorporated $^{14}\text{CO}_2$ are five times higher in the presence of $(\text{NH}_4)_2\text{SO}_4$ than in its absence. Hence $(\text{NH}_4)_2\text{SO}_4$ induces an increase of the total amount of

Fig. 2. Effect of $(\text{NH}_4)_2\text{SO}_4$ on the carboxylation of d-B.G.P. and F L E E L. The carboxylation of both substrates was monitored after several intervals of time in the presence and absence of 1 M $(\text{NH}_4)_2\text{SO}_4$: (●-●), F L E E L (4 mM); (○-○), F L E E L (4 mM) + $(\text{NH}_4)_2\text{SO}_4$; (■-■) d-B.G.P. (0.03 mM); (□-□), d-B.G.P. (0.03 mM) + $(\text{NH}_4)_2\text{SO}_4$.

carboxylation events that can be accomplished under our experimental conditions. From these type of experiments we have calculated the efficiency of the carboxylation reaction and it turned out that at limiting substrate concentrations 0.008 mole of CO_2 is bound per mole of F L E E L. For d-B.G.P. this value is 0.16.

Fig. 3. The carboxylation of F L E E L and d-B.G.P. in various carboxylating systems. The initial reaction rate was measured for the carboxylation of F L E E L (10 mM, A) and d-B.G.P. (0.05 mM, B). The incubations were performed in standard reaction mixtures containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The sources of carboxylase were bovine liver (●-●), kidney (○-○), testis (■-■) and aorta (□-□). Note the different scales for A and B.

We have also checked whether d-B.G.P. is carboxylated in non-hepatic systems. In fig. 3 the carboxylation of F L E E L and that of d-B.G.P. are compared in carboxylating systems from liver, kidney, testis and aorta and it is clear that both substrates are carboxylated in all systems.

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

DISCUSSION

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

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

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

We have compared the optimal reaction conditions for the carboxylation of FLEEL and d-B.G.P. and no differences were found. Also the addition of $(\text{NH}_4)_2\text{SO}_4$ to the reaction mixtures stimulated the carboxylation of both substrates to the same extent. Therefore we recommend the use of d-B.G.P. as a substrate for vitamin K-dependent carboxylase.

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C H A P T E R 5

DISCOVERY OF A 4-CARBOXYGLUTAMIC ACID-CONTAINING PROTEIN IN HUMAN SPERMATOOZOA

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SUMMARY

In this paper we describe the identification of a 4-carboxyglutamic acid-containing protein in human spermatozoa. After thermal decarboxylation the protein is a good substrate for vitamin K-dependent carboxylase from various origins. A quick purification procedure for the decarboxylated protein is presented and in a preliminar characterization we have established its molecular weight (28000 - 30000) and its amino acid composition.

INTRODUCTION

Vitamin K-dependent carboxylase has been demonstrated in liver as well as in a number of non-hepatic tissues (1,2). The enzyme system is involved in the conversion of glutamic acid (Glu) residues into 4-carboxyglutamic acid (Gla) residues and hence carboxylase-containing tissues are expected to be involved in the synthesis of Gla-containing proteins. Whereas the hepatic Gla-containing proteins have been well identified (3), the nature of the non-hepatic ones is less clear. Since relatively high amounts of carboxylase were found in testes, it seemed plausible that one or more Gla-containing proteins are present in sperm. Indeed we have found such a protein and its purification and preliminar characterization are reported in this paper.

MATERIALS AND METHODS

Chemicals.

Vitamin K₁ was obtained from Hoffmann-La Roche and vitamin K hydroquinone was prepared as described earlier (4). Dithiothreitol benzamidine and 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) were from Sigma and the pentapeptide Phe-Leu-Glu-Glu-Leu (F L E E L) was from Vega Biochemicals. NaH¹⁴CO₃ (40-60 Ci/mol) was purchased from Amersham. QAE-Sephadex, and the molecular weight marker proteins were from Pharmacia Fine Chemicals. All other chemicals were from Merck.

Preparation of crude sperm Gla-protein.

Fresh human sperm was collected from healthy donors and diluted 10 fold with a buffer containing 0.25 M sucrose, 0.10 M NaCl and 0.05 M Tris-HCl, pH 8.0. Sperm count and sperm quality were checked routinely and they were within the normal range. The spermatozoa were sedimented by centrifugation for 15 minutes at 600 g and 0 °C and homogenized in a buffer containing 10 mM benzamidine, 0.5 M KCl and 50 mM EDTA, pH 8.0. The suspension was subsequently centrifuged for 1 h at 100000 g and the supernatant was removed, diluted 5-fold with distilled water and extracted batchwise with QAE-Sephadex (1 ml of slurry per 100 ml of diluted supernatant). The Sephadex was washed with 0.1 M NaCl in 0.01 M Tris-HCl (pH 7.4) and the sperm Gla-protein was eluted with 1 M NaCl. The preparation was subsequent-

ly desalted by size-exclusion chromatography on a Sephadex G-25 column in water, brought to pH 2.0 with 1 M HCl and lyophilized. In some cases the lyophilized material was decarboxylated by heating at 110 °C for 24 h ([5]).

Purification of decarboxylated sperm Gla-protein.

Decarboxylated Sperm Gla-protein (d-S.G.P.) was purified from the lyophilized material by high performance liquid chromatography (HPLC) using a Beckman 112 gradient system. The first purification step was on a mono-Q anion-exchange column (Pharmacia, 50 x 5 mm). The lyophilized proteins (20 mg) were dissolved in a 0.02 M Tris-HCl buffer (pH 8.5) containing 0.1 M KCl (buffer A), adsorbed on the ion-exchange gel in the same buffer, and then eluted with a linear gradient from 0.1 to 1 M KCl in 20 mM Tris-HCl, pH 8.5 at room temperature. The flow rate was adjusted to 60 ml/h, the absorbance was monitored at 229 nm, and fractions of 0.5 ml were collected. The presence of d-S.G.P. in the various fractions was determined by incubating 0.1 ml aliquots in carboxylating reaction mixtures (0.25 ml) containing microsomes from normal bovine liver (see below). The amount of $^{14}\text{CO}_2$ incorporated into the exogenous substrate was taken as a measure for the amount of d-S.G.P. present in the fractions. The peak fractions were pooled, concentrated and purified further on a TSK-3000 SW size-exclusion column (Beckman, 300 x 7.5 mm) in buffer A.

Various assays.

The vitamin K-dependent incorporation of $^{14}\text{CO}_2$ was measured by incubating reaction mixtures (0.25 ml) containing 4 mg of microsomal protein, 0.15 M KCl, 1 M $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 0.4 mM vitamin K hydroquinone, 0.4% (w/v) CHAPS, 12% (v/v) ethylene glycol, 0.01 mCi $\text{NaH}^{14}\text{CO}_3$ and exogenous substrate as indicated. After 1 h at 25 °C 1 ml of 5% (w/v) trichloroacetic acid was added to the mixtures, which were then boiled for 1 min and counted in Atomlight (NEN).

Protein-bound Gla-residues were determined after alkaline hydrolysis of the proteins and analysis of the hydrolysate on a Nucleosil 5-SB column (Chrompack 250 x 4.6 mm) as described by Kuwada and Katayama (6). Polyacrylamide gel electrophoresis was performed in slab gels as described by Laemmli (7) and protein concentrations were measured according to Lowry (8). Amino acid analysis was performed on an LKB 4400 Analyzer fitted with a 4.6 x 200 mm stainless steel column packed with Ultropac 8 cation exchange resin. The column was eluted with lithium-containing buffers according to the manufacturer's prescriptions.

RESULTS

Fresh human sperm was separated into spermatozoa and seminal plasma and the sperm cells were extracted with 50 mM EDTA in 0.5 M KCl. After alkaline hydrolysis. Gla could be directly identified in the crude sperm cell

Fig. 1.

4-carboxyglutamic acid determination in sperm cell protein

The presence of 4-carboxyglutamic acid (Gla) was established in alkaline hydrolysates of a crude cell extract before (panel A) and after (panel B) thermal decarboxylation of the protein. The Gla-determination was performed by HPLC analysis as described in Methods. Peaks 1 are Glu and peak 2 is Gla.

extract (fig. 1A). Although varying amounts of Gla (10-30%) were also found in the seminal plasma, we have focussed our attention on the Gla-containing protein derived from the spermatozoa. When the crude sperm cell extract mentioned above was submitted to thermal decarboxylation, the Gla-residues were readily converted into Glu (fig. 1B). A second observation was that - in contrast to the starting material - the decarboxylated sperm Gla-protein

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Fig. 2.

HPLC analysis of the decarboxylated 4-carboxyglutamic acid-containing protein from sperm

- A: Crude decarboxylated sperm Gla-protein (d-S.G.P., 0.5 mg) was added to normal cow liver carboxylase and incubated in the presence of vitamin K hydroquinone and $\text{NaH}^{14}\text{CO}_3$ under standard conditions. After 1 h 1 ml of trichloroacetic acid (5% w/v) was added and after centrifugation the supernatant was neutralized with 1 M NaOH. Sodium dodecyl sulphate was added to a final concentration of 1% (w/v). A sample of 0.05 ml of this preparation was applied to a TSK-3000 SW column in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5 and 0.1% (w/v) sodium dodecyl sulphate. The flow rate was 1 ml/min. Fractions of 1 ml were collected and counted. The data in this diagram are corrected for the various dilutions and represent the total $^{14}\text{CO}_2$ incorporation in 0.5 mg d-S.G.P.
- B: Crude d-S.G.P. (20 mg) was applied to a mono-Q anion exchange column as described in Materials and Methods. Fractions of 1 ml were collected and aliquots thereof (0.05 ml) were tested for the presence of d-S.G.P. in the bovine liver carboxylase assay.
- C: The proteins eluting between 4 and 6 min were concentrated and applied to a TSK-3000 column in buffer A. The peaks were collected and tested for the presence of d-S.G.P. as described above.
- D: The protein peak eluting between 5 and 6 min was collected, concentrated and rechromatographed in buffer A on the same column.

(d-S.G.P.) could be used as an exogenous substrate for the in vitro carboxylating enzyme system from normal bovine liver. Human liver carboxylase could not be used because of its high level of endogenous substrate (8). After the reaction mixtures had been incubated for 1 h at 25 °C they were supplemented with trichloroacetic acid. The carboxylated d-S.G.P. was recovered in the supernatant and gel filtration chromatography (HPLC) in sodium dodecyl sulphate showed the label to be exclusively present in a protein peak with a molecular mass of about 34 000 (fig. 2A).

Since the Gla-determination is a technique too laborious for the screening of series of column fractions, we decided to decarboxylate the sperm Gla-protein in an early stage of its purification and to use the carboxylase assay for the detection of d-S.G.P. in the various fractions. The purification of d-S.G.P. was accomplished by high performance liquid chromatography using ion-exchange and size-exclusion columns (fig. 2B and C). This procedure resulted in a preparation which migrated as a single peak during gel-filtration chromatography in the absence and presence of sodium dodecyl sulphate (1%, w/v, fig. 2D). The recovery of d-S.G.P. from 20 ml of semen is shown in table I. SDS polyacrylamide gel electrophoresis

Table I

Purification of the decarboxylated 4-carboxyglutamic acid-containing protein from sperm

Step	protein (mg)	recovery (%) of		purification (-fold)
		Gla	substrate	
1. Ejaculate (20 ml)	3600	100	-	1
2. Spermatozoa	640	72	-	4
3. EDTA-extract	46	45	-	35
4. QAE-eluate	20	40	-	72
5. Decarboxylated 4	20	-	100	72
6. Mono Q	3	-	135	648
7. TSK-3000 SW	0.6	-	61	1464
8. TSK-3000 SW rechromatography	0.4	-	56	2016

Since the thermal decarboxylation of the sperm Gla-protein routinely occurred after step 4, only the recovery of protein-bound Gla-residues was measured during the purification steps 1-4. The purification factor (72-fold) is based on the assumption that only one Gla-containing protein is present in the starting material. In step 5 the preparation was decarboxylated and could serve as a substrate for hepatic carboxylase. The latter assay was used to measure the recovery of decarboxylated sperm Gla-protein.

of the purified material was performed in the absence and presence of 20 mM dithiothreitol (fig. 3). Under non-reducing conditions a single band was obtained, with an apparent molecular mass of 28000. Under reducing conditions a low molecular weight peptide chain was dissociated from the heavy chain. From the fact that this dissociation apparently does not cause a substantial decrease of the molecular mass of the heavy chain, we conclude that the light chain may be smaller than indicated by its position in the gel. From these experiments we concluded that d-S.G.P. is a two-chain molecule with an estimated molecular mass of approximately 30000.

The aminoacid composition of purified d-S.G.P. was determined after acid hydrolysis and is given in table II. Based on the assumption that only one methionine residue is present per molecule d-S.G.P., a molecular mass

Fig. 3.

SDS polyacrylamide gel analysis of the decarboxylated 4-carboxyglutamic acid-containing protein from sperm

Lanes A and E: marker proteins, reduced; lanes B,C, and D: decarboxylated sperm Gla-protein, reduced (20, 15 and 10 μ g, respectively); lanes F,G,H, and I: decarboxylated sperm Gla-protein, non-reduced (25, 20, 15 and 10 μ g, respectively).

of 28000 was calculated from the given aminoacid composition, which is in good agreement with our estimate from the HPLC gel filtration and polyacrylamide gel electrophoresis experiments. After the purification procedure for d-S.G.P. had been established we have also tried to purify non-decarboxylated Sperm Gla Protein and to determine its Gla-content. Although the presence of Gla could be clearly established in the purified protein, for unknown reasons its Gla-content varied from batch to batch, so that it was impossible for us to make a good estimate of the amount of Gla-residues per molecule of native protein.

Table II

Aminoacid composition of the decarboxylated 4-carboxyglutamic acid-containing protein from sperm

Aminoacid residue	Concentration in hydrolysate (μ M)	Number of residues per protein molecule
Asx	444	22.2
Thr	261	13.1
Ser	359	18.0
Glx	777	38.9
Pro	156	7.8
Gly	283	14.2
Ala	137	6.9
Val	196	9.8
Cys	432	21.6
Met	20	1.0
Ile	183	9.8
Leu	216	10.8
Tyr	105	5.3
Phe	64	3.2
Lys	306	15.3
His	162	8.1
Arg	121	6.1
Trp	n.d.	n.d.

The aminoacid composition of the purified protein was determined after acid hydrolysis. The number of residues per protein molecule was calculated on base of the assumption that one residue of Met is present per molecule. The data are not corrected for eventual hydrolysis losses. Trp was not determined because it is destroyed during acid hydrolysis.

DISCUSSION

The presence of carboxylase has been demonstrated in the testis of rat (2,10), steer (1), goat, ram and stallion (own unpublished results) thus suggesting that testicular carboxylation systems are indeed very common to

mammalian species, including man. Though there was no experimental proof, it seemed plausible that the endogenous substrate of testicular carboxylase might be a constituent of spermatozoa. Indeed Gla was detected in the sperm cell wash and after thermal decarboxylation the preparation contained a protein that could serve as a substrate for bovine hepatic and testicular carboxylase. The isolation procedure resulted in the purification of a hitherto unknown protein, the nascent form of which was designated as sperm Gla-protein. Initial characterization of its decarboxylated form (d-S.G.P.) was accomplished by determining its molecular weight and its aminoacid composition (table II). Striking is the very high content of Glx. It is likely, therefore, that the native protein contains a high amount of Glu and Gla residues and that it thus bears a considerable negative charge. Possibly this negative charge destabilizes the Gla-residues to some extent so that they are partially lost during various steps of purification of the sperm Gla-protein. This would explain the batch to batch variation in the Gla-content of purified sperm Gla-protein. Further research concerning the function of the sperm Gla-protein is in progress in our laboratory.

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C H A P T E R 6

ISOENZYMES OF VITAMIN K-DEPENDENT CARBOXYLASE

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SUMMARY

Vitamin K-dependent carboxylase was prepared from bovine liver, kidney, lung and testis and it was checked that these enzyme systems obeyed the laws of normal enzyme kinetics. Four carboxylatable substrates were obtained from different sources and the apparent Michaelis constants of the various carboxylases for these four substrates were measured. From the results thus obtained, we conclude that carboxylase is a group-name for a number of isoenzymes which are present in hepatic as well as in various non-hepatic tissues.

INTRODUCTION

About ten years ago, vitamin K-dependent carboxylase was discovered in the liver, where it mediates in the post-translational carboxylation of a number of proteins involved in blood coagulation (1-3). Only recently, similar enzyme systems were detected in non-hepatic tissues such as kidney, spleen, lung, testis, arterial vessel wall and bone (4,5). Since it is a common characteristic of all these enzyme systems that they use vitamin K as coenzyme, it is to be expected that all vitamin K antagonists (e.g. warfarin, dicoumarol) will inhibit carboxylase more or less to the same extent. This hypothesis is supported by the observation of Roncaglioni et al. (6) that under mild oral anticoagulant treatment the enzyme systems in liver, lung, spleen and testis are all affected to some degree.

The question remains, however, whether the carboxylases from different tissues are indistinguishable from each other. Contrarily, they also might form a family of isoenzymes. Since the various carboxylases are involved in the carboxylation of different substrates, it seemed plausible that if differences would exist, they might be found at the substrate-binding site rather than at the vitamin-K-binding site. We therefore tried to answer this question by preparing carboxylase from four different types of bovine tissue and by measuring the kinetic constants of substrates from different origin in these systems. The results of our investigation are presented in this communication.

MATERIALS AND METHODS

Chemicals.

Vitamin K₁ (konakion^R) was obtained from Hoffmann-La Roche (Basel, Switzerland) and chemically reduced to the hydroquinone form (7). Warfarin, dithiothreitol, CHAPS (3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate) and subtilisin were from Sigma (Saint Louis, U.S.A.), and the pentapeptide Phe-Leu-Glu-Glu-Leu (F L E E L) from Vega (Tucson, U.S.A.). NaH¹⁴CO₃ (40-60 Ci/mol) was purchased from Amersham (U.K.) and Atomlight from New England Nuclear (Dreieich, F.R.G.). QAE-Sephadex and Sephadex G-25 were from Pharmacia (Uppsala, Sweden). All other chemicals were from Merck (Darmstadt, F.R.G.).

Preparation of carboxylase.

The various organs were obtained from normal cows within half an hour after slaughtering and they were quickly cooled with ice-cold buffer A (0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.005 M EDTA and 30% (v/v) ethylene glycol). Crude microsomes were prepared from about 200 g of tissue as described earlier (7) and washed three times by resuspension in 600 ml of buffer A, followed by centrifugation for 1 h at 105 000 g. Subsequently the microsomes were washed twice with 1 M KCl in buffer A and after the last centrifugation step they were solubilized in 1% (w/v) CHAPS in buffer A, to a final concentration of 40 mg of protein per ml. The preparations were stored at -80 °C until use.

Carboxylase assay.

Unless stated otherwise, the incubation mixtures (0.25 ml) contained: 2.5 mg of microsomal protein, 0.25% (w/v) CHAPS, 0.1 mM vitamin K hydroquinone, 5 mM dithiothreitol, 12% (v/v) ethylene glycol, 1.5 M $(\text{NH}_4)_2\text{SO}_4$, 0.005 M $\text{NaH}^{14}\text{CO}_3$ and substrate as indicated. The reaction mixtures were incubated at 25 °C and at the indicated time points the reaction was stopped by adding 2 ml of 5% (w/v) trichloroacetic acid. Traces of non-bound label were removed by gentle boiling for 1 min before 10 ml of Atomlight were added. The samples were counted in a Packard Tricarb Scintillation counter.

Determination of kinetic constants.

Reaction mixtures containing varying substrate concentrations were incubated for 0, 5, 10, 15, 20, 25 and 30 min. During this period the reaction rates were linear in all experiments described in this paper. Blank values (without vitamin K hydroquinone) and the data obtained without substrate were subtracted from the data obtained in the presence of substrate. From the Lineweaver-Burk plot, constructed with these data, the apparent Michaelis constant (K_m) and maximal reaction rate ($V_{\max}$) were calculated.

Preparation of substrates.

Osteocalcin was prepared from bovine bone (8) and partly purified by batchwise adsorption to QAE-Sephadex in 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4 followed by a stepwise elution with 1 M NaCl before desalting, thermal decarboxylation and purification by preparative high performance liquid chromatography (8). Descarboxyprothrombin fragment 13-29 was prepared by the proteolytic cleavage of purified bovine descarboxyprothrombin with

subtilisin and purified with the aid of ion exchange and size exclusion chromatography (9). The Gla-containing protein from human sperm, the characterization of which will be reported elsewhere (B.A.M. Soute et al., FEBS Lett. 190: 137-141, 1985), was prepared by homogenizing spermatozoa from 400 ml of semen in a buffer containing 1 M KCl, 20 mM EDTA and 20 mM Tris-HCl, pH 7.4. The membrane remnants were spun down (10 min, 1500 g), and the supernatant was deluted 10-fold, adsorbed batchwise to 50 ml of QAE-Sephadex slurry and eluted stepwise with 1 M NaCl. The eluate was desalted on a Sephadex G-25 column (85x60 cm) in water and was supplemented with 0.1 M HCl to a final pH of 2.0 and lyophilized. The preparation was then thermally decarboxylated (24 h, 100 °C in vacuo) and could serve as a substrate for carboxylase. The decarboxylated sperm Gla-protein (d-S.G.P.) was then dissolved in a 0.02 M Tris-HCl buffer (pH 8.5) containing 0.1 M KCl and purified further by high-performance liquid chromatography. The first purification step was on a mono-Q anion exchange column (Pharmacia, 50x5 mm) and d-S.G.P. was eluted with a linear gradient from 0.1 to 1 M KCl at a flow rate of 60 ml/h. Fractions of 0.5 ml were collected and the presence of d-S.G.P. was assayed by its ability to serve as a substrate for hepatic carboxylase. The amount of $^{14}\text{CO}_2$ incorporated into the exogenous substrate was taken as a measure for the amount of d-S.G.P. in the various fractions. The peak fractions were pooled, concentrated and subjected to a final purification step on a TSK-3000 SW size exclusion column (Beckman, 300x7.5 mm) in 0.1 M KCl, 0.02 M Tris-HCl, pH 7.4.

Protein concentrations were determined according to Lowry et al. (10)

RESULTS

Washed, solubilized microsomes were prepared from bovine liver, kidney, lung and testis and used as a source of carboxylase. The reliability of the enzyme systems was checked in a set of experiments in which the kinetic constants were measured at varying concentrations of microsomes. The substrate chosen for these experiments was the pentapeptide F L E E L; all other reaction conditions were carefully kept constant. The results are presented in the form of Lineweaver-Burk plots (fig. 1). It is clear that the diagrams are linear and that the apparent Michaelis constants (K_m) are

Fig. 1.

Lineweaver-Burk plots at different enzyme concentrations.

Carboxylase was prepared from liver (A), kidney (B), lung (C) and testis (D) and the initial carboxylation rate of P L E E L was measured at microsomal protein concentrations of 4, 6, 10 and 16 mg/ml. At each protein concentration, the concentration of P L E E L was 10, 5, 2.5, 2, 1.25 and 1 mM. The initial carboxylation rates were calculated from time-courses (see Methods), which were made in triplicate, and blank values (minus vitamin K and minus exogenous substrate) were subtracted. The carboxylation rates are expressed as the amount of label (dpm) incorporated into P L E E L per minute incubation time. Explanation of symbols: ●—●, 4 mg; ○—○, 6 mg; ■—■, 10 mg and □—□, 16 mg of microsomal proteins per ml incubation mixture.

independent of the enzyme concentration. The maximal reaction rates ($V_{\max}$) were calculated from the intersections with the ordinates and they are shown in fig. 2. Obviously there is a linear relationship between the $V_{\max}$ and the enzyme concentration. We conclude that reliable measurements could be made within a rather wide range of enzyme concentrations and we have arbitrarily chosen to perform our experiments using 2.5 mg of microsomal protein per reaction mixture (0.25 ml).

After having defined our enzyme systems, we prepared and purified the following carboxylatable substrates: bovine descarboxyprothrombin fragment

Fig. 2.

V_{max} at different enzyme concentrations.

The maximal reaction rates (V_{max}) were calculated from the intersections of the ordinates in figure 1 and they are plotted against the concentration of microsomal proteins. Explanation of symbols: ●-●, liver; ○-○, kidney; ■-■, lung and □-□, testis.

13-29, decarboxylated osteocalcin (from bovine bone) and decarboxylated sperm Gla-protein (from human sperm). The apparent K_m values for these substrates and for the pentapeptide F L E E L were measured in the various enzyme systems and the results of these experiments are summarized in table I. The carboxylase preparations may now be compared by calculating the ratio of the apparent K_m values for two substrates in the various systems. When we take the data obtained with decarboxyprothrombin fragment 13-29 and decarboxylated sperm Gla-protein, for instance, this ratio is 0.014 in

lung, 0.035 in liver and even 2.3 in testis carboxylase. It has to be concluded, therefore, that differences exist between various carboxylases with respect to their substrate specificity.

Table I

Apparent Michaelis constants in various systems.

Apparent K_m (mM) in carboxylase from				
	Liver	Kidney	Lung	Testis
F L E E L	2.6	7.1	5.3	6.5
d-osteocalcin	0.028	0.027	0.008	0.011
fragment-Su	0.003	0.006	0.003	0.028
d-S.G.P.	0.085	0.158	0.204	0.012

The incubations were performed in standard reaction mixtures (0.25 ml) containing 2.5 mg of microsomal proteins. D-osteocalcin stands for decarboxylated osteocalcin and d-S.G.P. for decarboxylated sperm Gla-protein.

DISCUSSION

Although hepatic carboxylase has been discovered more than ten years ago, its purification from the crude microsomal fraction has not yet been completed (11,12). Non-hepatic carboxylases have even not been purified beyond the step of solubilization of the microsomes. The lack of a purified enzyme has been a serious drawback for investigations concerning the mechanism of the carboxylation reaction. On the other hand, if the appropriate controls are made, some information can also be obtained from the non-purified systems. In this way we have compared the substrate specificities of carboxylases derived from different types of tissue. Before we could start our investigations, we had to make sure that the kinetics of these systems were reliable, which means that: a) the K_m for a given substrate is independent of the enzyme concentration and b) a linear relationship exists between the V_{max} and the enzyme concentration. It

turned out that -provided that the microsomes were thoroughly washed- these conditions were met within a rather wide range of enzyme concentrations and we have arbitrarily chosen to perform our experiments using 2.5 mg of microsomal proteins per reaction mixture (0.25 ml). In this way we have measured the K_m values of carboxylase from various types of tissue for four different carboxylatable substrates. As is shown in table I, all substrates were carboxylated in each system, but the K_m values for a given substrate differed from one system to another. Decarboxyprothrombin fragment 13-29, for instance, is a rather good substrate (low K_m) in liver and in lung carboxylase, but it is worse in testis carboxylase. Decarboxylated sperm Gla-protein, on the other hand, has a low K_m in the testicular system, but a 7-15 fold higher K_m in carboxylase from liver, lung and kidney. Therefore it seems that carboxylases from different types of tissue may differ from each other with respect to their substrate selection and thus may be regarded as isoenzymes. The results shown in table I also show that some primary or secondary aminoacid structure is required for an efficient enzyme/substrate interaction since it is evident, that the K_m for the smallest substrate F L E E L is high in all four enzyme systems.

It has been shown, that vitamin K antagonists inhibit hepatic as well as non-hepatic carboxylases (5,6), possibly because the vitamin K-binding sites of all enzyme systems are closely similar. Now it turns out that differences may exist at the substrate-binding sites, it would -at least in principle- be possible to construct more specific inhibitors, which interfere with one type of carboxylase much stronger than with the others. Obviously these inhibitors should be non-carboxylatable substrate analogs such as those synthesized by Gaudry et al. (13) and by Rich et al. (14). Although it was demonstrated that these substrate analogs are potent inhibitors of hepatic carboxylase, they have unfortunately not been tested in non-hepatic enzyme systems. Obviously the development of these and other substrate-analogs will help us to get more insight into the mechanism of the substrate selection by vitamin K-dependent carboxylase.

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C H A P T E R 7

VITAMIN K-DEPENDENT CARBOXYLASE: THE CARBOXYLATION OF EXOGENOUS SUBSTRATES IN DIFFERENT SYSTEMS

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SUMMARY

Two types of solid-phase carboxylase, SPC-II and SPC-X have been prepared from the livers of warfarin treated cows. Their enzymatic activities were compared with substrate-free carboxylase in microsomes from normal cows and substrate-bound carboxylase in microsomes from warfarin treated cows. A number of exogenous substrates for carboxylase have been purified and tested. We found that large substrates, such as descarboxy-prothrombin, are only carboxylated by substrate-free carboxylase and not by the substrate-bound enzyme. No differences in apparent K_m values between SPC-II and SPC-X were observed.

INTRODUCTION

Vitamin K-dependent carboxylase is a microsomal enzyme system involved in the posttranslational modification of proteins (1,2). Initially, the system was thought to be uniquely present in liver, but later on carboxylase was also detected in other tissues such as lung, spleen, kidney, testis and arterial vessel wall (3,4). Whether all these carboxylases are identical is still a matter of research. Contrarily they also might form a family of isoenzymes and it cannot even be excluded, that different isoenzymes are present in one type of tissue.

Hepatic carboxylase is used in most studies concerning the vitamin K-dependent reaction. The characteristics of the enzyme preparation may depend on the donor animal pretreatment and the enzyme preparation procedure. Crude microsomal extracts from normal animals contain only a very small amount of endogenous substrate and therefore the preparation is called substrate-free carboxylase (5), whereas substrate-bound carboxylase is obtained from the livers of warfarin-treated animals (5,6). The endogenous substrate mainly consists of precursors of the coagulation factors II (prothrombin) and X (7). When the solubilized substrate-bound microsomes are extracted with Sepharose-linked antibodies against factor X (7) or prothrombin (8), two types of Solid-phase carboxylase (SPC) are obtained. They are designated as SPC-X and SPC-II, respectively.

We have prepared these four types of carboxylase from bovine liver and we have compared their respective properties. The results of our investigations are presented in this paper.

MATERIALS AND METHODS

Chemicals.

Vitamin K₁ (Konakion^R) was obtained from Hoffmann-La Roche (Switzerland) and vitamin K hydroquinone was prepared as described previously (9). The synthetic substrate Phe-Leu-Glu-Glu-Leu (F L E E L) was obtained from Vega-Biochemicals (U.S.A.), NaH¹⁴CO₃ (40-60 Ci/mol) from Amersham (U.K.) and Atomlight from New England Nuclear (F.R.G.). Warfarin, benzamidine, dithiothreitol (DTT), bovine albumin, Triton-X 100 and 3-[(3 cholamido-

propyl)dimethylammonio]-1-propane sulfonate (CHAPS) were purchased from Sigma (U.S.A.). CNBr-activated Sepharose, Sephadex G-25, G-100, G-200 and DEAE Sephadex were obtained from Pharmacia (Sweden). The proteases subtilisin and chymotrypsin were obtained from Boehringer. All other chemicals were from Merck (F.R.G.).

Preparation of antibodies. Bovine prothrombin was prepared as described by Owen et al. (10) and antibodies against the purified antigen were raised in goats. Anti-prothrombin antibodies were specifically extracted from the immune serum with the aid of Sepharose-bound prothrombin and eluted with 3 M KCNS. The purified antibodies gave single precipitin lines against normal reference plasma in an Ouchterlony diffusion test (11). After dialysis against 0.15 M NaCl, 20 mM Tris-HCl pH 7.5, the antibodies were bound to CNBr-activated Sepharose (10-15 mg of protein per g of dry Sepharose). Bovine factor X was prepared as described by Fujikawa et al. (12) and Sepharose-bound anti-factor X antibodies were prepared in a similar way as described for antiprothrombin. The Sepharose-bound anti-prothrombin was able to extract prothrombin from normal bovine plasma without affecting the level of factor X and vice versa.

Preparation of microsomal carboxylase. Cows were anticoagulated during one week by the oral administration of warfarin (10 mg/kg body weight daily). Crude microsomes were prepared from the livers of normal and warfarin treated cows as described earlier (5). After three washing cycles the microsomes were suspended in buffer A (1 M NaCl, 0.05 M Tris/HCl pH 7.5, 1 mM EDTA, 20% ethyleneglycol) to a concentration of 50 mg protein per ml and stored at -80 °C.

Preparation of solid-phase carboxylase (SPC). Microsomes from warfarin-treated cows were suspended in buffer A (50 mg protein/ml) and solubilized by the addition of CHAPS to a final concentration of 1% (w/v). Non-solubilized material was spun down (90 min at 100000 g) and the supernatant was supplemented with an equal volume of distilled water and 1 mg sodium azide per 100 ml. 40 ml of this preparation was incubated with 10 ml Sepharose-bound antibodies against either prothrombin or factor X; this suspension was rotated end over end at 4 °C. A maximal amount of carboxylase activity was bound to the Sepharose after 24 h for SPC-X and after 3 x 24 h for SPC-II. In the latter case the solubilized microsomes were refreshed after each period of 24 h. After the complete incubation,

the Sepharose-beads were washed with buffer B (0.1 M NaCl, 0.05 M Tris-HCl pH 7.5, 1 mM EDTA), suspended in an equal volume of buffer B and stored until use at -80°C .

Measurement of carboxylase activity. The vitamin K-dependent incorporation of $^{14}\text{CO}_2$ was measured by incubating 0.25 ml reaction mixtures in buffer B containing 0.1 ml SPC (0.05 mg protein) 0.01 mCi $\text{NaH}^{14}\text{CO}_3$, 8 mM DTT, 0.2 mM vitamin K hydroquinone and exogenous substrate as indicated. The tubes were sealed and incubated in a shaking water bath for 1 hour at 25°C unless indicated otherwise. The incubation was stopped by adding 0.25 ml ice-cold buffer A followed by centrifugation at 2000 g for 10 min at 4°C . An aliquot (0.25 ml) of the supernatant was transferred to a counting vial and non-bound $^{14}\text{CO}_2$ was removed by adding 2 ml trichloroacetic acid (5%) and boiling the solution for two minutes. The samples were supplemented with 10 ml Atomlight and counted in a Packard Tricarb scintillation counter. To measure the amount of endogenous substrate carboxylation, no exogenous substrate was added and after incubation the complete reaction mixture was transferred to a counting vial and treated further as described above. When microsomes from normal or warfarin-treated cows were used (5 mg protein per 0.25 ml) 0.4% CHAPS (w/v) and 1 M $(\text{NH}_4)_2\text{SO}_4$ were added to the reaction mixture as described for solid phase carboxylase. In all experiments, parallel incubations were performed in the absence of vitamin K hydroquinone and DTT; these blank values were subtracted.

Preparation of substrates. Descarboxyfactor II and descarboxyfactor X were prepared from the blood of warfarin-treated cows, according to the methods of Stenflo and Ganrot (13) and Lindhout et al. (14), respectively. Fragment-Su, the subtilisin fragments 13-29, were prepared by digesting the descarboxyfactors with Sepharose-bound subtilisin; the fragments were purified by ion-exchange and size exclusion chromatography (15). The chymotryptic fragments 1-41 (16) were obtained by digestion of the descarboxyfactors with Sepharose-bound chymotrypsin and partly purified by ion-exchange chromatography. Decarboxylated bovine osteocalcin (49 aminoacid residues) was prepared as described earlier (17).

Determination of apparent K_m values. Solid phase carboxylase reaction mixtures containing varying substrate concentrations were incubated for 0, 15, 30, 45 and 60 min. During this period the reaction rates were linear in all experiments described in this paper. At least five different substrate

concentrations were used in the range of 0.5 - 10 mM for F L E E L, 1 - 100 μ M for the descarboxy factors II and X and for decarboxylated osteocalcin and 0.1 - 10 μ M for the subtilisin fragments of the descarboxy factors. The amounts of incorporated $^{14}\text{CO}_2$ in the absence of added substrate were subtracted. The inverse of the initial reaction rates thus obtained was plotted against the inverse substrate concentration. From the resulting Lineweaver Burk plot the apparent Michaelis constant (K_m) was calculated according to the method of Eisenthal and Cornish-Bowden (18).

Other methods. Protein concentrations were determined according to Lowry et al. (19). The protein content of solid phase carboxylase was measured after its elution from the Sepharose beads with 6 M urea in 2% sodium dodecyl sulphate. Protein-bound Gla-residues were determined as described by Kuwada and Katayama (20).

RESULTS

SPC-II and SPC-X were prepared as described in Materials and Methods. The optimal reaction conditions for carboxylation were similar to those in the crude microsomal systems except for the fact that in the solid phase systems, detergents, even at low concentrations, acted strongly inhibitory. Although the two SPC's presumably contain different substrates no differences between the complexed carboxylases could be observed. In both types of enzyme the endogenous substrates were carboxylated preferentially during the first 15-20 minutes and after this period exogenous substrates such as F L E E L were carboxylated with a constant reaction rate for more than 2 h. The lag-phase in carboxylation of exogenous substrates may be circumvented by preincubation of SPC in the presence of vitamin K hydroquinone and non-labeled NaHCO_3 for 1 h. In the experiments described below, pre-incubated SPC was used.

To compare the affinities of the Sepharose-bound enzymes, a number of carboxylatable substrates were prepared. Descarboxy factors II and X were isolated from plasma of warfarin treated cows and purified. Since thermal decarboxylation (21,22) of normal prothrombin and factor X could not be reproducibly repeated we have used descarboxy factors throughout our experiments. Smaller substrates were obtained by subtilisin cleavage of descar-

Table I

Kinetic constants of various substrates for SPC II and SPC-X

Substrate	Km (mM)	
	SPC II	SPC X
D-II	n.m.	n.m.
D-X	n.m.	n.m.
D-II fr. Su	0.0014	0.0013
D-X fr. Su	0.0013	0.0016
D-osteocalcin	0.036	0.045
F L E E L	3.1	2.7

The apparent Km values were calculated from the initial carboxylation rates at various concentrations of the substrate, as described in Materials and Methods. Abbreviations used: D-II, descarboxyfactor II; D-X, descarboxyfactor X. fr. Su, subtilisin fragment; D-osteocalcin, decarboxylated osteocalcin; n.m. not measurable.

boxy factors which yields a carboxylatable peptide of 17 amino acid residues (fragment Su). Furthermore osteocalcin was isolated from bovine bone and decarboxylated in vitro. In none of these preparations Gla could be detected.

No differences between SPC II and SPC X were found (table I). Moreover the large substrates were not carboxylated at all. To exclude the possibility that the descarboxy factors were bound by free antibodies on the Sepharose the SPC's were pre-incubated with either bovine prothrombin or factor X before addition of the descarboxy factors. This treatment had no effect on the amount of carboxylation measured.

Since both SPC's are substrate-bound enzyme systems, we have checked whether the endogenous substrates might hamper the carboxylation of large exogenous substrates. For that purpose we have compared the properties of substrate-free and substrate-bound carboxylase in detergent-solubilized microsomes from normal and warfarin treated cows, respectively. Except for

Table II

Carboxylation of exogenous substrates by substrate-free microsomes, substrate-bound microsomes and solid-phase carboxylases.

Substrate	Conc. (mM)	$^{14}\text{CO}_2$ incorporated (dpm/h)			
		substrate-free microsomes	substrate-bound microsomes	SPC-II	SPC-X
none		820	16200	90	110
D-II	0.01	900	16080	90	105
D-II	0.1	4020	16150	110	140
D-X	0.01	880	16200	100	110
D-X	0.1	3870	16000	110	120
D-II fr. Su	0.01	25430	51380	4120	6970
D-X fr. Su	0.01	24340	48660	4530	6340
D-II fr. C.T.	0.01	20060	38300	2260	2850
D-X fr. C.T.	0.01	18680	27700	2120	2730
D-osteocalcin	0.01	14920	35020	3740	4750
F L E E L	0.01	830	16200	100	110
F L E E L	1.6	35800	88600	5880	10720

The carboxylation reaction was performed under standard conditions. The values are the means of triplicate measurements. fr. C.T. stands for chymotryptic fragment; for other abbreviations, see the text to Table I.

the substrates mentioned in table I we have also used the carboxylatable chymotryptic cleavage product (41 residues) of the descarboxy factors. The results of these experiments are shown in table II. It is clear that the peptide substrates (up to 49 amino acid residues) were good substrates in both systems, whereas the protein substrates could only be carboxylated in substrate-free carboxylase.

DISCUSSION

It has been shown earlier, that with the aid of insolubilized antibodies against blood coagulation factor X, 65% of the substrate-bound carboxylase could be extracted from solubilized microsomes (7). From the supernatant no more complexes could be bound with insolubilized antifactor X, but still 21% with antiprothrombin. Together with the analyses of the extracted proteins (7), these experiments strongly indicate that no prothrombin precursors occur in SPC-X and vice versa. No indications were obtained for any difference between the carboxylases present in the two solid phase systems, however.

On the other hand we did find a difference between substrate-free and substrate-bound carboxylase. Large substrates such as descarboxyprothrombin and descarboxyfactor X were carboxylated, albeit poorly, in substrate-free carboxylase, whereas in the substrate-bound systems (in neither solubilized microsomes nor SPC) these protein substrates were not carboxylated. From our work with SPC-X we know, that even after completion of the carboxylation reaction the enzyme and its carboxylated endogenous substrate remain firmly complexed, although the carboxylation of small peptides such as F L E E L occurs uninhibited. The experiments shown in this paper clearly indicate that in substrate-bound carboxylase, possibly because of sterical hindrance by the endogenous substrate, the carboxylation of exogenous substrates is hampered. Therefore substrate-free carboxylase (from the liver of normal, non-treated animals) is the enzyme system of choice when the affinity of carboxylase for various substrates is investigated.

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CHAPTER 8

DIRECT MEASUREMENT OF VITAMIN K-DEPENDENT ENZYMES IN VARIOUS ISOLATED AND CULTURED TUMOR AND NON-TUMOR CELLS

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SUMMARY

A modification of the assay for vitamin K-dependent carboxylase is described with which the enzyme could be detected in relatively low amounts of cells ($n = 10^6$). Using this assay, we could demonstrate vitamin K-dependent carboxylase activity in hepatocytes, renal tubular cells, osteoblasts, endothelial cells and macrophages, but not in lymphocytes or platelets. The cultured tumor cells UMR-106, B16 and 5583 also contained vitamin K dependent carboxylase activity. Vitamin K epoxide reductase activity was demonstrated only in cells where vitamin K-dependent carboxylase activity was present. The tumor cells possessed remarkably less K epoxide reductase activity than the normal cells. When cells were cultured in medium containing warfarin, the K epoxide reductase activity was found to be decreased and the amount of non-carboxylated precursor proteins had increased, suggesting an analogous vitamin K mechanism as in liver.

INTRODUCTION

Vitamin K acts as a coenzyme in the carboxylation of peptide-bound glutamic acid residues to 4-carboxyglutamic acid residues (Gla), which strongly bind Ca^{2+} -ions. The active cofactor, vitamin K hydroquinone (KH_2), is formed via the reduction of vitamin K quinone (K) by one or more reductases. During the carboxylation reaction, KH_2 is converted into vitamin K epoxide (KO) which is reduced again to K by KO reductase. Anticoagulants of the coumarin type interfere with the formation of Gla by inhibiting the reductase enzymes (for reviews see refs. 1,2,3). It has been suggested that coumarin derivatives may also inhibit the formation of tumor metastases, but the antimetastatic activity may not necessarily occur via interference with the vitamin K cycle (4).

Vitamin K dependent carboxylase activities have been demonstrated in the microsomal fractions of various tissues, including tumor tissue (2,3,5). Recently we have also shown the presence of coumarin sensitive KO reductases in rat hepatic and various non-hepatic tissues (6). Obviously all these preparations contain a variety of cells. To study the role of vitamin K-dependent enzymes in tumor metastasis and in other processes, it is necessary to know in which type of cells these enzymes are present. Using the technique commonly employed for the determination of vitamin K-dependent carboxylase in microsomal fractions, a fairly large quantity of cells is needed. Therefore we adapted this method to measure directly vitamin K-dependent carboxylase activity in a relatively small number of cells. Various types of cells were examined subsequently for their vitamin K-dependent enzymatic activities.

MATERIALS AND METHODS

Reagents:

Water-solubilized vitamin K (Konakion^R) was obtained from Hoffman-La Roche (Switzerland) and vitamin KH_2 was prepared as described earlier (7). Pure vitamin K was obtained from Merck (F.R.G.) and vitamin KO was prepared from it according to the method of Tischler (8). Warfarin, dithiothreitol (DTT), tocopherolacetate, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate (HEPES)

and 3-([3-cholamidopropyl] dimethylammonio)-1-propane sulfonate (CHAPS) were purchased from Sigma (U.S.A.). The synthetic substrate Phe-Leu-Glu-Glu-Leu (F L E E L) was obtained from Vega Biochemical Co. (U.S.A.), $\text{NaH}^{14}\text{CO}_3$ (50-60 Ci/mol) from Amersham (U.K.) and Atomlight from New England Nuclear (F.R.G.). Fetal calf serum (FCS) was obtained from Seromed (F.R.G.) and Dulbecco's MEM culture medium and Hank's balanced salt solution were from Gibco (U.K.). All other chemicals were obtained from Merck (F.R.G.)

Cells:

The cell lines UMR-106 (rat osteosarcoma), 5583-83 (human colon carcinoma) and B 16-F10 (mouse melanoma) were propagated in our laboratory as monolayers in Falcon T 25 tissue culture medium plus respectively 5% FCS, 10% FCS and 10% FCS plus 25 mM HEPES. The other cells were kindly provided by various laboratories. Endothelial cells were isolated from human umbilical veins and cultured as described by Willems et al. (9). Renal tubular cells from monkey and from dog were isolated and cultured according to van Wezel et al. (10). Osteoblasts were isolated from rat calvaria and cultured as described by Boonekamp et al. (11). For some experiments cells were cultured in the presence of warfarin (100 ug/ml medium). After harvesting, the cells were washed 3-4 times with PBS and counted before the cell pellets were frozen at -80°C . Hepatocytes were isolated from perfused rat livers according to Groen et al. (12). The purity of these cells was >95%. Three batches of hepatocytes (from three different livers) were mixed and used in the experiments described below. Macrophages were isolated from rat spleens as described by Muller et al. (13). Platelets and lymphocytes were isolated from the buffy coats obtained from blood of healthy male donors according to Bevers et al. (14). After counting, all cells were frozen at -80°C . Before freezing the viability of the cells, as estimated by occlusion of trypan blue, was >90%. After thawing all cell pellets were resuspended in buffer A (1 M NaCl, 50 mM Tris/HCl, pH 7.4, 20% ethylene-glycol) to a final concentration of 10^7 cells/ml. The cell mixtures were sonicated before use.

Vitamin K-dependent carboxylase assay:

The vitamin K-dependent carboxylation of F L E E L was measured by incubating $\pm 10^6$ lysed cells in buffer A with F L E E L (10 mM), vitamin KH_2 (0.2 mM), DTT (4 mM), $\text{NaH}^{14}\text{CO}_3$ (0.02 mCi), $(\text{NH}_4)_2\text{SO}_4$ (1 M), phosphatidylcholine (50-200 μg) and the detergent CHAPS (50-200 μg). The

optimal phosphatidylcholine and CHAPS concentrations had to be checked for each batch of cells. Phosphatidylcholine was added as mixed micelles of the phospholipid with cholate (1:1, w/w). Incubation was performed in 0.25 ml reaction mixtures at 25 °C for various time intervals. The reaction was stopped and non-bound $^{14}\text{CO}_2$ was removed by adding 1 ml trichloroacetic acid (5% w/v) followed by degassing the reaction mixture for 3 min at elevated temperatures. The samples were supplemented with 10 ml Atomlight and counted in a Beckman scintillation counter. To measure the amount of non-carboxylated precursor proteins (endogenous substrate) $2-3 \cdot 10^6$ cells were incubated with only KH_2 , DTT and $\text{NaH}^{14}\text{CO}_3$ in buffer A for 60 min. In all experiments, parallel incubations were performed in the absence of KH_2 and DTT. These blank values were subtracted.

Vitamin KO reductase assay:

The KO reductase activity was measured essentially as described earlier (6) with adaptations for the use of a small amount of cells per incubation (15). In short, $0.5 - 1 \cdot 10^6$ lysed cells were pre-incubated in buffer A in 0.1 ml mixtures with DTT (10 mM) for 3 min at 37 °C. The reaction was started by adding 4 µg KO in 2 µl propanol-2. After 1 h the reaction was stopped by adding 0.9 ml propanol-2 containing 2.5 µg tocopherolacetate as an internal standard. The mixture was extracted with 0.6 ml hexane and 0.5 ml 0.01 M AgNO_3 to prevent co-extraction of DTT. The hexane phase was washed with 0.5 ml 0.4% NaCl and evaporated to dryness at 35 °C under a stream of nitrogen. The residue was dissolved in 50 µl propanol-2 and the vitamin K content was determined using HPLC analysis.

RESULTS

To investigate the presence of vitamin K-dependent systems in cultured cells we adapted our vitamin K-dependent carboxylase assay in such a way that the vitamin K-dependent $^{14}\text{CO}_2$ incorporation could be measured directly in an amount as small as 10^6 cells. The optimal CHAPS and phosphatidylcholine concentrations may vary per type of cell. In general, both inhibit at higher concentrations. The addition of $(\text{NH}_4)_2\text{SO}_4$ stimulates the $^{14}\text{CO}_2$ incorporation into F L E E L at least four times. A time course of the vitamin K-dependent carboxylation by various types of cells is shown in fig. 1.

Fig. 1.
Time course of vitamin K dependent incorporation of $^{14}\text{CO}_2$ into FLEEL by various cells. The carboxylation reaction was performed for various time intervals under standard conditions. Explanation of symbols:

▼-▼ hepatocytes; ●-● renal tubular cells
◆-◆ UMR cells △-△ B16 cells
○-○ endothelial cells

From a number of different cells the vitamin K-dependent-carboxylase activity was measured (table I). It will be obvious that vitamin K-dependent carboxylase is present in all cells tested, except in platelets and lymphocytes. In these cells vitamin K-dependent carboxylase activity could not even be demonstrated when $\pm 5 \cdot 10^7$ cells were used for one incubation.

To investigate the presence of other enzymes of the vitamin K cycle in cells, we also measured vitamin KO reductase activity. Vitamin KO reductase

Table I

Vitamin K dependent carboxylase activity in various cells

type of cell	$^{14}\text{CO}_2$ incorporation into F L E E L (dpm/ 10^6 cells)	
	+KH ₂	-KH ₂
hepatocytes (rat)	42900	330
renal tubular cells (monkey)	4700	280
renal tubular cells (dog)	3810	250
osteoblasts (rat)	6785	270
macrophages (rat)	1320	260
endothelial cells (man)	19700	330
lymphocytes (man)	350	330
platelets (man)	400	410
UMR (rat)	13125	260
B16 (mouse)	8060	280
5583 (man)	4125	200

Vitamin K-dependent carboxylation was performed under standard conditons in the presence and absence of vitamin KH₂ and DTT. Carboxylase activity is expressed as dpm $^{14}\text{CO}_2$ incorporated in F L E E L per 30 min per $\pm 10^6$ cells. The values are the average of triplicate measurements.

activity was only demonstrated in cells where vitamin K-dependent carboxylase activity was present (table II). In general, tumor cells produced a lesser amount of vitamin K quinone than the non-tumor cells, indicating that their vitamin KO reductase activity is less active. No correlation was found between the relative values of vitamin K-dependent carboxylase activity and vitamin KO reductase activity as measured in vitro. Such a correlation was neither found in the microsomal fractions of various rat tissues (6).

Table II

Vitamin KO reductase activity in various cells

Type of cell	pmol vitamin K formed
hepatocytes	250-300
renal tubular cells (ape)	185-240
renal tubular cells (dog)	220-235
endothelial cells	90-180
UMR	9- 12
B16	7- 17
5583	11- 20

Vitamin KO reductase activity was measured in $0.5-1.10^6$ cells. The enzymatic activity is expressed as pmol vitamin K formed per 10^6 cells per hour. The data give the range between various batches of cells.

When animals are treated with warfarin an accumulation of non-carboxylated precursor proteins can be detected in liver and in various non-hepatic tissues. These precursor proteins are called the "endogenous substrate" for carboxylase. We cultured some types of cells in the presence of warfarin and compared their content of non-carboxylated precursor proteins with the amount in corresponding normal cultured cells (table III). In all cases a small but distinct increase of the amount of non-carboxylated precursor proteins could be observed. The values are in the same order of magnitude as the accumulation found for non-hepatic rat tissues after coumarin treatment. In liver, however, at least a tenfold increase in non-carboxylated precursor proteins has been reported (6).

To determine whether the incorporated label could be identified as Gla, the various ^{14}C -labeled reaction products were heated at 110°C in 6 M HCl for 24 h. Since Gla-residues are acid-labile, the theoretical loss of $^{14}\text{CO}_2$ during this treatment is 50%. The observed losses were 44% in the case of B16 cells, 57% with UMR-106 cells, 59% with 5583 cells and 48% with the endothelial cells. From these results we concluded that under our experimental conditions Gla-residues had been formed by all four cell lysates.

Table III

Influence of warfarin in cell culture medium on the amount of non-carboxylated precursor proteins

type of cell	accumulation (%) (amount without warfarin = 100%)	
B16	185	(170-201)
UMR	320	(230-405)
5583	140	(114-163)
endothelial cells	170	(148-192)

The amount of non-carboxylated precursor proteins was measured in $2-3 \cdot 10^6$ cells. The values after culturing on warfarin-containing medium (100 ug warfarin per ml) are expressed as a percentage of the corresponding amounts in the same quantity of normal cultured cells. The data are the mean of duplicate measurements using two parallel batches of cells. The range between the different preparations is given in parentheses.

Table IV

Influence of warfarin in cell culture medium on the vitamin KO reductase activity

type of cell	vitamin KO reductase activity (%) (activity without warfarin = 100%)	
B16	28	(18-37)
UMR	35	(29-42)
5583	30	(28-33)
endothelial cells	70	(59-80)

Vitamin KO reductase activity was measured in $\pm 10^6$ cells. The values obtained from cells cultured on a warfarin containing medium are expressed as a percentage of the vitamin KO reductase activity found in a same quantity of corresponding cells cultured on a normal medium. Data are the mean of duplicate measurements. The range between the different preparations is given in parentheses.

As warfarin is thought to block vitamin K₀ reductase in liver, we investigated the activity of this enzyme in cells cultured on a warfarin-containing medium. In all cases the vitamin K₀ reductase activity was diminished as compared to the corresponding values in normal cultured cells (table IV). Tumor cells appeared to be more sensitive for warfarin than normal endothelial cells.

DISCUSSION

In this paper we describe a method for measuring vitamin K-dependent carboxylase activity directly in a relatively small quantity of cells ($n =$ about 10^6). Using this method, we have shown that vitamin K-dependent carboxylase is present in various types of isolated and cultured cells. We could also detect vitamin K₀ reductase in these cells. As the experimental error in counting of the cells is about 20%, the values given are only indicative. These observations confirm the presence of vitamin K-dependent enzymes in many non-hepatic tissues as was shown before (3,6). In general, the function of the vitamin K-dependent enzymes in the non-hepatic cells is not yet completely clear. Probably all these enzymes will produce Gla-containing proteins. As yet the synthesis and secretion of Gla-containing proteins have been described for only some types of cells. Osteoblasts are suggested to produce osteocalcin, a protein that might be involved in the regulation of bone mineralization (16). Nishimoto and Price demonstrated that this bone-Gla protein is also produced by rat osteosarcoma cells (17). Endothelial cells are believed to produce the Gla-containing protein S (18). Protein S is also secreted by platelets (19), however we could not detect any vitamin K-dependent enzymatic activity in platelets. Therefore, protein S may be synthesized in the megakaryocytes before platelets are produced by fragmentation. Macrophages, which contain carboxylase in small but detectable amounts, show procoagulant activity (13) and the synthesis of the clotting factors II, VII and X has recently been demonstrated in these cells (20).

The proteins which may be synthesized in the other cells have not yet been detected. Gla-containing proteins dissimilar of the coagulation factors and osteocalcin however, have been demonstrated in renal stones

(21), in urine (22), in sperm cells (23), in calcified skin nodules (24) and in atherosclerotic plaques (25). Further research concerning these non-hepatic Gla-containing proteins and their place of synthesis are in currently progress in our laboratory.

Buchtal et al. previously showed vitamin K-dependent carboxylase activity in microsomes from tumor tissue (5). This activity was not necessarily due to tumor cells, but could also be ascribed to for instance vitamin K-dependent activity in the vessel wall. Our results clearly confirm now the presence of vitamin K-dependent enzymes in various tumor cells.

The relation between the inhibition of tumor metastases by warfarin and the vitamin K-dependent enzymes is not clear at this moment. In this connection it may be worth noting that tumor cells showed a remarkably lower vitamin K epoxide reductase activity than the non-tumor cells, while the vitamin K-dependent carboxylase activities were found to be within the same range. Moreover the tumor cells seemed to be slightly more sensitive for warfarin treatment as compared to the endothelial cells. A comparison between vitamin K-dependent reactions in tumor- and non-tumor cells from the same origine will probably give us more insight in the significance of the vitamin K-dependent enzymes for tumor metastases.

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CHAPTER 9

VITAMIN K-DEPENDENT ENZYMES IN CULTURED CELLS FROM HUMAN ARTERIAL AND VENOUS VESSEL WALL

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SUMMARY

The presence of vitamin K-dependent carboxylase is demonstrated in both human arteries and veins. The enzymatic activity per mg microsomal protein in atherosclerotic arteries (N=8) was less than 25% of the activity in non-affected arteries (N=3). Vitamin K-dependent carboxylase activity was also present in cultured arterial and venous endothelial cells, and in cultured smooth muscle cells. Also vitamin K epoxide reductase activity and vitamin K quinone reductase activity were found in these cells, which indicates that vitamin K can be recycled in the cells. When venous endothelial cells were cultured on a warfarin-containing medium the reductase activities had decreased and the amounts of non carboxylated precursor proteins had increased, indicating that coumarins inhibit the vitamin K-dependent carboxylation in human endothelial cells.

INTRODUCTION

Vitamin K is involved in the carboxylation of specific glutamic acid residues (Glu) to 4-carboxyglutamic acid residues (Gla). The active cofactor in the carboxylation reaction, vitamin K hydroquinone (KH_2) is formed via the reduction of vitamin K quinone (K) by one or more K reductases. In vitro DTT may serve as a cofactor for these enzymes. During carboxylation, vitamin KH_2 is converted to vitamin K epoxide (KO) which is reduced again to vitamin K by the DTT-dependent KO reductase. The only known function of Gla is a strong interaction with Ca^{2+} ions (1-3). The coagulation factors II, VII, IX and X and the coagulation inhibiting proteins C and S are examples of Gla-containing proteins. During the last 10 years other Gla-containing proteins, dissimilar to the (anti-)coagulation factors have been detected. One of them is osteocalcin which may play a role in bone mineralization. This protein, purified from the bone of various species and from the media of cultured rat osteosarcoma cells, has been well characterized (4,5). In vitro it prevents the precipitation of calcium phosphate from a supersaturated solution, while the decarboxylated form does not, suggesting that Gla-residues are involved in this inhibitory effect (6,7). Other Gla-containing proteins which, like osteocalcin, may play a role in the deposition of calcium salts have been demonstrated in kidney stones (8), skin noduli (9) and atherosclerotic plaques (10). The in vivo function of these proteins is not yet clear and neither we know the place where they are synthesized. Probably also in these proteins the formation of Gla-residues occurs via a vitamin K-dependent process. Vitamin K-dependent carboxylase activity has been demonstrated earlier in the microsomal fractions of various tissues of different species, including the bovine aorta (11). Here we report the presence of the vitamin K-dependent enzymes in human arteries and veins. Recently we described a modification of our vitamin K-dependent carboxylase assay to measure directly vitamin K-dependent $^{14}\text{CO}_2$ incorporation in a small amount of cells (12). Using this method we also investigated the presence of vitamin K-dependent enzymes in cultured cells which normally form part of the vessel wall i.e. endothelial cells and smooth muscle cells.

MATERIALS AND METHODS

Chemicals:

Pure vitamin K was obtained from Merck (F.R.G.) and vitamin K₀ was prepared from it according to the method of Tischler (13). Water-solubilized vitamin K (Konakion^R) was obtained from Hoffmann-La Roche (Switzerland) and vitamin K_{H₂} was prepared as described earlier (14). The synthetic substrate Phe-Leu-Glu-Glu-Leu (F L E E L) was purchased from Vega Biochemical Co (U.S.A.), NaH¹⁴CO₃ (50-60 Ci/mol) was from Amersham (U.K.) and Atomlight was from New England Nuclear (F.R.G.). Fetal calf serum was obtained from Seromed (F.R.G.) and Dulbecco's MEM culture medium and Hank's balanced salt solution were from Gibco (U.K.). Warfarin, dithiothreitol (DTT), tocopherolacetate, and 3-([3-cholamidopropyl]dimethyl ammonio)-1-propane sulfonate (CHAPS) were purchased from Sigma (U.S.A.). All other chemicals were obtained from Merck (F.R.G.).

Methods:

Pieces of human aorta, vena cava and varicose veins were obtained from the departments of Surgery and Pathology of the Academic Hospital in Maastricht and the Slotervaart hospital in Amsterdam. It was ascertained that all material was frozen at -80 °C immediately after surgery and at last within 3 hours after death. The aortae were divided into atherosclerotic and non-atherosclerotic ones according to the pathologist's opinion. Three non-atherosclerotic tissues were obtained from young men, who died after traffic accidents. Most of the tissues were obtained from aged people. The varicose veins (n=3) were obtained from people aged between 35 and 50 years. After thawing, the tissues (10-30 g each) were stripped of fat and cut into small pieces before they were homogenized in a Breda Scientific blender, with 2-3 volumes of buffer A (0.1 M NaCl, 50 mM Tris/HCl, pH 7.4, 20% ethyleneglycol). The mixture was centrifuged for 10 min at 8000 g at 10 °C. The supernatant was kept and the precipitate was mixed again with 2 volumes of buffer A and recentrifuged for 10 min at 8000 g. The joined supernatants were used for the preparation of the microsomal fraction by centrifuging the mixture for 1 h at 100000 g at 4 °C. The microsomal pellet was resuspended in buffer A and centrifuged again until the supernatant was colourless (1-2 washing cycles). A final wash step was performed in buffer B (buffer A, containing 1 M NaCl instead of 0.1 M NaCl). Afterwards the

microsomal pellet was resuspended in a small volume of buffer B to a protein concentration of 7-12 mg per ml, frozen in liquid nitrogen and stored at -80°C until use. No measurable loss in activity was found for at least 2 months.

All cells were kindly provided by various laboratories. Endothelial and smooth muscle cells were isolated from human umbilical cord arteries and veins and cultured as described by Willems et al. (15). For some experiments, cells were cultured in the presence of warfarin (100 $\mu\text{g/ml}$ medium). Endothelial cells from canine femoral arteries and veins were isolated and cultured as described by Groenewegen et al. (16). After harvesting, the cells were washed 3-4 times with PBS and counted before the cell pellets were frozen at -80°C . The viability, as estimated by occlusion of trypan blue, was >90% in all cells tested. Hepatocytes were isolated from perfused rat livers according to Groen et al. (17), counted and frozen at -80°C . Their purity was >95%. After thawing, all cell pellets were resuspended in buffer B to a final concentration of 10^7 cells/ml. The cell mixtures were sonicated before use.

Vitamin K-dependent carboxylation was performed as described earlier for microsomes (18) and cells (12), by measuring the incorporation of $^{14}\text{CO}_2$ into the synthetic substrate F L E E L. Per incubation 1 mg microsomal proteins or 10^6 cells were used. The carboxylation reaction was started by adding either vitamin KH_2 or vitamin K + DTT. As vitamin K has to be converted into KH_2 before carboxylation can take place, the activity of the DTT dependent K reductase enzyme can be measured via this reaction. To measure the amount of membrane-bound non-carboxylated precursor proteins (the so-called endogenous substrate) the KH_2 -dependent carboxylation was performed in the absence of the exogenous substrate F L E E L. The amount of $^{14}\text{CO}_2$ incorporated in the trichloroacetic acid-precipitable fraction is considered as a measure for the number of the membrane bound precursor proteins. In all carboxylation experiments, parallel incubations were performed in the absence of K and DTT. These blank values were subtracted. The DTT-dependent KO reductase activity was measured essentially as described earlier (12,18). One mg of microsomal proteins or 10^6 cells were incubated for 30 min at 30°C in the presence of 4 μg KO and 10 mM DTT and the amount of vitamin K quinone formed is considered to be a measure for the enzymatic activity. Protein concentrations were determined according to Sedmak and Grossberg (19).

RESULTS

KH_2 -dependent $^{14}\text{CO}_2$ incorporation was measured in the microsomal fractions from human arteries and veins. A typical time course for both tissues is shown in fig. 1. In the presence of the synthetic substrate F L E E L, the reaction proceeded in a linear way until 30 min. A rather small amount of endogenous substrate could be demonstrated in both tissues (fig. 1).

Fig. 1.
Time course of the carboxylation by microsomal preparations from human arteries (●-●) and veins (▲-▲). The KH_2 stimulated carboxylation was performed in the absence (open symbols) and presence (closed symbols) of the exogenous substrate F L E E L (10 mM). Blank values, derived from parallel incubations without KH_2 and DTT ranged between 200 and 400 dpm and were subtracted.

No substantial differences were found between the carboxylation activities in microsomal preparations from the vena cava (600 ± 100 dpm/min per mg microsomal protein, $n=4$) and from the varicose veins (570 ± 80 dpm/min per mg microsomal protein, $n=3$).

A substantial difference in the KH_2 -dependent carboxylase activity per mg microsomal protein was found between non-atherosclerotic and atherosclerotic arteries. The enzymatic activities in the microsomal fractions from the last ones ($n=8$) were less than 25% of the activities found in the preparations from three non-affected tissues (fig. 2A). Differences were also found in KO reductase activities (fig. 2B).

Fig. 2.

Comparison between microsomal preparations from atherosclerotic and non-atherosclerotic tissues.

A: KH_2 stimulated carboxylation of F L E E L; open bar: non-atherosclerotic tissue ($n=3$), grey bar: atherosclerotic tissue ($n=8$). Median and extreme values are given.

B: KO reductase activity, open bar: non-atherosclerotic tissue ($n=3$); grey bar: atherosclerotic tissue ($n=5$). Median and extreme values are given.

To detect which type of cells in the vessel wall contain vitamin K-dependent enzymes, cultured endothelial cells and smooth muscle cells both derived from umbilical cord arteries and veins, were examined on their vitamin K-dependent enzymatic activities. In all cells tested, KH_2 -dependent carboxylase activity was present (table I).

Table I

Vitamin K-dependent enzymatic activities in cultured arterial and venous cells

type of cells	KH_2 -driven carboxylation ($^{14}\text{CO}_2$ -bound)	[K+DTT]-driven carboxylation ($^{14}\text{CO}_2$ -bound)	KO reduction (pmol K formed)
Arterial endo- thelial cells	6270	2400	94
Venous endo- thelial cells	11290	5270	141
Arterial smooth muscle cells	7110	2810	110
Venous smooth muscle cells	8980	3670	117
Rat hepatocytes	24400	10980	300

The KH_2 -stimulated carboxylase activities and the $^{14}\text{CO}_2$ [K +DTT] stimulated carboxylase activities are given as the amount of $^{14}\text{CO}_2$ incorporated into F L E E L in 30 min. Blank values, obtained from parallel incubations without K and DTT, ranged between 200 - 400 dpm and were subtracted. The KO reductase activities are given as the amount of pmol K formed during 30 min of incubation. All reaction mixtures contained 10^6 cells. The data are the average of triplicate measurements. Variances between the experiments were less than 15%.

We could also demonstrate DTT-dependent KO reductase activity and [K + DTT]-stimulated carboxylase activity in these cells (table I). From these results we concluded that all cells tested contain the enzymes required for

an efficient recycling of vitamin K. Most of the experiments concerning vitamin K-dependent enzymes have been performed with rat or bovine liver microsomes. Therefore we have included in table I the vitamin K-dependent enzymatic activities as measured in a same quantity of isolated rat hepatocytes. The hepatocytes contained 2.5 to 4 times more vitamin K-dependent enzymatic activities than the cells from vessel wall. These data are comparable with those found in hepatic and non-hepatic tissues (18).

Not only cells derived from the vessels of the umbilical cord contain vitamin K-dependent enzymes. Also endothelial cells cultured from canine femoral arteries and veins showed KH_2 -dependent carboxylase activity: the venous cells contained $\pm$ 35% and the arterial cells $\pm$ 50% of the enzymatic activity in rat hepatocytes.

Coumarin drugs like warfarin prevent the formation of Gla-residues in proteins produced by liver tissue. The coumarins inhibit the DTT-dependent KO reductase and the DTT-dependent K reductase steps in the vitamin K cycle, resulting in a decreased production of the active cofactor in carboxylation, KH_2 . To investigate the effect of warfarin on the vitamin K-dependent enzymes in venous endothelial cells, warfarin was added to the culture medium (100 $\mu\text{g}/\text{ml}$) for 3 to 5 days. This warfarin concentration did not apparently alter the normal pattern of cell growth. At higher concentrations ($> 200 \mu\text{g}/\text{ml}$) the cells stopped growing and finally died. As compared to the activities in normal cultured cells, the DTT-dependent KO reductase activity and the $[\text{K} + \text{DTT}]$ -stimulated carboxylase activity were decreased, while the KH_2 -dependent carboxylase activity was increased (table II). We also found an increase in the amount of endogenous substrate. To determine whether ^{14}C -Gla was formed in the precursor proteins, incubated samples of warfarin-treated cells were heated at 110°C in 6 M HCl for 24 h. This treatment resulted in a loss of 48-54% of the bound ^{14}C . As the theoretical loss of ^{14}C from ^{14}C Gla, labeled in one of the carboxylgroups, would be 50% we concluded that Gla-residues had been formed by the endothelial cell preparations.

Table II

Effects of warfarin in the culture medium on the vitamin K-dependent activities in endothelial cells

parameters measured	quantities (percentage of normal values)
KO reductase activity	70- 72%
[K + DTT]-driven carboxylase activity	63- 74%
KH ₂ -driven carboxylase activity	134-143%
amount of endogenous substrate	178-206%

In parallel experiments, cells were cultured with and without warfarin (100 µg/ml culture medium). All data obtained from cells after warfarin treatment, are expressed as a percentage of the corresponding normal values. The ranges between a triplicate series of experiments are given.

DISCUSSION

Vitamin K dependent enzymes were shown to be present in arterial and venous endothelial cells and in smooth muscle cells. From the data in this paper, it can be concluded that warfarin influences the vitamin K cycle in human endothelial cells in a similar way as it inhibits the vitamin K-dependent enzymes in bovine and rat liver. Low dose oral coumarin treatment of rats resulted in a decrease of DTT-dependent KO reductase activities in liver and in several non-hepatic tissues (18). It seems probable, therefore, that also in patients under coumarin-anticoagulant therapy, the DTT dependent KO- and K reductase activities will be decreased in endothelial cells. As comparable vitamin K dependent systems are present in smooth muscle cells, we presume that the carboxylation of proteins in these cells will also be affected by coumarin treatment. Before we can speculate about possible effects of this inhibition we have to know which proteins are carboxylated in vivo by these cells. Endothelial cells are believed to produce the anti-coagulation factor protein S (20), but the protein(s) which may be synthesized by smooth muscle cells have not yet been detected. Whereas in an earlier paper vitamin K-dependent carboxylase

could only be demonstrated in arteries and not in veins (11), improved homogenization procedures and a far more sensitive carboxylase assay have enabled us also to demonstrate unequivocally the presence of the enzyme in veins.

Until now, we have no explanation for the fact that atherosclerotic tissues seem to contain less KH_2 -dependent carboxylase activity per mg microsomal protein than the non-affected tissues. However, the rather low carboxylase activity may result in an undercarboxylation of secreted Gla-containing proteins, which may influence their Ca^{2+} -binding capacity. In analogy with osteocalcin, the undercarboxylation might interfere with the prevention of Ca-salts from a supersaturated solution. In vivo this might contribute to the formation and/or maintenance of atherosclerotic plaques.

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C H A P T E R 10

SOME CHARACTERISTICS OF VITAMIN K-DEPENDENT CARBOXYLASE IN HUMAN TISSUES

M.A.G. de Boer-van den Berg and C. Vermeer

SUMMARY

Vitamin K hydroquinone-dependent carboxylase activity is demonstrated in the microsomes from human kidney, lung, spleen and testis and the characteristics of these systems are compared with those of the human liver enzyme. The pentapeptide F L E E L, descarboxyprothrombin fragment 13-29, decarboxylated sperm-Gla-protein and decarboxylated osteocalcin could be used as exogenous substrates for all five types of carboxylase, but the relative carboxylation rates differed from one tissue to another. DTT-dependent vitamin K reductase was tested by replacing vitamin K hydroquinone by vitamin K quinone + DTT in the carboxylase assay and the presence of the enzyme was established in all tissues examined. In the presence of vitamin K quinone + DTT the carboxylation reaction was inhibited for 50% by warfarin concentrations between 2 and 15 μ M. NADH did not enhance the [vitamin K quinone + DTT]-stimulated carboxylation reaction.

INTRODUCTION

Vitamin K-dependent carboxylase is a microsomal enzyme system involved in the posttranslational conversion of glutamic acid residues into 4-carboxyglutamic acid (Gla) residues in proteins (1-3). Vitamin K in its hydroquinone form (KH_2) is the active cofactor in this reaction and during the carboxylation it is converted into vitamin K 2,3 epoxide (KO). A second enzyme, the dithiothreitol-dependent KO reductase (4) is able to reduce KO into vitamin K quinone (K), which is subsequently reduced further to KH_2 by two or more K reductases, one of which is -at least in vitro- dithiothreitol (DTT) dependent, the other one(s) NADH dependent. The physiological counterpart of DTT in the two DTT-dependent enzymes mentioned above is presently unknown. Coumarin derivatives like warfarin primarily inhibit the DTT-dependent reductases, which results in a decreased production of KH_2 and thus in an impaired Gla formation.

For a long time it has been thought that vitamin K-dependent carboxylase was exclusively located in the liver, where it is involved in the synthesis of the blood coagulation factors II, VII, IX and X and the anticoagulation proteins C and S. Later on it was demonstrated in experimental animals that the formation of Gla-residues is a far more common process, occurring in many other tissues as well (5-7). Evidently the results of these animal studies should be verified with human tissues. Until now the only data available have been obtained from human liver (8,9) and placenta (8,10), skin (11) and vessel wall (12). In this paper we compare the vitamin K-dependent enzymes from human liver with those from human kidney, lung, spleen and testis.

MATERIALS AND METHODS

Chemicals

Vitamin K(Konakion^R) was obtained from Hoffmann-La Roche (Switzerland) and vitamin KH_2 was prepared by chemical reduction (13). $\text{NaH}^{14}\text{CO}_3$ (50-60 Ci/mol) was purchased from Amersham (U.K.) and Atomlight from New England Nuclear (F.R.G.). NADH and subtilisin were obtained from Boehringer Mannheim (F.R.G.) and warfarin, dithiothreitol (DTT) and 3-([3-cholamido-

propyl]dimethyl ammonio)-l-propane sulfonate (CHAPS) from Sigma (U.S.A.). The pentapeptide Phe-Leu-Glu-Glu-Leu (F L E E L) was from Vega Biochemical Co (U.S.A.). Human prothrombin was kindly supplied by dr. G. Tans from our institute. Antibodies against native human blood coagulation factors II, VII, IX and X as well as those against the native human proteins C, S and Z were generous gifts from dr. R. Bertina (Leiden, The Netherlands). The antibodies had been immuno-purified and linked to CNBr-activated Sepharose. All other chemicals were of the highest quality commercially available.

Protein substrates

Decarboxylated osteocalcin was prepared from bovine bone (14) and decarboxylated sperm-Gla-protein from human spermatozoa (15). The proteolytic subtilisin split product fragment 13-29 (fragment Su) was prepared from human prothrombin in a similar way as it was described for bovine decarboxyprothrombin (16). The peptide was then converted into a carboxylatable substrate by thermal decarboxylation of its Gla-residues (17), which facilitated its detection during the subsequent purification steps.

Preparation of microsomes.

Piece of human liver, kidney, spleen, lung and testis (10 g each) were obtained from the Departments of Pathology of the University Hospital in Maastricht and the Slotervaart Hospital in Amsterdam. All material was obtained from obduction at last within 3 hours after death. Tissues were obtained and experiments performed after written permission from the Ethical Board of the University of Maastricht. After thawing, the tissues were carefully cleaned and cut into small pieces before they were homogenized in a Breda Scientific mixer with 2-3 volumes of buffer A (0.1 M NaCl, 50 mM Tris/HCl, pH 7.4, 20% ethyleneglycol, 2% Trasylol). Corresponding tissues from five different donors were pooled before homogenization. The microsomal fractions were prepared by different centrifugation (12). The final washed microsomal pellet was resuspended in buffer B (buffer A, containing 1 M NaCl instead of 0.1 M NaCl), frozen in liquid nitrogen and stored at -80°C until use. No measurable loss in activity was found for at least 6 months.

Vitamin K-dependent activities.

Vitamin K-dependent carboxylation of exogenous substrates was performed as described earlier (7). Vitamin KH_2 was used as a coenzyme and all reaction mixtures contained 1 mg of microsomal proteins, 8 mM DTT, 0.2% CHAPS (w/v),

0.01 mCi $\text{NaH}^{14}\text{CO}_3$, 0.2 mM vitamin KH_2 and an exogenous substrate, e.g. F L E E L (4 mM); decarboxylated osteocalcin (0.02 mM); decarboxylated subtilisin split product 13-29 from prothrombin (0.02 mM) or decarboxylated sperm-Gla-protein (0.2 mM). The enzymatic activity is expressed as dpm incorporated CO_2 per min per mg protein.

DTT dependent vitamin K reductase activity was measured in a similar way by replacing vitamin KH_2 by vitamin K and by using 4 mM F L E E L as the exogenous substrate.

In some experiments 2 mM NADH was added to the vitamin K containing reaction mixture, the DTT concentration was then lowered to 5 mM or omitted.

Non-carboxylated precursor proteins were quantitated by performing the vitamin KH_2 -dependent reaction in the absence of exogenous substrate. Under these conditions the amount of $^{14}\text{CO}_2$ fixed may be regarded as a measure for the amount of endogenous substrate present in the microsomes. In all carboxylation experiments, parallel incubations were performed in the absence of vitamin K and DTT. These blank values were subtracted.

Other methods

Protein concentrations were determined according to Sedmak and Grossberg (18).

Sephacrose bound antibodies against human native coagulation factors II, VII, IX and X and protein C, S and Z were used to extract the endogenous substrate proteins from the hepatic microsomal fraction, after vitamin KH_2 dependent $^{14}\text{CO}_2$ incorporation had occurred. The extraction procedure was essentially similar as described before for bovine liver microsomes (19).

RESULTS

Washed microsomes were prepared from human liver, lung, spleen, kidney and testis. In these samples we have tried to identify and partially characterize two enzymatic reactions, which both play a role in the formation of Gla-residues in proteins.

KH_2 -dependent carboxylase was tested in the presence of vitamin K hydroquinone and F L E E L and the carboxylation rate observed under these conditions was arbitrarily taken as a measure for the amount of carboxylase

Table I

Amounts of vitamin KH_2 -dependent carboxylase activity and amounts of endogenous substrate in different tissues

Tissue	Carboxylase activity (dpm/min per mg protein)	Endogenous substrate (dpm per mg protein)
liver	2800	1900
kidney	720	370
lung	540	425
spleen	100	385
testis	535	370

Vitamin KH_2 -dependent carboxylase activities were measured in the microsomal fraction of various tissues. The values are expressed as dpm $^{14}\text{CO}_2$ incorporated into F L E E L per min per mg microsomal protein. Blank values, ranging between 6 and 17 dpm per min per mg microsomal protein were subtracted. The amounts of endogenous substrate were measured by performing the carboxylation reaction in the absence of exogenous substrate. The values are expressed as dpm $^{14}\text{CO}_2$ incorporated per mg microsomal protein. Blank values, ranging between 10 and 30 dpm per mg microsomal protein, were subtracted. All data are the means of duplicate measurements. Variations were less than 10%.

present. In all cases the reaction occurred with a constant rate for at least 30 min. The data obtained are summarized in table I and it is clear that vitamin KH_2 -dependent carboxylase activity was present in all tissues examined.

Microsome-bound non-carboxylated precursor proteins which in vitro function as an endogenous substrate for carboxylase, may be detected under similar conditions as described above, but in the absence of F L E E L. As is shown in table I also the presence of endogenous substrate could be established in all preparations. We have tried to identify the carboxylatable proteins in human liver microsomes in a similar way as it was performed for bovine liver (19): after the in vitro carboxylation of endogenous substrate had been completed the reaction mixtures were extracted with Sepharose-bound immuno-purified antibodies against each of the native human coagulation factors II, VII, IX and X as well as the proteins C, S and Z, and it was investigated to which extent the

Table II

Vitamin KH_2 -dependent carboxylase activity towards three different substrates by various tissues

Tissue	d-osteocalcin	d-fragment Su	d-sperm Gla-protein
liver	100%	100%	100%
kidney	37	37	27
lung	15	29	18
spleen	7	30	11
testis	13	43	22

Vitamin KH_2 -dependent carboxylase activity was measured towards three different substrates. The data are expressed as a percentage of the corresponding carboxylase activity in liver microsomes. The carboxylase activities in liver, expressed as the amount of $^{14}\text{CO}_2$ incorporated per min per mg microsomal protein are 760 dpm for d-osteocalcin, 325 dpm for d-fragment Su and 425 dpm for d-sperm-Gla-protein, fragment Su stands for the subtilisin split product of human prothrombin, containing the aminoacids 13-29; d- stands for decarboxylated. Blank values, ranging between 4 and 16 dpm per min per mg microsomal protein were subtracted. The data are the average of duplicate measurements, in which the variations were less than 12%.

Table III

Inhibition by warfarin of the vitamin K + DTT dependent carboxylation

Tissue	[K + DTT]-dependent carboxylase activity (dpm/min, per mg protein)	Warfarin concentration (μM) required for 50% inhibition)
Liver	1800	3
kidney	230	15
lung	215	15
spleen	85	5
testis	230	2

Vitamin K + DTT-dependent carboxylase activities were measured in the microsomal fractions of various tissues. The values are expressed as dpm $^{14}\text{CO}_2$ incorporated into F L E E L per min per mg microsomal protein. Blank values, ranging between 4 and 10 dpm per min per mg microsomal protein were subtracted. Warfarin was added in a concentration range between 1.0 and 32 μM . The data are the mean of duplicate measurements. Variations were less than 10%.

^{14}C -labeled reaction products had bound to the various immobilized antibodies. In contrast to the bovine system, however, the labeled human precursor proteins exhibited no affinity towards one of the antibodies used.

Since substantial differences have been found between the substrate specificities of carboxylase from different bovine tissues (20), we have also checked if a similar phenomenon could be established for the human enzymes.

We therefore have measured the substrate specificity of the five types of carboxylase using three different protein substrates: decarboxylated sperm Gla-protein, decarboxylated prothrombin fragment 13-29 (both of human origin) and decarboxylated osteocalcin from bovine bone. Although some differences were found between the substrate carboxylation in the various microsomes (table II), the amounts of tissue available did not allow us to quantify these differences by determining the K_m values for each of the substrates in all carboxylase preparations.

For the detection of DTT-dependent vitamin K quinone reductase (another enzyme of the vitamin K-cycle), the carboxylase assay was modified by replacing vitamin KH_2 by vitamin K + DTT. In the system thus obtained vitamin K has first to be reduced to vitamin KH_2 before it can serve as a cofactor for carboxylase. In this way also the DTT-dependent K reductase could be demonstrated in all tissues examined (table III). Vitamin K-antagonists of the coumarin type, like warfarin, are well known inhibitors of DTT-dependent K reductase in liver and we have investigated if the reductases from other tissues were similarly sensitive towards this drug. Obviously the IC-50 values for warfarin were measured in the [vitamin K + DTT]-dependent system and the results are summarized in table III. As compared to the data in bovine systems (5) the IC-50 values obtained with the human microsomes were rather low. Even at high warfarin concentrations (1 mM), however, in all tissues a residual carboxylase activity (ranging from 10-20% of the non-inhibited enzymes) was found.

Finally we have also investigated the presence of NADH-dependent vitamin K reductase, using the conditions described by Wallin (9). In the experimental set-up the [vitamin K + DTT]-dependent carboxylation is performed in the presence and in the absence of NADH and the difference between the two activities is defined as the NADH-dependent reductase

activity. In none of the microsomal preparations this enzymatic activity could be detected, however. The activity could neither be demonstrated when the carboxylation was performed in the presence of vitamin K + NADH only.

DISCUSSION

In this paper we describe the occurrence of vitamin KH_2 -dependent carboxylase and DTT-dependent K reductase in the microsomal fractions of human liver, kidney, lung, spleen and testis. If expressed per mg of microsomal protein both enzymes are most abundantly present in the liver. In the non-hepatic tissues, the KH_2 -dependent carboxylase activity ranged between 10 and 30% from those in liver, dependent on the substrate used, whereas the DTT-dependent K reductase activity was found to be 5-15% from the activity measured in liver. It is striking that in spleen microsomes, the carboxylation of the endogenous substrate as well as that of descarboxy subtilisin fragment 13-29 was comparable with that of the other non-hepatic tissues, whereas the carboxylation of the other substrates (F L E E L, d-osteocalcin and d-sperm Gla-protein) is 5-10 times lower. This might suggest that also in man vitamin KH_2 -dependent carboxylases from different tissues have different substrate specificities.

It was checked that the donors of the tissues had not been treated with vitamin K-antagonists. Nevertheless non-carboxylated endogenous substrate was found to be present in the various microsomal fractions. This phenomenon was demonstrated earlier for rat tissues (7).

All DTT-dependent K reductases were inhibited by warfarin. The warfarin concentration required for 50% inhibition of the [K + DTT] stimulated carboxylation ranged between 2 and 15 μM . In general less warfarin was needed for the in vitro inhibition of human K reductases than for the corresponding bovine enzymes (5). It is noteworthy that we were unable to completely inhibit the reductase reactions. Even at warfarin concentrations as high as 1 mM a residual enzymatic activity of 10-20% was found.

We could not confirm the results of Wallin and Martin (9) who recently reported the existence of a NADH-dependent vitamin K reductase in human liver microsomes. These authors found a stimulation of the [vitamin K +

DTT]-driven carboxylation after adding 2 mM NADH to their reaction mixtures. We have not found such a stimulation in one of the tissues even not at low DTT concentrations. The different results obtained by both groups may be the result of different procedures used for the preparation of the microsomes. After our extensive washing procedure no NADH-dependent K reductase activity was found in microsomes from human tissues, so we may assume that this enzyme originates from the cytosol instead from the microsomal membrane.

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C H A P T E R 11

THE IN VIVO EFFECTS OF 4-HYDROXYCOUMARIN DERIVATIVES ON HUMAN HEPATIC AND NON-HEPATIC VITAMIN K-DEPENDENT ENZYMES

M.A.G. de Boer-van den Berg and C. Vermeer

SUMMARY

The in vivo effects of oral anticoagulant therapy with 4-hydroxycoumarins on various vitamin K-dependent enzyme systems in man were compared. In hepatic microsomes obtained from donors who had been treated with 4-hydroxycoumarins for more than 6 months, the vitamin K 2,3 epoxide reductase activity and the DTT-dependent vitamin K quinone reductase activity, were diminished to 35% and 20% of the corresponding normal values. In the non-hepatic tissues, only a small decrease in vitamin K 2,3 epoxide reductase activities could be demonstrated, while no differences were found in the vitamin K quinone reductase activities. In none of the tissues, a significant increase of non-carboxylated precursor proteins was observed, whereas also vitamin K hydroquinone-dependent carboxylase activities seemed to be unaffected by the anticoagulant treatment.

INTRODUCTION

Vitamin K is involved in the posttranslational conversion of specific glutamic acid residues into 4-carboxyglutamic acid (Gla) residues (1-3). This reaction is catalyzed by vitamin K-dependent carboxylase, located at the luminal side of the endoplasmic reticulum. The active cofactor in the carboxylation reaction, vitamin K hydroquinone (KH_2), can be formed from vitamin K quinone by two or more reductases, one of which is -in vitro- dithiothreitol (DTT)-dependent, the other(s) NADH dependent. During carboxylation vitamin KH_2 is converted into vitamin K 2,3 epoxide (KO) which is subsequently reduced to vitamin K by vitamin K epoxide reductase which in vitro may use DTT as a reducing cofactor. The physiological counterpart of DTT in these reactions is not yet known. 4-Hydroxycoumarin derivatives are believed to inhibit the DTT-dependent reductase-steps (4). The result of this inhibition is that the supply of vitamin KH_2 is exhausted, which leads to a decrease of the carboxylation rate. In vivo, this sequence of events results in the appearance of non-carboxylated coagulation factors in the blood stream. These descarboxy factors do not contain the Ca^{2+} binding Gla-residues and hence they have no biological activity.

For many years it has been thought that vitamin K-dependent carboxylase exclusively occurred in the liver where it is involved in the formation of the coagulation factors II, VII, IX and X. Administration of 4-hydroxycoumarin drugs has been used frequently for the control of blood coagulation during thrombogenic episodes. In the last 10 years it became evident from animal studies that vitamin K-dependent carboxylation also occurred in many non-hepatic tissues like kidney, lung, testes and spleen (5,6). Recently, we also demonstrated the presence of vitamin K-dependent carboxylase activity in non-hepatic tissues from human origin (7-9). Probably, all these non-hepatic vitamin K-dependent enzymes will produce Gla-containing proteins, some of which have already been isolated and characterized e.g.: osteocalcin, isolated from human bone (10) and sperm-Gla-protein isolated from human spermatozoa (11).

In an earlier study we have reported the effects of the administration of low doses of warfarin, acenocoumarol and phenprocoumon to rats (12). The 4-hydroxycoumarin treatment resulted in a decrease of vitamin KO reductase

activity in the various non-hepatic tissues, whereas also a small increase in the amounts of non-carboxylated precursor proteins was observed. In liver, both effects were more pronounced than in the non-hepatic tissues. From these data it seems probably that 4-hydroxycoumarins will also affect the non-hepatic vitamin K-dependent enzymes in man and hence may lead to an undercarboxylation of the non-hepatic Gla-containing proteins. We have tested this hypothesis and the results of our investigations are presented in this paper.

MATERIALS AND METHODS

Chemicals.

Water-solubilized vitamin K (Konakion^R) was obtained from Hoffmann-La Roche (Switzerland) and vitamin K hydroquinone was prepared by chemical reduction (13). Pure vitamin K was obtained from Merck (F.R.G.) and vitamin K 2,3-epoxide was prepared from it according to the method of Tishler et al. (14). $\text{NaH}^{14}\text{CO}_3$ (50-60 Ci/mol) was purchased from Amersham (U.K.) and Atomlight from New England Nuclear (F.R.G.). Dithiothreitol (DTT), tocopherolacetate and 3-([3-cholamidopropyl]dimethyl-ammonio)-1-propane sulfonate (CHAPS) were obtained from Sigma (U.S.A.). The synthetic pentapeptide Phe-Leu-Glu-Glu-Leu (F L E E L) was purchased from Vega Biochemical Co (U.S.A.). All other chemicals were of the highest quality commercially available.

Preparations of microsomes.

Pieces of human liver, kidney, lung, spleen, testis, artery, vene, heart and femoral muscle tissue were obtained from the departments of Pathology of the Slotervaart Hospital in Amsterdam, the University Hospital in Maastricht and the St. Josef Hospital in Kerkrade. All material was obtained and frozen at -80°C , at last within 3 hours after death. Tissues (10-20 g each) were obtained from 7 donors who had been treated with acenocoumarol (n=5) or phenprocoumon (n=2). Tissues were also obtained from 11 non-treated donors. The arteries obtained were free from atherosclerotic lesions, according to the pathologist's opinion. Tissues were obtained and experiments were performed after written permission of the Ethical Board from the University. After thawing, the tissues were carefully cleaned and

homogenized in a mixer with 2-3 volumes of buffer A (0.1 M NaCl, 50 mM Tris/HCl, pH 7.4, 20% ethylenglycol, 2% Trasylol). The microsomal fractions were prepared by differential centrifugation as described before (9). After an extensive washing procedure, the microsomal pellet was resuspended in buffer B (buffer A, containing 1 M NaCl instead of 0.1 M NaCl) frozen in liquid nitrogen and stored at -80°C until use. No substantial loss of activity was observed for at least 6 months.

Vitamin K-dependent enzymes.

Vitamin KH_2 -dependent carboxylase activity was measured as described earlier (12). The reaction mixtures contained 1 mg of microsomal protein, 0.2 mM vitamin K hydroquinone, 4 mM F L E E L, 8 mM DTT, 0.2% CHAPS (w/v), 0.01 mCi $\text{NaH}^{14}\text{CO}_3$ and 1 M $(\text{NH}_4)_2\text{SO}_4$ in buffer B. Incubations were performed at 25°C for 30 min. It was checked that the reaction rates were constant during the whole incubation period. The carboxylase activity is expressed as dpm incorporated $^{14}\text{CO}_2$ per min per mg microsomal protein. Similar reaction conditions were used to measure the DTT-dependent vitamin K reductase activity but in that case vitamin KH_2 was replaced by vitamin K quinone. The amount of non-carboxylated precursor proteins (endogenous substrate) was measured by performing the KH_2 -dependent carboxylase assay in the absence of F L E E L and $(\text{NH}_4)_2\text{SO}_4$. Under these conditions the amount of $^{14}\text{CO}_2$ fixed may be regarded as a measure for the quality of non-carboxylated precursor proteins present in the microsomes. In all experiments, parallel incubations were performed in the absence of vitamin KH_2 and DTT. These blank values were subtracted. The DTT-dependent KO reductase activity was measured as described earlier for rat tissues (12). The number of pmol K formed after 30 min incubation of 1 mg microsomal protein in the presence of vitamin KO (4 μg) and DTT (10 mM) was used as a measure for the enzymatic activity.

Protein concentrations were determined according to Sedmak and Grossberg (15) and statistical analyses were performed using a one tailed Wilcoxon test.

RESULTS

Microsomal fractions were prepared from human liver, lung, spleen, kidney, testis, artery, vene, muscle and heart from normal donors and from donors who had been treated with 4-hydroxycoumarins for a continuous period longer than 6 months immediately preceeding their decease. In these preparations we tried to measure the effects of in vivo oral anticoagulant therapy on vitamin K-dependent reactions.

Table I

Vitamin KH_2 -dependent carboxylase activity in tissues from normal and 4-hydroxycoumrin treated donors

Tissue	vitamin KH_2 -dependent carboxylase activity (dpm per min per mg microsomal protein)	
	non-treated donors	anticoagulant treated donors
liver	3052 $\pm$ 708 (n= 6)	2806 $\pm$ 770 (n=5)
kidney	885 $\pm$ 412 (n=10)	878 $\pm$ 445 (n=6)
lung	454 $\pm$ 127 (n= 6)	524 $\pm$ 80 (n=3)
spleen	117 $\pm$ 53 (n= 8)	187 $\pm$ 141 (n=5)
testis	498 $\pm$ 199 (n= 4)	418 $\pm$ 28 (n=3)
artery	457 $\pm$ 75 (n= 3)	464 $\pm$ 87 (n=3)
vene	615 $\pm$ 108 (n= 3)	748 $\pm$ 89 (n=3)
muscle	345 $\pm$ 44 (n= 3)	371 $\pm$ 50 (n=3)
heart	118 $\pm$ 11 (n= 3)	121 $\pm$ 26 (n=3)

Vitamin KH_2 dependent carboxylase activities were measured in the microsomal fractions of various tissues from normal donors and from donors treated with 4-hydroxycoumarin drugs. Blank values, ranging between 5 and 15 dpm per min per mg microsomal protein, were subtracted. From each series of tissues the mean values $\pm$ SD are given. The numbers of tissues examined are given in parentheses.

Table II

Effect of 4-hydroxycoumarin treatment on the amount of non-carboxylated precursor proteins

Tissue	amount of non-carboxylated precursor proteins (dpm per mg microsomal protein)	
	non-treated donors	anticoagulant-treated donors
liver	1890 $\pm$ 277	2695 $\pm$ 1045
kidney	404 $\pm$ 157	750 $\pm$ 402
lung	354 $\pm$ 82	389 $\pm$ 88
spleen	332 $\pm$ 112	368 $\pm$ 92
testis	290 $\pm$ 68	335 $\pm$ 106
artery	257 $\pm$ 75	264 $\pm$ 87
vene	478 $\pm$ 134	583 $\pm$ 70
muscle	410 $\pm$ 71	459 $\pm$ 58
heart	253 $\pm$ 47	284 $\pm$ 35

The amounts of non-carboxylated precursor proteins were measured in the microsomal fractions of various tissues from normal donors and from donors treated with 4-hydroxycoumarin drugs. Blank values, ranging between 20 and 50 dpm per mg microsomal protein, were subtracted. From each series of tissues the mean values $\pm$ SD are given. The number of tissues examined are as indicated table I.

In a first experiment, the presence of vitamin KH_2 -dependent carboxylase activity was measured in the various fractions. No substantial differences in activity were found between the mean values obtained in normal and anticoagulant-treated donors (table 1). However, we found a substantial variation between the individual values in each group. These interindividual variations could not be ascribed to the preparation of microsomes (which all had been prepared parallelly) or the carboxylase assay (the variation between duplo experiments is always less than 10%).

We also investigated the amounts of non-carboxylated precursor proteins in human microsomes (table 2). Although, as compared to the data obtained

Table III

Effect of 4-hydroxycoumarin treatment on the DTT-dependent vitamin K reductase activities.

tissue	[K+DTT]stimulated carboxylase activity (dpm per min per mg microsomal protein)	
	non-treated donors	anticoagulant-treated donors
liver	2340 $\pm$ 793	495 $\pm$ 175
kidney	342 $\pm$ 302	303 $\pm$ 129
lung	269 $\pm$ 49	317 $\pm$ 32
spleen	91 $\pm$ 33	78 $\pm$ 41
testis	255 $\pm$ 90	287 $\pm$ 66
artery	58 $\pm$ 38	64 $\pm$ 35
muscle	192 $\pm$ 71	183 $\pm$ 60

The DTT-dependent K reductase activities were measured as [K+DTT] stimulated carboxylase activities in the microsomal fractions of various tissues from normal donors and from donors treated with 4-hydroxycoumarin drugs. Blank values ranging between 5 and 15 dpm per min per mg microsomal protein, were subtracted. From each series of tissues the mean values $\pm$ SD are given. The number of tissues examined are as indicated in table I. The amounts of microsomes obtained from vene and heart tissue, did not permit us to include them in these experiments.

from the normal donors, the average values of endogenous substrate in microsomes from anticoagulant-treated donors appeared to be slightly increased in all tissues, this increase was not statistically significant.

As 4-hydroxycoumarins are thought to inhibit both, KO-reductase and the DTT-dependent K-reductase, we also investigated the effects of oral anti-coagulant therapy on these reductase-activities. From the experiments it results that the level of DTT-dependent vitamin K reductase was normal for all tissues examined except for liver (table 3). The average value of the activity in liver microsomes from anticoagulant-treated donors was less than 25% of the corresponding average values from the non-treated ones ($p < 0.05$).

Table IV

Effect of 4-hydroxycoumarin treatment on the vitamin K₀ reductase activities

tissue	vitamin K ₀ reductase activity (pmol K formed per min per mg microsomal protein)	
	non-treated donors	anticoagulant-treated donors
liver	50.5 $\pm$ 11.3	17.7 $\pm$ 3.2
kidney	18.4 $\pm$ 6.1	16.8 $\pm$ 5.7
lung	26.1 $\pm$ 8.3	16.6 $\pm$ 3.4
spleen	13.4 $\pm$ 6.4	11.0 $\pm$ 5.7
testis	37.6 $\pm$ 17.3	24.6 $\pm$ 7.2
artery	15.9 $\pm$ 4.3	10.7 $\pm$ 2.8
vein	11.8 $\pm$ 6.4	10.6 $\pm$ 4.1
muscle	12.3 $\pm$ 3.1	11.8 $\pm$ 2.6
heart	16.5 $\pm$ 2.0	12.1 $\pm$ 3.7

Vitamin K epoxide reductase activities were measured in the microsomal fractions of various tissues from normal donors and from donors treated with 4-hydroxycoumarin drugs. From each series of tissues the mean values $\pm$ SD are given. The number of tissues examined are as indicated in table I.

In a subsequent experiment the presence of vitamin K₀ reductase in the various tissues was investigated. As is shown in table 4 the enzymatic activity was present in all tissues examined. The mean values of the vitamin K₀ reductase activities in the non-hepatic microsomes from anticoagulant-treated donors showed a consistent tendency to be lower than in the corresponding tissues from normal donors, but this decrease was not statistically significant. On the other hand, in liver a substantial decreased vitamin K₀ reductase activity ($p < 0.05$) was demonstrated in the microsomes from the anticoagulant treated donors.

DISCUSSION

In this study the presence of vitamin K-dependent enzymes in various human tissues has been confirmed. In general, the relative carboxylation rates are similar to those found for rat tissues; only human spleen tissue contained a remarkable lower carboxylase activity than the rat spleen tissue (12). The vitamin KO reductase activities in microsomes from human tissues were substantially higher than those in corresponding rat tissues (12).

The effects of in vivo oral anticoagulant therapy in man appeared to be most pronounced on the DTT-dependent reductases in liver. In spite of large interindividual variations, both reductase activities were significantly decreased after 4-hydroxycoumarin treatment. The effects of this treatment on non-hepatic reactions are less pronounced. DTT-dependent K reductases were not inhibited. The KO-reductase activities in the anticoagulant treated group showed a consistent tendency to be lower than those in the non-treated group. However, possibly due to the large interindividual variations, this decrease did not reach the level of significance. The finding that in non-hepatic tissues the KO-reductase activities were less decreased than in liver, is in agreement with data earlier reported for rats (12,16). The 10-fold increase of rat hepatic endogenous substrate after 4-hydroxycoumarin treatment was not observed in human tissue, while the accumulation of endogenous substrate in non-hepatic tissues was comparably low. In agreement with rat experiments, no remarkable differences were found between acenocoumarol and phenprocoumon treatment. However, the number of tissues investigated was too small to be conclusive.

From the data presented, we may conclude that, if oral anticoagulant therapy with 4-hydroxycoumarins affects non-hepatic vitamin K-dependent systems in man, the decrease in reductase activities may be expected to be small. However, even a small inhibition of the vitamin K-dependent system may lead to an undercarboxylation of Gla-containing proteins and hence to a loss of biological activity.

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C H A P T E R 12

SUMMARY AND GENERAL DISCUSSION

Vitamin K is involved in the posttranslational carboxylation of specific peptide-bound glutamic acid (Glu) residues to 4-carboxyglutamic acid (Gla) residues. This reaction is catalyzed by vitamin K-dependent carboxylase, an enzyme which is located at the luminal side of the endoplasmic reticulum. The active cofactor in this carboxylation reaction is the hydroquinone form of vitamin K. Vitamin K is thought to be absorbed in the intestine and transported to the liver in its quinone form. In vitro vitamin K quinone may be reduced to vitamin K hydroquinone by reductases, one of which is dithiothreitol (DTT)-dependent; the others are NADH-dependent. During carboxylation, vitamin K hydroquinone is converted into vitamin K 2,3 epoxide which is then reduced again to vitamin K quinone by the DTT-dependent vitamin K epoxide reductase. The physiological counterpart of DTT in these reactions is not yet known. The DTT-dependent reductases and the vitamin K-dependent carboxylase are all present in the microsomal fraction of tissue homogenates. 4-Hydroxycoumarin derivatives such as warfarin, inhibit the DTT-dependent reductases. This results in an exhaustion of the vitamin K hydroquinone supply, and hence the carboxylation rate slows down. In vivo, this sequence of events results in the

appearance in blood of descarboxycoagulation factors (PIVKA's), which differ from the normal coagulation factors in such a way that they do not contain the Ca^{2+} binding Gla-residues. Hence they have no biological activity. The literature on vitamin K-dependent reactions has been briefly summarized in chapter 1.

For many years it has been thought that vitamin K-dependent carboxylase occurred exclusively in the liver where it is involved in the formation of the biologically active coagulation factors II, VII, IX and X and the anticoagulant proteins C and S. Administration of 4-hydroxycoumarin drugs has been used frequently for the control of blood coagulation during thrombogenic episodes. Nowadays, it is known that vitamin K-dependent carboxylation also occurs in non-hepatic tissues. In this thesis we report the presence of vitamin K-dependent carboxylase activity in several non-hepatic tissues: skin (chapter 2), brain (chapter 3), muscle (chapters 3 and 11) and venes (chapter 9). Furthermore we confirm the presence of vitamin K-dependent carboxylase in kidney, testis, lung, spleen and heart tissue (chapters 3, 10 and 11) and arterial vessel wall (chapter 9). If expressed per mg microsomal protein, the vitamin K-dependent carboxylase activities in skin and venes are about 20% and those in brain and muscle are less than 10% from that in liver, while other non-hepatic vitamin K-dependent carboxylase activities ranged between 4 and 30% from the hepatic activity. Skin tissue from nude mice was separated into epidermal and dermal tissue. In both tissues, vitamin K-dependent carboxylase activity was detected (chapter 2). The presence of DTT-dependent K reductase and K epoxide reductase in non-hepatic tissues (chapters 2, 3 and 11) could also be demonstrated. This indicates that also non-hepatic tissues contain an enzymatic mechanism to recycle vitamin K.

From the present data it cannot be excluded that at least some enzymatic activity, measured in the various tissues, originated from the vitamin K-dependent enzymes present in vessel wall. To overcome the drawback of a mixed population of cells (which is always the case if whole tissue homogenates are used) we have investigated the presence of vitamin K-dependent carboxylase in small quantities of cultured or isolated cells. For this purpose the carboxylation assay was adapted and using this modified assay, vitamin K-dependent carboxylase activity could be demonstrated in hepatocytes, renal tubular cells, osteoblasts, macrophages,

endothelial cells, smooth muscle cells and in the investigated tumor cells: rat osteosarcoma, human colon carcinoma and mouse melanoma (chapters 8 and 9). In all these cells, reductase activities could also be demonstrated. As compared to the normal cells, the tumor cells showed a remarkably low vitamin K epoxide reductase activity.

The preparation of a substrate for carboxylase, a bone-Gla-protein which was isolated from bovine bone, is described in chapter 4. In its decarboxylated form, this protein appeared to be a good substrate for hepatic and non-hepatic vitamin K-dependent carboxylase. In bovine liver carboxylase, the apparent K_m for decarboxylated osteocalcin was about 200-fold lower than that for F L E E L.

Another Gla-containing protein was isolated from human spermatozoa: the sperm Gla-protein (chapter 5). The amino acid composition was established and the molecular weight was found to be about 30000 D. After thermal decarboxylation, this protein also appeared to be a good substrate for carboxylase from various tissues. The function of the sperm-Gla-protein is still unknown.

The results in chapter 6 strongly suggested that vitamin K-dependent carboxylases originating from bovine liver, kidney, lung and testis, may belong to a family of isoenzymes. This conclusion was derived from kinetic studies using 4 different substrates, including decarboxylated osteocalcin and decarboxylated sperm-Gla-protein. From these studies it was clear that the substrate specificities of the various carboxylases are different.

It was also investigated if different types of carboxylase might occur in one type of tissue. For this purpose we isolated from anticoagulated bovine liver, carboxylase enzymes bound to prothrombin precursors as well as carboxylase enzymes bound to precursors of factor X. The technique of the separation of the two enzyme-substrate complexes is based on the observation that in microsomes from warfarin treated animals the carboxylase enzyme appeared to be firmly bound to its endogenous substrate. In liver this endogenous substrate is mainly formed by precursors of the coagulation factors X and II. Using Sepharose-linked antibodies against native coagulation factor X and prothrombin, the precursor-carboxylase complex could be extracted from the microsomal preparation. The resulting Sepharose-bound carboxylase complexes are designed as SPC-X and SPC-II respectively and both showed carboxylase activity. A number of exogenous

substrates for carboxylase has been tested but no differences were observed in the apparent K_m values using either SPC-X or SPC-II. These experiments were hampered by the fact that only small substrates could be used. SPC-X and SPC-II are both complexed to their endogenous substrate. Probably because of sterical hindrance, large (protein) substrates are not carboxylated by these enzymes, but exclusively by substrate-free carboxylase.

In most studies, tissues of animal origin were used. Evidently, the results obtained with experimental animals should be verified in man. Therefore we have investigated in various human tissues, the presence of vitamin K-dependent enzymes and some of their properties (chapters 2, 9 and 10). In general, the results obtained were comparable with those found in animal studies. If different exogenous substrates were used, the relative carboxylation rates varied, which suggest that also in man the carboxylase from different tissues may differ in their substrate specificity (chapter 10). In vitro, the DTT-dependent K reductase activities were inhibited by low warfarin concentrations.

The effects of 4-hydroxycoumarin drugs on non-hepatic vitamin K-dependent enzymes have been studied. In rats the effects of a chronic low-dose treatment with three types of 4-hydroxycoumarins (acenocoumarol, phenprocoumon and warfarin) on hepatic and non-hepatic vitamin K-dependent systems were compared (chapter 3). Although the plasma concentrations of the three coumarins differed largely, these differences were not reflected in the microsomal coumarin contents. Non-hepatic microsomes contained per mg protein less than 20% of the coumarin found in liver microsomes. No substantial differences were observed between the effects of the three anticoagulants. Non-hepatic vitamin K epoxide reductase activities (kidney, lung, spleen, brain and testis) were between 45 and 65% of normal, while the hepatic activity was diminished to about 35% after the in vivo 4-hydroxycoumarin treatments. In liver microsomes, a 15 fold accumulation of non-carboxylated precursor proteins was detected; though less pronounced a similar effect was found in the non-hepatic microsomes. An increase of the non-carboxylated precursor proteins was also found in dermis and epidermis after warfarin treatment of nude mice (chapter 2). Moreover, if cells were cultured in the presence of warfarin, the amount of endogenous substrate increased while the reductase activities decreased.

The in vivo effects of 4-hydroxycoumarin drugs on human vitamin K-dependent systems in liver and in non-hepatic tissues are reported in chapter 11. In this study, large individual variations were observed between the enzymatic activities in corresponding tissues from normal donors. Vitamin K-dependent enzymatic activities were also determined in tissues obtained from donors, who had been treated with 4-hydroxycoumarin drugs for at least 6 months immediately before their decease. In their hepatic microsomes, the K epoxide reductase activity and the DTT-dependent K reductase activity were diminished to about 35% and 20% of the corresponding normal values. The effects of the anticoagulant therapy on non-hepatic reactions were less pronounced. The K epoxide reductase activities in the anticoagulant treated tissues showed a consistent tendency to be lower than those in the non-treated tissues. However, possibly due to the large interindividual variations, this decrease did not reach the level of significance. No differences were found between the DTT-dependent K reductase activities in the corresponding tissues from normal and anticoagulated donors. In all tissues, the amounts of non-carboxylated precursor proteins were slightly or not increased, while no differences were observed in vitamin K-dependent carboxylase activities after 4-hydroxycoumarin treatment.

Further research in this field will be required to estimate the importance of the effects of 4-hydroxycoumarin drugs on the synthesis of non-hepatic Gla-containing proteins.

SAMENVATTING

Vitamin K is betrokken bij de posttranslationele carboxylering van specifieke peptide-gebonden glutaminezuurresiduen (Glu) tot 4-carboxyglutaminezuurresiduen (Gla). Deze reactie wordt gekatalyseerd door vitamine K-afhankelijk carboxylase, een enzym dat gelokaliseerd is in de binnenmembraan van het endoplasmatisch reticulum. De hydroquinon vorm van vitamin K is de actieve cofactor in deze carboxyleringsreactie. Algemeen wordt aangenomen dat vitamin K in quinon vorm wordt geabsorbeerd in de darmen en getransporteerd naar de lever. In vitro kan vitamine K door reductases tot vitamine K hydroquinon gereduceerd worden. Eén van deze reductases wordt in vitro door dithiothreitol (DTT) gestimuleerd; de andere worden door NADH gestimuleerd. Tijdens de carboxylering wordt vitamin K hydroquinon omgezet in vitamin K 2,3 epoxide; dit wordt vervolgens door DTT-afhankelijk vitamin K epoxide reductase gereduceerd tot vitamine K quinon. De fysiologische tegenhanger van DTT in genoemde reactie is niet bekend. De DTT-afhankelijke K en K epoxide reductases en de vitamine K-afhankelijk carboxylase komen allen voor in de micosomale fraktie van weefselhomogenaten. 4-Hydroxycoumarine derivaten zoals warfarine, remmen de DTT-afhankelijke reductases. Deze remming resulteert in een drastisch tekort aan vitamine K hydroquinon en als gevolg daarvan neemt de carboxyleringssnelheid af.

In vivo leidt dit tot het verschijnen van descaboxyfactoren (PIVKA's) in bloed. Deze eiwitten bezitten geen Ca^{2+} -bindende Gla-residuen, zoals de normale stollingsfactoren hebben. De descaboxystollingsfactoren hebben dan ook geen biologische aktiviteit. De literatuur betreffende vitamine K-afhankelijke reacties is samengevat in hoofdstuk 1.

Gedurende vele jaren is verondersteld dat de vitamine K-afhankelijke carboxylering uitsluitend plaats vindt in de lever. Daar is de reactie noodzakelijk voor de vorming van de biologisch actieve stollingsfactoren II, VII, IX en X en de antistollingswiwitten proteïne C en proteïne S. 4-Hydroxycoumarine bevattende medicijnen worden veel gebruikt om ongewenste bloedstolling te voorkomen. Tegenwoordig weten we dat vitamine K afhankelijke carboxylering ook plaatsvindt in niet-hepatische weefsels. In het in dit proefschrift beschreven onderzoek stelden we de aanwezigheid van vitamine K-afhankelijke carboxylase activiteit vast in diverse niet-hepatische weefsels en wel in huid (hoofdstuk 2), in hersenen (hoofdstuk 3), in spierweefsel (hoofdstukken 3 en 11) en in venen (hoofdstuk 9). Tevens bevestigden we de aanwezigheid van vitamine K afhankelijke carboxylase activiteit in nier-, testis-, long-, milt- en hartweefsel (hoofdstukken 3, 10 en 11) en in de arteriële vaatwand (hoofdstuk 9). Uitgedrukt per mg microsomaal eiwit bevatten huid en venen ongeveer 20% van de hepatische vitamine K-afhankelijke carboxylase-activiteit; voor hersenen en spierweefsel is dit percentage minder dan 10%, terwijl deze activiteit in andere niet-hepatische weefsels varieerden van 4 tot 30% van de hepatische enzymactiviteit. Huidweefsel van naakte muizen werd gescheiden in dermaal en epidermaal weefsel. In beide typen weefsel kon vitamine K-afhankelijke carboxylase activiteit aangetoond worden (hoofdstuk 2). In niet-hepatische weefsels werden naast de vitamine K-afhankelijke carboxylase activiteit ook DTT-afhankelijke K reductase- en K epoxide reductase activiteit aangetoond (hoofdstukken 2, 3 en 11). Deze resultaten duiden erop, dat ook niet-hepatische weefsels een enzymatisch mechanisme bevatten dat hergebruik van vitamine K mogelijk maakt.

Op grond van de huidige gegevens kan niet uitgesloten worden dat wellicht een deel van de enzymatische activiteit welke wordt gemeten in diverse weefsels, afkomstig is van de in de vaatwand gelokaliseerde vitamine K-afhankelijke enzymen. Wanneer weefselhomogenaten worden gebruikt, is er altijd sprake van een gemengde populatie van cellen. Om dit probleem te omzeilen is de aanwezigheid van vitamine K-afhankelijk carboxylase onderzocht in kleine hoeveelheden gekweekte of geïsoleerde cellen. Hiertoe werden de condities waaronder de carboxyleringstest werd uitgevoerd, aangepast. Op deze wijze kon vitamine K-afhankelijke carboxylase-activiteit aangetoond worden in hepatocyten, niertubulus cellen, osteo-

blasten, macrofagen, endotheelcellen, gladde spiercellen en in de onderzochte tumorcellen: ratte-osteosarcoom-, humaan colon carcinoom- en muize-melanoomcellen (hoofdstuk 8 en 9). In alle genoemde cellen werd tevens reductase aktiviteit aangetoond. De gemeten K epoxide reductase aktiviteit was in tumorcellen aanzienlijk lager dan in normale cellen. De bereiding van een substraat voor carboxylase, geïsoleerd uit runderbotten, het Gla-bevattende eiwit osteocalcine, is beschreven in hoofdstuk 4. In gedecarboxyleerde vorm bleek dit eiwit een goed substraat voor zowel hepatisch als niet-hepatisch vitamine K-afhankelijk carboxylase. De gemeten K_m -waarde van gedecarboxyleerd osteocalcine voor runderlevercarboxylase was en faktor 200 lager dan de gemeten K_m waarden van het synthetisch peptide substraat F L E E L voor dit carboxylase.

Een ander Gla-bevattend eiwit werd uit humaan sperma geïsoleerd en aangeduid als sperma-Gla-eiwit (hoofdstuk 5). Hiervan werd de aminozuursamenstelling vastgesteld; het molecuulgewicht werd bepaald op ongeveer 30000. Na decarboxylering bleek dit eiwit eveneens een goed substraat voor carboxylase in diverse weefsels. De functie van het sperma-Gla-eiwit is niet bekend.

In hoofdstuk 6 staat beschreven dat de carboxylases aanwezig in runderlever, -nier, -long en -testis waarschijnlijk isoenzymen zijn. Uit kinetische studies met 4 verschillende substraten, inclusief gedecarboxyleerd osteocalcine en gedecarboxyleerd sperma-Gla-eiwit, werd geconcludeerd dat de diverse carboxylases een verschillende substraatspecificiteit bezitten. Tevens werd onderzocht of één type weefsel verschillende typen carboxylases bevat. Hiertoe isoleerden we uit lever van geantistolde runderen, carboxylase-enzymen gebonden aan precursors van prothrombine, én carboxylase enzymen gebonden aan precursors van faktor X. De techniek om deze enzym-substraat complexen te scheiden is gebaseerd op de waarneming dat, in microsomen van met warfarine behandelde dieren, het carboxylase enzym stevig gebonden zit aan endogeen substraat. In lever bestaat dit endogeen substraat voornamelijk uit precursors van de stollingsfactoren X en II. Door gebruik te maken van de Sepharose-gebonden antistoffen tegen de natieve stolfactoren X en II, werd het precursor-carboxylase-complex uit de microsomen geëxtraheerd. De zo ontstane Sepharose-gebonden carboxylase-complexen worden aangeduid met SPC-X en SPC-II. Beide complexen bezitten carboxylase aktiviteit. Er werden een aantal exogene substraten voor

carboxylase getest. Er konden echter geen verschillen aangetoond worden in de gemeten K_m waarden. In deze experimenten bleken alleen kleine substraten gecarboxyleerd te worden. In SPC-X en SPC-II zijn de carboxylases gecomplexxerd met endogeen substraat. Grotere substraten worden, wellicht door sterische hindering, niet gecarboxyleerd door gecomplexeerde enzymen, maar uitsluitend door substraat-vrij carboxylase.

In vrijwel alle studies zijn weefsels van dierlijke oorsprong gebruikt. Het zal duidelijk zijn, dat de resultaten verkregen uit dierexperimentele studies geverifieerd dienen te worden in de humane situatie. Om een eerste aanzet hiertoe te geven zijn in verschillende humane weefsels de aanwezigheid van vitamine K-afhankelijke enzymen en enkele eigenschappen daarvan onderzocht (hoofdstuk 10). In grote lijnen komen de resultaten overeen met de gegevens uit dierexperimentele studies. Wanneer verschillende exogene substraten werden gebruikt, bleken de relatieve carboxyleringssnelheden te variëren, hetgeen suggereert dat er ook bij de mens een verschil in substraatspecificiteit kan bestaan tussen de verschillende weefselcarboxylases. In vitro werden de DTT-afhankelijke K reductase activiteiten geremd door lage warfarine concentraties.

Het effect van 4-hydroxycoumarine derivaten op niet-hepatische vitamine K-afhankelijke enzymen werd bestudeerd. In ratten werden de effecten van een week durende behandeling met lage doses 4-hydroxycoumarinen (acenocoumarol, phenprocoumon en warfarine) op hepatische en niet-hepatische vitamine K-afhankelijke enzymen vergeleken (hoofdstuk 3). Ofschoon de plasmaconcentraties van de 3 gebruikte coumarine derivaten aanzienlijk verschilden, werden deze verschillen niet teruggevonden in de microsomale coumarine concentraties. Niet-hepatische microsomen bevatten aan coumarinen per mg eiwit minder dan 20% van de hoeveelheid in levermicrosomen. Er werden geen essentiële verschillen in de effecten van de drie anticoagulantia onderling gezien. Na in vivo behandeling met 4-hydroxycoumarine waren de vitamine K epoxide reductase activiteiten in de niet-hepatische weefsels (nier, long, milt, hersenen en testis) gedaald tot waarden tussen 45% en 65% van niet geantistolde controles, terwijl de activiteit in lever was teruggelopen tot ongeveer 35% van normaal. In lever microsomen nam de hoeveelheid niet-gecarboxyleerd precursor eiwit na behandeling met 4-hydroxycoumarines toe met een faktor 15; in de niet-hepatische weefsels werd eveneens een ophoping van endogeen substraat

gevonden, maar deze toename was minder groot. Ook in de dermis en epidermis van naakte muizen werd een verhoogde hoeveelheid niet-gecarboxyleerd precursor eiwit aangetoond nadat deze met warfarine waren behandeld (hoofdstuk 2). Ook wanneer cellen werden gekweekt in aanwezigheid van warfarine, nam de hoeveelheid endogeen substraat toe, terwijl de reductase aktiviteit afnam.

In Hoofdstuk 11 is aandacht besteed aan de in vivo effecten van 4-hydroxycoumarine derivaten op humane vitamine K-afhankelijke enzymen in de lever en in niet-hepatische weefsels. In deze studie werden grote individuele verschillen gesignaleerd in enzymatische aktiviteiten in overeenkomstige weefsels van normale donoren. Vitamine K-afhankelijke enzym aktiviteiten werden ook bepaald in weefsels afkomstig van donoren, die gedurende 6 of meer maanden voorafgaande aan hun overlijden met 4-hydroxycoumarine derivaten waren behandeld. In het leverweefsel van deze donoren waren de K epoxide reductase aktiviteit en de DTT-afhankelijke K reductase aktiviteit gedaald tot ca. 35%, resp. 20% van de normaalwaarden. De effecten van antistollingstherapie op niet-hepatische vitamine K-afhankelijke reakties zijn minder geprononceerd. De K epoxide reductase aktiviteit in weefsels van donoren onder antistollingstherapie lijken in het algemeen verlaagd te zijn ten opzichte van de overeenkomstige aktiviteiten in normale weefsels. Dit verschil was echter niet significant, hetgeen wellicht een gevolg is van de grote interindividuele spreiding. Er werden geen verschillen gezien tussen normale en geantistolde weefsels in de DTT-afhankelijke K reductase aktiviteit, noch in de vitamine KH_2 afhankelijke carboxylase aktiviteit. De hoeveelheid endogeen substraat na antistollingsbehandeling was slechts weinig toegenomen.

Verder onderzoek op dit gebied zal nodig zijn om meer inzicht te krijgen in de effecten die 4-hydroxycoumarine derivaten hebben op de synthese van niet-hepatische Gla-bevattende eiwitten.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 7 juli 1950 te Amsterdam. Zij behaalde het diploma gymnasium β in 1968 aan het Pius X Lyceum te Amsterdam. Daarna trad zij in dienst bij het Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandse Rode Kruis, eveneens in Amsterdam, alwaar zij tot augustus 1974 als analist werkzaam was. In de avonduren studeerde zij achtereenvolgens voor het HBO-B diploma biochemie, dat zij in 1971 verkreeg, en voor het MO-A diploma in de scheikunde en natuurkunde, welke opleiding zij in 1975 met succes afrondde. Daarna studeerde zij aan de Universiteit van Amsterdam en deed het kandidaats-examen in de richting scheikunde in 1976. Het doctoraalexamen werd in 1979 afgelegd met als hoofdvak biochemie en als bijvakken neurofysiologie en voedingsleer. Tevens werd een le graads onderwijsbevoegdheid in de richting scheikunde behaald. In de periode augustus 1974 - juli 1975 en de periode augustus 1978 - november 1982 was zij als docent scheikunde, natuurkunde, klinische chemie en biochemie werkzaam op diverse scholen in het voortgezet onderwijs en hoger beroepsonderwijs. Vanaf december 1982 tot september 1986 was zij als wetenschappelijk medewerker verbonden aan de capaciteitsgroep Biochemie van de Rijksuniversiteit Limburg te Maastricht. Momenteel is zij werkzaam bij de Haagse Hogeschool voor Beroepsonderwijs als docent chemie en als coordinator van de laboratorium-opleidingen in de medisch-chemische richting.

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