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REVIEW ARTICLE

γ-Carboxyglutamate-containing proteins and the vitamin K-dependent carboxylase

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INTRODUCTION

Vitamin K functions as a cofactor during the post-translational modification of proteins. The reaction in question is the carboxylation of glutamate (Glu) residues into γ-carboxyglutamate (Gla) and it is depicted in Fig. 1. Because Glu is only a weak Ca^{2+} chelator and Gla a much stronger one, the vitamin K-dependent step substantially increases the Ca^{2+}-binding capacity of a protein. The discovery of vitamin K by Henrik Dam in 1935 [1] and the identification of Gla in the early 1970s [2–4] have been reviewed elsewhere and will not be detailed in this paper. Here we intend to summarize the recent advances in vitamin K research.

During the last decade our knowledge concerning vitamin K-dependent processes has been substantially increased, and laboratory techniques have been developed which enable a thorough investigation of vitamin K action on a molecular level. This will become clear if one realizes that: (*a*) the enzymic reaction in which vitamin K serves as a coenzyme is precisely known; (*b*) carboxylating enzyme systems have been developed *in vitro* giving a linear dose–response relation for vitamin K; (*c*) methods are available with which the three well-known metabolites of vitamin K can be readily quantified in tissues, biological fluids and reaction mixtures *in vitro*; (*d*) the products of vitamin K action (protein-bound Gla residues) are easily detectable, even in preparations which are less than 5% pure. On these points vitamin K compares favourably with most other vitamins.

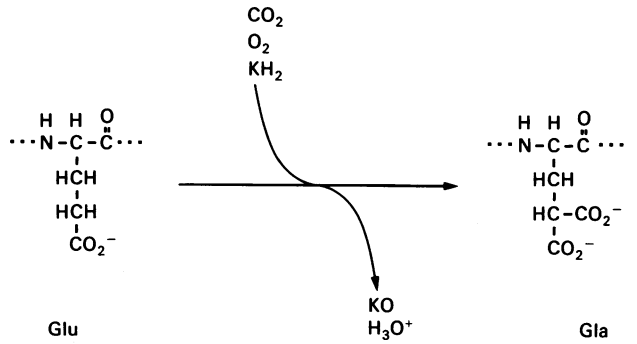

Fig. 1. Conversion of peptide-bound glutamic acid into γ-carboxyglutamic acid

The enzyme involved is called carboxylase. During the carboxylation reaction vitamin K hydroquinone (KH_2) is converted to vitamin K epoxide (KO).

DETECTION OF Gla RESIDUES IN PROTEINS

Historically, the identification of Gla was hampered by its decarboxylation under the acid conditions generally applied for protein hydrolysis and subsequent amino acid analysis. After the discovery of Gla, methods were developed to protect the structure of its malonic acid moiety by diborane reduction into dihydroxyleucine [5,6]. The latter product is stable during acid hydrolysis, but on a standard amino acid analyser it is not well separated from threonine and aspartic acid. Therefore [^{3}H]diborane was used for the reduction step, resulting in ^{3}H-labelled alcohols, which could be identified in the column effluent by liquid-scintillation counting.

Later on it was realized that Gla is stable under alkaline conditions and methods were developed for the detection of Gla in alkaline hydrolysates of proteins. The procedure described by Hauschka *et al.* [7] is a modified standard amino acid analysis with ninhydrin detection and is accurate and sensitive but time-consuming. Other methods for Gla detection include a direct colorimetric assay [8], gas chromatography [9] and mass spectroscopy [10]. Extremely simple and reproducible techniques were developed with the aid of h.p.l.c., first including post-column derivatization [11], but afterwards also using precolumn derivatization with either *o*-phthalaldehyde [12] or phenylisothiocyanate [13] followed by either anion exchange [12] or reversed phase chromatography [13,14]. In our laboratory the method described by Kuwada & Katayama [12] is used and has proven to be reliable. An example will be given below (Fig. 2). In all procedures mentioned above it is important that the identity of the material eluting at the Gla position is verified by an additional method. Parallel to the alkaline hydrolysate, for instance, an acid hydrolysate may be prepared in which Gla is transformed into Glu. Consequently the peak eluting at the position of Gla should be absent from these samples. Its shift to the Glu position will generally be masked by the large amount of original Glu unless a radiolabel has been specifically incorporated into the Gla residues [15].

The usefulness of having a sensitive technique available for Gla detection may be demonstrated by investigations in which the bone Gla-protein osteocalcin was extracted from fossil bones. After it was reported that detectable amounts of osteocalcin could be isolated from moa bones approx. 7000 years old [16], Ulrich *et al.* [17] reported the preservation of osteocalcin in fossil bovide bones ranging from 12000 to 13 million years old. The amounts of osteocalcin that could be recovered from the ancient bones varied considerably, probably because of

Abbreviations used: Gla, γ-carboxyglutamate; MGP, matrix Gla-protein; BGP, bone Gla-protein; PGP, plaque Gla-protein.

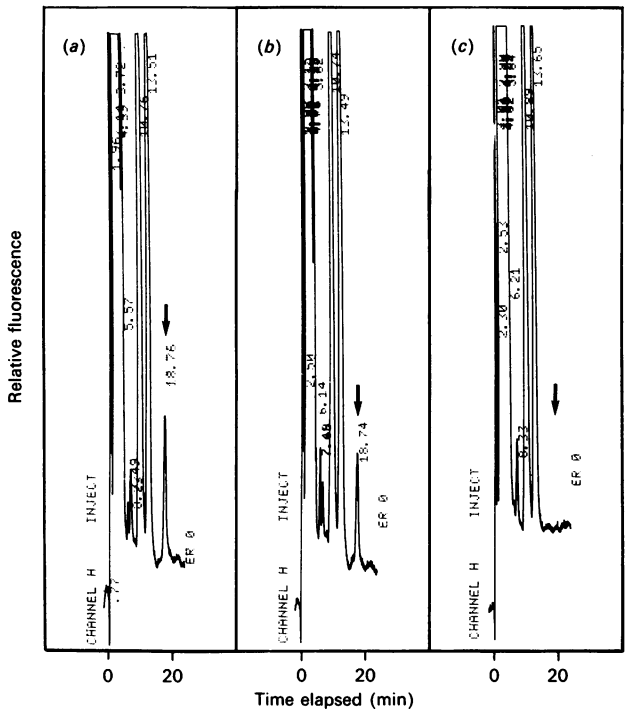

Fig. 2. Detection of Gla in bone extracts

The bone samples were powdered mechanically and extracted with 1 M-EDTA [17]. Non-dissolved material was discarded and the dissolved proteins were subjected to alkaline (*a* and *b*) or acid (*c*) hydrolysis. Gla analysis was performed as in [12]. The samples were from recent bovine bone (*a*) and from *Caprotragoides sehlini* with an estimated age of 13 million years (*b* and *c*). Gla elutes at 18.7 min (arrows) and Glu at 10.8 min.

the widely different preservation conditions the bones had experienced (humidity, pH), but in most cases 1–10 g of the bone samples contained sufficient amounts of osteocalcin to permit its detection by techniques based on antigen–antibody recognition (radioimmunoassay and e.l.i.s.a.). To exclude the possibility that unknown processes during the long geological history of the material would give rise to false-positive results in these assays, Gla analysis was performed as a second, independent test demonstrating the authenticity of fossil osteocalcin. An example of the results obtained is shown in Fig. 2, in which we compare the Gla analysis in crude extracts from recent bovine bone (*a*), and fossil bovide bone after alkaline (*b*) and acid (*c*) hydrolysis. The osteocalcin from fossil bones could be partly purified by h.p.l.c. and coeluted with recent osteocalcin during anion exchange and gel permeation chromatography. If sufficient amounts of osteocalcin can be extracted from fossil bones and adequately purified, attempts could be made to determine its amino acid sequence. Such sequence data could offer a new approach to the phylogenetic study of extinct taxa.

OCCURRENCE, FUNCTION AND ORIGIN OF Gla-CONTAINING PROTEINS

Vitamin K-dependent carboxylase has been found in such diverse tissues as liver, testis, skin, lung and kidney [18–21]. In fact, the only tissues in which no carboxylase could be identified thus far are brain and tendon. Because the tissues mentioned above each contain various types of cells, methods were developed for the detection of carboxylase on a micro-scale. With these techniques the enzyme could be detected in preparations containing as few as 10^6 isolated or cultured cells such as hepatocytes, renal tubule cells, osteoblasts, fibroblasts, endothelial cells as well as in various cell lines: osteosarcoma, hepatoma, melanoma and colon carcinoma [22,23]. Because carboxylase is present in almost every tissue investigated, one would expect to find numerous Gla-containing proteins produced by these systems. This turned out to be not the case, however. Instead the number of well-characterized Gla-proteins has remained surprisingly low thus far. On the basis of their origin they may be classified as follows.

(*a*) The Gla-proteins mainly found in blood plasma. The unusual amino acid Gla was discovered in prothrombin [2–4], one of the blood coagulation factors. At that time the high degree of sequence similarity between prothrombin and the coagulation factors VII, IX and X had already been established. As a logical consequence the latter proteins were also subjected to Gla analysis and all appeared to contain 10–12 Gla residues per molecule [24,25]. A few years later three new plasma proteins were discovered, all with a substantial sequence similarity to prothrombin [26–28]. This similarity included the number and position of the Gla residues. The proteins C and S are now known to play an important role in the regulation of the blood coagulation process. Their function is to inactivate the activated coagulation factors V and VIII by limited proteolytic degradation [29,30]. The function of protein Z is presently unknown.

(*b*) Gla-proteins occurring in calcified tissues. Gla-containing proteins have been found in various calcified tissues such as bone [31,32], dentine [33], renal stones [34], hardened atherosclerotic plaques [35] and coral [36]. Because of the very high affinity of Gla-proteins for insoluble calcium salts [37], it is not necessarily true that these proteins are synthesized at or near the place where they are found: the protein found in calcified atheromatous plaques, for instance, may be either produced by the vessel wall or adsorbed from the blood stream. Similar doubts exist with respect to the proteins present in renal stones and coral, and definite conclusions with respect to the nature and origin of these proteins await further research and the elucidation of their primary structure. On the other hand, the two Gla-proteins found in bone have been characterized to a sufficient extent. They are called matrix Gla-protein (MGP) and osteocalcin or bone Gla-protein (BGP). MGP was initially discovered in bone [38], but its mRNA has now been detected in tissues varying as much as lung, kidney and vessel wall [39]. Hence we must assume that MGP is expressed in all these tissues and probably in others as well. Osteocalcin is the most abundant Gla-protein in man. It was detected about 15 years ago in bone [31,32] and appeared to be identical with the Gla-protein isolated from dentine. The synthesis of osteocalcin occurs exclusively in osteoblasts and odontoblasts and after its secretion most of the protein is bound to the hydroxyapatite matrix of the bone. A small fraction, however, is set free in the blood stream, where it may be detected by radioimmunoassay. It has been suggested that the serum osteocalcin level may be used as a marker for osteoblast activity and several bone diseases [40–42]. Although the two bone Gla-proteins were discovered many years ago,

their function is presently not well understood. For a number of years it was thought that the coumarin-induced bone abnormalities, observed in human fetuses [43] and in young rats [44], were due to incomplete carboxylation of osteocalcin, but after the discovery of MGP the defects may be related to the latter protein as well. Osteocalcin has a very strong inhibitory effect on the precipitation [45] and crystallization [46] of various calcium salts from supersaturated solutions *in vitro*, but in the same papers it was shown that this is a general property of most Gla-proteins including the blood coagulation factors. It is doubtful, therefore, whether inhibition of mineralization is a primary function of osteocalcin and/or MGP *in vivo*. Others have postulated that osteocalcin may function as a chemoattractant on the bone resorbing cells (osteoclasts), thereby promoting not only recruitment, but also the formation and activation of the osteoclasts and/or osteoclast progenitor cells [47,48].

(*c*) Other Gla-containing proteins have been found in spermatozoa [49], urine [50,51], lung surfactant [52] and snake [53,54] and snail [55] venoms. Except the latter one, which is a highly potent neurotoxin [56] produced by the Conidae family, these proteins have not been characterized to the level of amino acid sequence, which hampers investigations with respect to their possible relations to or sequence similarity with other Gla-proteins.

VITAMIN K-DEPENDENT γ-GLUTAMYLCARBOXYLASE

The vitamin K-dependent carboxylase has all characteristics of an integral membrane protein, and it is concentrated at the luminal side of the rough endoplasmic reticulum of various cells [57]. After homogenization of these cells or tissues, the enzyme is recovered in the microsomal fraction, from which it can be solubilized by various detergents [58,59]. Discoveries which may be regarded as real landmarks in vitamin K research were: (*a*) the observation that $NaH^{14}CO_3$ may be used as a source of CO_2, so that the progress of the carboxylation reaction can be measured by counting the incorporated label [60], and (*b*) the synthesis of short, Glu-containing substrates, which may be carboxylated *in vitro* [61,62]. The impact of these discoveries has recently been reviewed elsewhere [63–65]. In this section I will discuss: (*a*) the purification of carboxylase, (*b*) the substrate selection of carboxylase, and (*c*) the mechanism of the carboxylation reaction.

Purification

The purification of carboxylase has proven to be extremely difficult. Two strategies have been employed, both leading to a purification of about 100-fold. De Metz *et al.* [66] used the livers of warfarin-treated cows as a starting material. In these livers non-carboxylated precursors of blood coagulation factors (mainly factor X) had accumulated and remained complexed to carboxylase. With the aid of Sepharose-bound antibodies against normal plasma factor X these enzyme–substrate complexes could be extracted from detergent-solubilized microsomes. The resulting product was called solid-phase carboxylase, and upon adding vitamin K the enzyme was able to carboxylate not only the complexed factor X-precursors, but also synthetic substrates like the pentapeptide FLEEL. Solid-phase carboxylase turned out to be extremely stable and was used, for instance, in studies demonstrating that phospholipids form an essential part of carboxylase [67]. A drawback of the system is that attempts to elute carboxylase from the Sepharose have not been successful. The fact that even after the carboxylation of all endogenous substrate the enzyme remains attached to its Gla-containing reaction product demonstrates that the mechanism by which the enzyme dissociates from its product is presently not well understood. Another purification method was developed, therefore, by Soute *et al.* [68], who used the livers from non-treated animals for the preparation of salt-washed microsomes. Carboxylase thus obtained is substrate-free, and it was solubilized by differential detergent extraction. Partial purification was accomplished by $(NH_4)_2SO_4$ precipitation and gel permeation chromatography. The resulting product turned out to be stable during freezing at −80 °C for more than 2 months and during storage on ice for at least 2 days. When analysed on SDS/polyacrylamide gels, the product of both purification schemes mentioned above still contained at least seven different proteins, none of which could be identified as carboxylase. Obviously one might expect the enzyme to consist of several subunits, but in that case the subunits should be present in nearly equimolar amounts, which was not the case. Recently a new approach has been reported resulting in the purification of carboxylase to homogeneity [69]. The procedure is based on the affinity chromatography of carboxylase using a Sepharose-bound peptide containing the complete pro-sequence of prothrombin. It is to be expected that this approach will lead to the breakthrough for which enzymologists working on carboxylase have been waiting and that in the next 1 or 2 years sufficient amounts of homogenous carboxylase will become available to allow a thorough investigation of this enzyme.

Substrate recognition

Concerning the mechanism of substrate selection by carboxylase, a lot of information has recently become available. All Gla-containing proteins are secretory proteins, which are synthesized in a precursor form that contains the signal sequence (required for translocation through the rough endoplasmic reticulum membrane), a propeptide and the mature protein. After translocation, the signal peptide is promptly cleaved by a signal peptidase on the luminal side of the rough endoplasmic reticulum. The propeptide, however, is removed at a later stage of post-translational modification, probably not until the precursor protein reaches the Golgi body [63]. If properly aligned, it becomes clear that some highly conserved amino acids are present in all mammalian Gla-containing proteins characterized today. The common residues are found both in the propeptide as well as in the *N*-terminal part of the mature protein (Fig. 3). In their prosequence all proteins contain Arg at position −1, and it marks the cleavage site of the propeptide. Yet it appears that also an Arg-rich region at positions −2 to −5 is required for proper cleavage of the propeptide: in matrix Gla-protein, which does contain Arg at −1, but which lacks Arg at positions −2 to −5, the propeptide is not removed but forms an integral part of the mature protein. It is remarkable that at both sides of this 'internal' prosequence carboxylation of Gla-residues may occur [85]. Additional evidence demon-

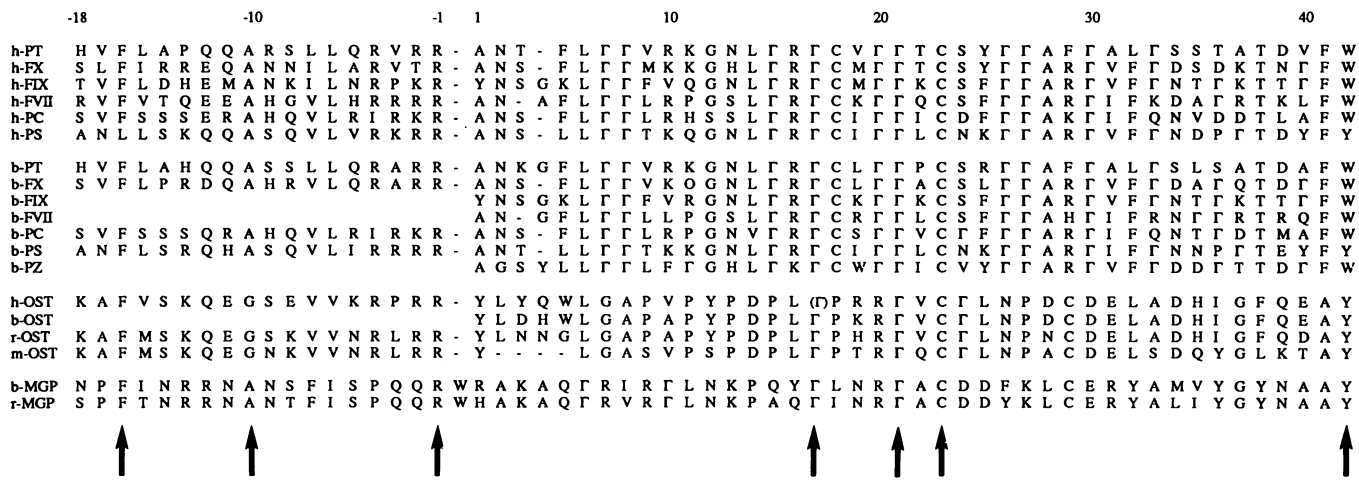

Fig. 3. One-letter code notation of the amino acid sequences in the propeptides (if known) and Gla domains of the mammalian vitamin K-dependent proteins characterized as yet

The sequences are aligned to give maximal similarity and number 1 corresponds to the first residue of mature prothrombin. Γ stands for Gla and residue 17 in human osteocalcin is in parentheses because it is only partly carboxylated. Abbreviations are: h, human; b, bovine; r, rat; m, mouse; PT, prothrombin; FX, factor X; FIX, factor IX; FVII, factor VII; PC, protein C; PS, protein S; PZ, protein Z; OST, osteocalcin; MGP, matrix Gla-protein. The structures are from [28] and [70–85]. The arrows indicate the residues discussed in the text.

strating the importance of the Arg-rich sequence for cleavage of the propeptide is obtained from structural analysis of naturally occurring human mutant proteins with a defect in the propeptide region. It was shown, for instance, that propeptide cleavage had not taken place in circulating factor IX Cambridge (Arg-1 → Ser [86]) and neither in factor IX Oxford 3 (Arg-4 → Gln [87]). The fact that in addition these mutants are poorly carboxylated is presently not well understood and could not be supported by experiments *in vitro* [88]. A second common residue in the prosequence of all mammalian Gla-proteins except human protein S is Phe at position −16, whereas closely related amino acid residues are found at position −10 (Gly or Ala) and −6 (Leu, Ile or Val). Furthermore it is at least striking that in all cases three to five residues immediately preceding position −10 as well as the residues −9 and −8 have either polar or charged side chains. Strong evidence in favour of the importance of the prosequence for γ-carboxylation was provided by molecular biology. The cDNA coding for human factor IX [89,90] and human protein C [91] was expressed in heterologous mammalian cells, and product analysis showed a substantial degree of carboxylation in both recombinant proteins. With the aid of site-specific mutagenesis it was subsequently demonstrated that forms lacking either the complete propeptide (residues −18 to −1) or parts of it (e.g. residues −18 to −11) were not carboxylated [91,92]. Also point mutations at position −16 (Phe → Ala) or −10 (Ala → Glu) nearly eliminated carboxylation of factor IX [92]. Based upon this knowledge various interesting peptides were constructed and tested for their role in carboxylating systems *in vitro*. Knobloch & Suttie [93] and Ulrich *et al.* [94] demonstrated that synthetic peptides similar to the propeptides of various human blood coagulation factors stimulate the carboxylation of small peptide substrates without being covalently linked to these substrates. The stimulation was rather modest, however (about 3-fold). In their paper Ulrich *et al.* also claimed that the covalent attachment of the propeptide to a carboxylatable substrate is a critical requirement for efficient carboxylation: proPT28 (a synthetic peptide similar to the sequence −18 to +10 in descarboxyprothrombin) is carboxylated with a K_m which is at least three orders of magnitude lower than that of a combination of the peptides −18 to −1 and +1 to +10. Hence peptides containing both the prosequence and a 'substrate sequence' with one or more carboxylatable Glu residues form a new class of substrates, highly preferable to relatively simple peptides like FLEEL or FLEEV, which are all characterized by K_m values ranging from 3 to 7 mM. Whether the presence of the prosequence is the only requirement for a Glu-containing peptide to be carboxylated is presently a matter of debate. To explore this question peptides could be constructed containing both the prothrombin prosequence and Glu-containing sequences from proteins that are normally not carboxylated, and to test their carboxylation *in vitro*. This kind of experiment has not yet been reported, however.

When looking at the primary structure of the mature Gla-proteins (Fig. 3), another sequence similarity is evident. This sequence (Gla-Xaa-Xaa-Xaa-Gla-Xaa-Cys at the positions +17 to +23) was recently discovered by Price *et al.* [85] and it is found in all known mammalian Gla-proteins. A possible role for this sequence in substrate recognition by carboxylase may be deduced from experiments *in vitro*. During the last decade a number of peptide substrates for carboxylase have been synthesized corresponding to the Gla-containing sequences in a number of coagulation factors, except that Gla had been replaced by Glu. These peptides were all poor substrates for carboxylase, with K_m values in the millimolar range [95]. None of these substrates contained the invariant Glu-Xaa-Xaa-Xaa-Glu-Xaa-Cys sequence, however. On the other hand, decarboxylated osteocalcin [96] and descarboxyprothrombin fragment 13–29 [97] were excellent substrates, with K_m values comparable to those for proPT28. Decarboxylated osteocalcin as well as

descarboxyprothrombin fragment 13–29 lacks a prosequence, but does contain the sequence Glu-Xaa-Xaa-Xaa-Glu-Xaa-Cys. Therefore, the latter sequence possibly plays an additional role in the substrate selection by carboxylase. The direct involvement of a sequence in the Gla-domain itself in its recognition by carboxylase opens the possibility that in one polypeptide chain, carboxylatable sequences are found with different affinities for carboxylase. This might explain why in some proteins one of the Gla residues is undercarboxylated. An interesting point is that cysteine forms part of the invariant sequence and that carboxylase also has been shown to contain an essential thiol group [64]. It has been suggested therefore by Price [98] that the invariant cysteine residue could form a disulphide bond with the essential thiol of carboxylase. This covalent bond might form upon substrate recognition and would serve to anchor the substrate to the enzyme during the carboxylation reaction. After formation of all necessary Gla-residues, altered product binding would then allow disulphide exchange, to yield directly the correct disulphide in the protein product [98]. This hypothesis implies that during the carboxylation reaction disulphide bonds are rapidly formed and broken, and that disulphide bond formation in the nascent polypeptide chain is linked with the last stage of carboxylation. Interestingly, an enzyme called protein disulphide isomerase (EC 5.3.4.1) has been isolated and purified from liver and pancreas microsomes [99]. As was shown by Lambert & Freedman [100], this enzyme is able to catalyse: (*a*) the formation of protein disulphide bonds from reduced proteins, (*b*) the isomerization of disulphide bonds in 'incorrectly' disulphide-bonded proteins, and (*c*) the reduction of protein disulphide bonds by simple thiol compounds such as dithiothreitol (DTT). Hence protein disulphide isomerase accelerates the formation and rearrangement of disulphide bonds by facilitating the following equilibrium:

$$\text{DTT-(SH)}_2 + \text{polypeptide-S}_2 \rightleftarrows \text{DTT-S}_2 + \text{polypeptide-(SH)}_2$$

The position of this equilibrium depends on the dithiothreitol and polypeptide concentrations. The fact that the optimal conditions for the carboxylation *in vitro* of peptide-bound Gla residues include the presence of reduced dithiols [63,64] is consistent with the putative linkage between protein disulphide isomerase and carboxylase activity. The question why the carboxylation of a nascent protein stops at a distance of approx. 40 amino acid residues apart from the prosequence has not yet been answered. An aromatic amino acid residue (Trp or Tyr) is found at position 42 in all proteins, but its possible role in terminating the series of carboxylation events seems to be contradicted by the presence of a non-carboxylated Glu residue at position 31 in osteocalcin and in MGP.

Mechanism of carboxylation

With respect to the mechanism of the carboxylation reaction on a molecular level there is consensus about a number of general principles, which will be summarized below. Many details remain to be clarified, however. In normal liver three forms of vitamin K are found. The active cofactor for carboxylase is vitamin K hydroquinone (KH_2) and all data presently available indicate that the oxidation of KH_2 to vitamin K epoxide (KO) by molecular oxygen provides the energy required for the carboxylation of Glu residues [63–65]. KO formation and carboxylation are not strictly coupled, however. Under reaction conditions *in vitro* KO formation frequently exceeds carboxylation by 5–10-fold [101,102], whereas the epoxidation continues even in the absence of either CO_2 or carboxylatable substrate [102,103]. It has also been shown that CN^- strongly inhibits carboxylation *in vitro*, whereas simultaneously the rate of KO formation is stimulated more than 2-fold [101,102]. On the other hand, carboxylation without the concurrent oxidation of KH_2 has never been reported. Therefore it seems as if the oxidation of KH_2 to KO is a more or less autonomous process, and that the energy released may be used as needed for the carboxylation reaction. Recently it has been shown in a homogenous carboxylase preparation, however, that both activities are exerted by the same enzyme [69].

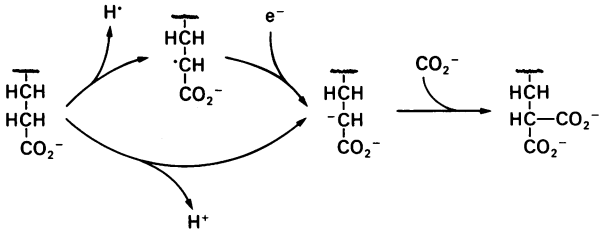

Fig. 4. Proposed pathways for vitamin K-dependent carboxylation of Glu into Gla

Details are discussed in the text.

In theory, the addition of a CO_2 to the γ-carbon in a Glu residue may occur either via an activation of CO_2 or via labilization of a γ-hydrogen. Current evidence strongly supports the latter hypothesis and most investigators have suggested that hydrogen removal precedes the addition of CO_2 [104–106]. This conclusion follows, for instance, from experiments showing a KH_2- or O_2-dependent exchange of ^3H from ^3H_2O into the γ-position of peptide-bound Glu residues [104]. Again there is no strict coupling between the two reactions involved in the carboxylation process: the extent of γ-carbon hydrogen abstraction exceeds by far the number of carboxylation events and even occurs in the absence of CO_2. Hence the carbon–hydrogen bond breaking cannot be the rate-limiting step of the reaction. Azerad *et al.* [107] and Dubois *et al.* [108] have demonstrated that the hydrogen abstraction is stereospecific and corresponds to the elimination of the pro-*S* hydrogen of glutamic acid. This justifies the expectation that CO_2 addition also occurs in a stereospecific way.

Both radical formation followed by a one-electron reduction and proton abstraction have been proposed as the pathway leading to a formal carbanion [103,105]. A carboxylation event would then be completed by the electrophilic attack of this carbanion by CO_2. The two possible pathways are depicted in Fig. 4. Further work is needed, preferably in much purer enzyme systems, to advance understanding of this complicated mechanism.

ENZYMES INVOLVED IN THE VITAMIN K CYCLE

Recycling of vitamin K

As was pointed out above, carboxylase activity seems to be related to the conversion of KH_2 to KO (epoxidase activity). Liver and other carboxylase-containing tissues

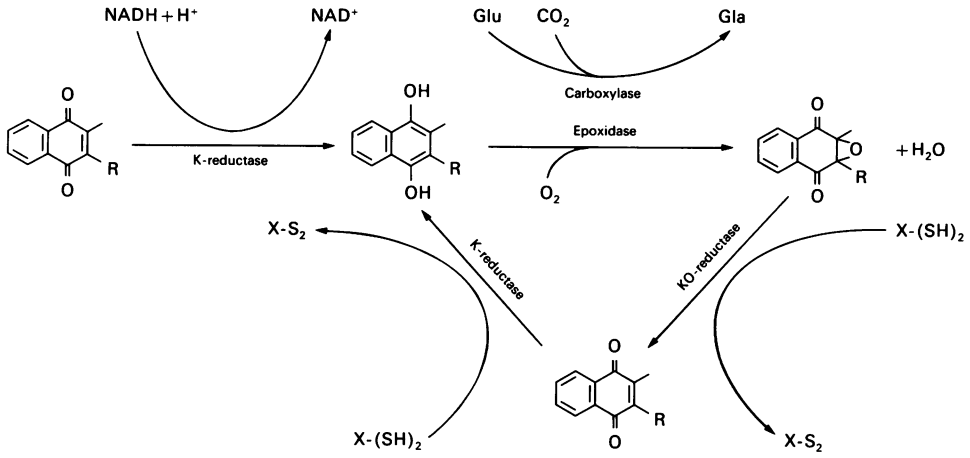

Fig. 5. The vitamin K cycle

$X\text{-}(SH)_2$ and $X\text{-}S_2$ stand for reduced and oxidized dithiols, respectively. Functional dithiols are dithiothreitol and thioredoxin. The NADH- and dithiol-dependent K-reductases are different enzymes. Only the dithiol-dependent reductases are inhibited by coumarin derivatives.

generally contain a number of other vitamin K-metabolizing enzymes, however [109,110]. Here we will shortly discuss the KO and K reductases. Together with the expoxidase these enzymes form the vitamin K cycle (Fig. 5). Strong arguments for this cycle being operational *in vivo* are: (1) in the absence of reductase inhibitors the carboxylation reaction *in vitro* may be initiated with either KH_2, K or KO [111]; in the presence of these inhibitors, however, only KH_2 is active as a coenzyme for carboxylase; (2) because in foods vitamin KH_2 (if present) is rapidly oxidized to vitamin K quinone by air, the dietary intake of the vitamin occurs solely as the quinone; (3) the low daily requirement of vitamin K as compared to Gla excretion requires that the vitamin is recycled several thousand times [112] before it is degraded via lactone and subsequent glucuronide formation [113]. It is striking that besides a number of K reductases only one KO reductase has been found in tissue homogenates. Therefore this enzyme is crucial for the recycling of KO [114,115]. KO reductase activity is dependent on the presence of dithiols and is extremely sensitive to the action of 4-hydroxycoumarin derivatives such as warfarin. Also K quinone may be reduced in a dithiol-dependent way [116]. It is not known whether the dithiol-dependent reduction of KO and K is exerted by one enzyme or by two. The fact that a number of variables (e.g. optimal dithiol concentration [114] and apparent K_i for warfarin [116]) were found to be closely similar seems to favour the former possibility. The strongest argument has been brought up by Fasco *et al.* [116] who showed that in warfarin-resistant rats both enzymic activities have a decreased sensitivity for warfarin. The low probability of a genetic alteration affecting two enzymes in a similar way argues against the possibility that the dithiol-dependent KO and K reductase are two separate enzymes.

Dithiol-dependent reductases

In vitro the dithiol-dependent reductase(s) are generally measured in the presence of dithiothreitol [63,65]. This, however, is a synthetic product and the physiological cofactor is unknown at this time. A possible candidate would be thioredoxin, a dithiol protein which is widespread in nature [117]. Thioredoxin is an abundant protein in many parenchymatous organs and has been detected in various subcellular fractions of calf liver, including nuclei, mitochondria and microsomes [118]. It was found that thioredoxin, together with thioredoxin reductase and NADPH, could replace dithiothreitol during the reduction of K [119] and KO [120]. In this way a system was obtained in which vitamin K was recycled solely by the action of natural compounds. Using a different assay system these results were confirmed by Silverman & Nandi [121]. The fact that thioredoxin may function as a reducing cofactor for KO and K reductase does not prove that it exerts a similar function *in vivo*, however. In this respect it is important to realize that thioredoxin has a broad specificity and that as yet there is no evidence for its occurrence at the luminal side of the rough endoplasmic reticulum. Both dithiol-dependent reductases are strongly inhibited by 4-hydroxycoumarin derivatives [122,123] via a mechanism which is only partly understood [124,125]. The drugs cause an accumulation of KO in the liver, thereby exhausting the supply of KH_2 and preventing further carboxylation events.

NADH-dependent reductase

Another enzyme is the NADH-dependent K reductase [115]. This enzyme is insensitive to coumarins, but will exclusively reduce K and not KO. Hence it is unable to complete the vitamin K cycle by itself. The enzyme is of vital importance, however, if the effect of coumarin drugs has to be abolished (e.g. in the case of intoxication). Because KO reductase remains blocked under these conditions, vitamin K (added in the quinone form) can be used only once and relatively large amounts of the vitamin are required to have a measurable effect. Recently it was shown that the osteoblast-like osteosarcoma UMR-106 is devoid of the NADH-dependent K reductase, whereas the other enzymes of the vitamin K cycle are abundantly present [126]. Apparently the same holds true for osteoblasts *in vivo*, because it was reported by Price *et al.* [127] that, by feeding rats with a mixture of vitamin K and warfarin, the production of normally carboxylated osteocalcin could be blocked, whereas their

blood coagulation was maintained at a normal level. A consequence of this discovery is that more insight may be obtained in the physiological function of osteocalcin and MGP by employing a rigorous warfarin protocol without affecting the blood coagulation system. Possibly this technique will prove to be adequate for investigating the function of other extrahepatic Gla-proteins as well.

Vitamin K-antagonists

As was mentioned above, a number of coumarin derivatives exhibit an anticoagulant activity by inhibiting the effective recycling of vitamin K. Presently these drugs are frequently used as rodenticides (mainly warfarin) and for the treatment and prophylaxis of thrombo-embolic diseases (warfarin, acenocoumarol and phenprocoumon). The use of coumarin anticoagulants for preventing a primary myocardial infarction in persons with angina pectoris may be questioned. Several studies have demonstrated, however, the beneficial effect of a long-term anticoagulant treatment for preventing a re-infarction in patients who have survived a primary infarction [126,127]. It was demonstrated that the therapeutic range is extremely narrow and is limited on one side by an unwarranted bleeding tendency of the patient (at INR values above 4.8) and at the other side by the efficacy of the therapy (INR values should be less than 2.1). In these studies large numbers of subjects were treated with coumarin derivatives for several years. Therefore it is remarkable that so few side-effects of this therapy are known [130]. It has been shown, for instance, that in rats [127] and in humans [131,132] the osteocalcin Gla content is strongly affected by coumarin derivatives. Yet no bone abnormalities have been reported in adult patients on long-term anticoagulant therapy. In this respect one has to realize, however, that the effects may be marginal and may require several decades to become clinically manifest.

In 1974 a second generation of 4-hydroxycoumarins became available, generally designated as 'superwarfarins' [133]. These compounds (e.g. difenacoum and brodifacoum) are more hydrophobic than warfarin and they are characterized by very long biological half-life times (up to 180 days). Because of their extreme toxicity they were only used as rodenticides, especially in those areas where warfarin-resistant rat populations have developed [134]. The production and application of these poisons includes the risk of accidents and ingestion by humans [135,136], the counteraction of which is extremely difficult. Another point of concern is the observation that also against the superwarfarins resistance has already developed [134]. Therefore it seems justified to adopt a hesitant attitude with respect to the large scale application of these drugs.

CELLULAR PROCESSING OF VITAMIN K-DEPENDENT PROTEINS

Production of factor IX-related antigen

Our present knowledge about the molecular biology of proteins involved in blood coagulation has recently been reviewed by Furie & Furie [137]. Here I will restrict myself to a specific topic in this field and discuss the progress which has been made in the production of recombinant coagulation factor IX. Native factor IX occurs in blood plasma and is required for normal haemostasis; its functional deficiency results in haemophilia B, a sex-linked bleeding disorder occurring in the Western population at a frequency of about 2 per 10^5. The production of recombinant factor IX is of clinical importance, because factor IX concentrates are required for substitution therapy in haemophilia B. As yet these concentrates are prepared from pooled human plasma, which includes the risk of transmitting viral agents responsible for diseases such as hepatitis B and AIDS. Recombinant DNA techniques may provide the means to produce the required proteins without exposing the patients to these risks and at lower costs.

The production of biologically active recombinant factor IX implies the correct post-translational processing of the intracellular precursor molecule. Vitamin K-dependent carboxylation of its first 12 *N*-terminal Glu residues still represents a significant challenge to the scientists working in this field. Because bacteria do not contain carboxylase, eukaryotic cell lines are generally used to express the cDNA coding for human factor IX. Suitable hosts are baby hamster kidney (BHK) cells [138] and Chinese hamster ovary (CHO) cells [139,140]. Provided that vitamin K is present in the culture media, these cells are able to excrete partially carboxylated factor IX. Although the levels of factor IX produced in these studies varied widely, its reported Gla content never exceeded 50–70% of that in plasma factor IX. These values even represent an upper limit because in most cases factor IX procoagulant activity was measured after barium citrate adsorption and subsequent elution of the factor IX-like material, a procedure which includes a strong selection for γ-carboxylated factor IX molecules. In contrast to factor IX, recombinant prothrombin was reported to be fully carboxylated [141]. As compared to recombinant factor IX the absolute level of Gla formation in the host cells (CHO) also was 5–10-fold higher during the production of recombinant prothrombin. It was proposed, therefore, that prothrombin or its prosequence may have some structural characteristics which make it a better substrate than factor IX for vitamin K-dependent carboxylase. To assess whether the marginal differences between the prosequence of factor IX and that of prothrombin are responsible for the higher carboxylation level of the latter, a chimaeric protein might be constructed by fusing the cDNA coding for the signal and prosequence of prothrombin with that coding for mature factor IX. The possibility to prepare this kind of fusion protein was initially described by Berkner *et al.* [142] who reported the expression of various mixed-type molecules constructed from factor VII and factor IX.

Cellular carboxylation of recombinant factor IX

One of the problems encountered in these studies is that a reasonable fraction of carboxylated (and hence biologically active) factor IX is only obtained at low expression levels. The production of factor IX antigen could be greatly improved by gene amplification [139]. It seems, however, as if the vitamin K-dependent carboxylase activity in the host cells is rapidly saturated, resulting in a ratio of carboxylated factor IX to total factor IX of 1% or less. Theoretically two procedures may be used to solve this problem: (*a*) the selection or construction of cell lines containing unusually high levels of the enzymes of the vitamin K cycle, and (*b*) the carboxylation *in vitro* of insufficiently carboxylated proteins. In many respects the first possibility is the most elegant one, but it requires that substantial technical

problems be solved. Most of the available cell lines have been screened for their carboxylase content, but even the use of hepatoma cells did not improve the Gla content of recombinant factor IX [143]. The cloning in the host cells and expression of the genes coding for the enzymes of the vitamin K cycle might be helpful, but remains a remote possibility as long as these proteins have not been purified. Also it remains to be seen whether these genes may be amplified in parallel with those coding for factor IX, whether they will be expressed as active enzymes and whether these enzymes will be incorporated at the luminal side of the rough endoplasmic reticulum membranes in the host cells.

Carboxylation *in vitro* of recombinant factor IX

The second possibility to prepare fully carboxylated recombinant factor IX at high expression levels was explored by Soute *et al.* [144], who reported the carboxylation *in vitro* of insufficiently carboxylated factor IX from a transformed CHO cell line. An explanation for the fact that the recombinant protein was recognized by carboxylase may be given by the observation that substantial amounts of the recombinant factor IX had been incompletely processed and still contained the prosequence. A similar kind of substrate for carboxylase was prepared by Suttie *et al.* [145], who expressed proprotein C in *Escherichia coli*, and who showed that only proteins containing a propeptide region were substrates for rat liver carboxylase. From these data it may be concluded that, before the carboxylation *in vitro* of incompletely carboxylated material may contribute to the preparative production of biologically active recombinant factor IX, an as yet putative propeptide peptidase will have to be isolated and used to dissociate the mature factor IX from its propeptide.

NUTRITIONAL REQUIREMENTS FOR VITAMIN K

Adults

In nature two classes of vitamin K occur. Both contain a functional naphthoquinone ring and an aliphatic side chain, which is phytol in the case of vitamin K_1, whereas in vitamin K_2 it is composed of a varying number of isoprene units. Vitamin K_1 (phylloquinone) is mainly found in green plants [146] and vitamin K_2 (menaquinone) is produced by bacteria (e.g. the microflora in the gut) [147]. The extent to which each of the K vitamins contributes to the carboxylation of blood coagulation factors is still unclear and the results that have been published thus far are confusing. Several investigators have demonstrated that menaquinones account for 80–95 % of the total hepatic vitamin K stores [148,149]. Yet a short period of vitamin K_1 restriction induced a substantial decrease of blood coagulation activity and prothrombin synthesis, both in human volunteers [150] and in rats [149]. It was concluded that menaquinone concentrations in liver do not reflect relative availability for carboxylase and that menaquinones do not appear to be capable of supporting coagulation factor synthesis in rat liver. These conclusions are inconsistent, however, with the reported therapeutical effect of vitamin K_2 in preventing haemorrhagic disease in the newborn [151].

The daily requirement of vitamin K is estimated at 1–2 μg/kg weight [63]. A normal 'mixed diet' contains about 300 μg of phylloquinone/day, from which about 50 % may be absorbed. These data show that there is a good balance between vitamin K intake and requirement. In rare occasions, however, this balance may be disturbed resulting in a vitamin K deficiency. In this respect two groups (notably newborns an elderly women) are at special risk, and they will be dealt with below.

Newborns

During the first few days after birth the gut is sterile and will not contain menaquinone-producing bacteria. Shearer and colleagues [152] have shown that in addition the plasma levels of vitamin K_1 in neonates are extremely low as compared with their mothers. Probably this phenomenon is related to the fact that the placenta is a relatively poor organ for the transmission of lipids and hydrophobic molecules like vitamin K [153]. On the other hand, the requirement for vitamin K in the newborn is high: besides the liver (for the production of the Gla-containing coagulation factors), also the rapidly growing bones will need substantial amounts of vitamin K for the synthesis of osteocalcin and MGP. A second well-known phenomenon is that at birth the plasma levels of the vitamin K-dependent coagulation factors are lower (30–60 %) than in adults, and that they may fall even further until the second or third day [154]. On many occasions it has been suggested that these low coagulation factor concentrations are related to the low plasma vitamin K levels, mentioned above. If such a relation would exist, however, one would expect that substantial amounts of non-carboxylated coagulation factors would occur in newborn plasma. This is definitely not the case. It was shown by several authors [151,155] that in 60–90 % of the plasma samples obtained from the umbilical cord and/or from healthy neonates, trace amounts of descarboxyprothrombin occur. The concentration was reported to be less than 1 % of the normal prothrombin concentration, and it is highly questionable whether the decrease of plasma coagulation factor concentrations by 1 % is of clinical relevance. Yet it seems that the vitamin K supply in newborns is only marginal, which makes them prone to developing vitamin K deficiency. It is my opinion that the routine administration of vitamin K to all healthy newborns is not warranted by the data presently available. Prophylactic administration of vitamin K is indicated, however, for at least the following groups of babies: (i) those whose mothers have experienced periods of vitamin K deficiency during pregnancy; the deficiency may be caused, for instance, by the malabsorption of fats, the use of antibiotics or by oral anticoagulants; (ii) newborns receiving parenteral nutrition, or suffering from diarrhoea, mucoviscidosis or an impaired fat resorption, and (iii) breast-fed children. It was shown by Haroon *et al.* [156] that the vitamin K concentration in human milk is much lower than in cow's milk or in instant formula foods. In this group a symptomatic vitamin K-deficiency may easily develop, which may lead to serious complications like intracranial haemorrhages. In all these cases the optimal means of application and dosage has to be established as yet.

Elderly subjects

Several reports have recently been published indicating that a second group at risk for vitamin K deficiency may be formed by postmenopausal women. It is well known that after the start of the menopause the loss of bone mass may increase considerably. This process is very

common, especially among Caucasian (white) women, and it is probably related to the hormonal shift which takes place at the onset of the menopause. The progress of demineralization may continue for periods of 20 years or longer, which makes the bones fragile and may result in spontaneous fractures of the femur neck and of the vertebrae. The stage at which the bone mass is significantly below normal is designated as osteoporosis.

Because at least two vitamin K-dependent Ca^{2+}-binding proteins are abundantly present in mammalian bones, it seemed indicated to investigate whether a relation could be found between the rate of bone loss and the vitamin K status of postmenopausal women. Indeed, Hart and colleagues [157] reported that the circulating level of vitamin K_1 in osteoporotic patients with fractured femur neck was about 30% of normal, and they concluded that this kind of patient seemed to have a deficit of circulating vitamin K_1 which may be relevant for the maintenance of their proper bone metabolism. A second observation was reported from Japan [158] where, in a small study, three patients with postmenopausal osteoporosis received a daily treatment with vitamin K. This treatment resulted in a decrease of calcium loss by 18–50%. Both studies were hampered by the fact that only small groups of subjects were studied. Therefore we decided 2 years ago to start more elaborate investigations concerning the effect of vitamin K on bone metabolism in humans. In these studies [159] it was found that: (i) in postmenopausal women the Ca^{2+}-binding capacity of the circulating osteocalcin is less than 50% of normal; (ii) the oral administration of vitamin K (1 mg/day for 2 weeks) to these subjects induces an increase of both the plasma osteocalcin level as well as its Ca^{2+}-binding capacity; (iii) the administration of vitamin K to fast losers of urinary calcium results in a 50% decrease of the Ca^{2+} loss; and (iv) in parallel also the urinary hydroxyproline excretion (a well-known marker for bone turnover) was reduced and a good correlation between both processes was found.

These data indicate that vitamin K may be one of the factors of potential importance to retard the loss of bone mass in elderly women. More elaborate investigations are required, however, to show whether vitamin K supplementation may form an additional tool to reduce the number and severity of the complications of osteoporosis.

VIEWS AND PERSPECTIVES

At this moment we are observing a number of rapid developments in the vitamin K field, which warrant the expectation that within 1 or 2 years our fundamental knowledge will increase considerably. In the first place real progress has been made concerning the purification of carboxylase, and it can be expected that by the technique of affinity chromatography using propeptide-containing substrates covalently linked to insoluble resins, homogenous carboxylase will become available in sufficient quantities to permit a thorough investigation of the reaction mechanism and to provide proper kinetic data. Synthetic peptides derived from the propeptide and the Gla-domain of a number of vitamin K-dependent proteins will show us the mechanism of substrate selection by carboxylase. It can also be expected that other Gla-containing proteins will be discovered. An example is PGP (plaque Gla-protein) which was recently isolated and purified from calcified arteriosclerotic plaques [160]. With the aid of polyclonal antibodies against PGP an assay was developed with which PGP was also detected in blood plasma from normal healthy subjects. Plasma from atherosclerotic patients seemed to be devoid of PGP. Whether plasma PGP may be used as a marker for atherosclerotic disease remains to be awaited, however.

A question which remained unsolved as yet concerns the source of the reducing equivalents entering the vitamin K cycle. We suggest to investigate whether reduced protein disulphide isomerase might fulfil the role of hydrogen donor. Arguments for this hypothesis are: (*a*) protein disulphide isomerase has been found in all tissues containing the enzymes of the vitamin K cycle; (*b*) *in vitro* the cyclic conversion of vitamin K seems to occur independent of the carboxylation reaction, and under most conditions KO formation exceeds Gla formation by far; (*c*) protein disulphide isomerase is located at the luminal side of the rough endoplasmic reticulum and contains four repeats of thioredoxin, a protein shown to be active as a reducing cofactor for KO and K reductase; (*d*) the constant stream of cysteine residues present in the nascent polypeptide chains would form an excellent source of hydrogen, and (*e*) the formation of disulphide bonds from free cysteine residues requires the removal of hydrogen, but the way in which the latter is oxidized has never been established.

Finally we want to mention that it is surprising that with respect to the extrahepatic vitamin K-dependent proteins most investigators have focussed their attention on the occurrence of the antigen and not its Gla content. This is quite different from the field of blood coagulation, where it is known that, irrespective of the vitamin K status of an individual, the circulating antigen concentration remains more or less constant, whereas the degree of carboxylation (and hence the biological activity) of the antigen is strictly dependent on vitamin K. We expect that more will become clear about the function of osteocalcin, MGP, PGP and other extrahepatic Gla-proteins if besides antigen concentrations also the Gla content or Ca^{2+}-binding capacity of these proteins will be studied in various patients. It seems plausible that, as in blood coagulation, the administration of vitamin K or vitamin K antagonists may form additional tools to prevent or to cure diseases related to an incorrect functioning of these proteins.

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