

The Vitamin K-dependent Carboxylase*

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Introduction

The only known biological function of Vitamin K (Fig. 1) in animals is as a required cofactor for the production of the unusual amino acid, γ -carboxyglutamate (Gla). This amino acid has a profound role in human blood coagulation. Several blood proteins require the presence of nine to thirteen Gla residues for normal function; these are the so-called Vitamin K-dependent (VKD) proteins. While some of the VKD blood proteins have a pro-coagulant function (prothrombin, and factors VII, IX, and X), others primarily serve anti-coagulant roles (proteins C, S, and Z). For all of the VKD blood proteins, however, the Gla residues are located in a homologous \approx 45 residue amino-terminal "Gla" domain (1-3). The presence of multiple Gla residues in this domain allow it to adopt a calcium-dependent conformation that promotes binding to a membrane surface (4, 5), such as found on damaged vascular endothelial cells or activated platelets. This interaction allows the localization of the VKD blood protein near the site of vascular injury, where it participates in reactions that either promote or regulate clotting (6-8). The importance of Gla in the catalytic function of these proteins is emphasized by the effect that administration of Vitamin K antagonists (e.g. coumadins, such as warfarin sodium) has on humans. Warfarin decreases the concentration of vitamin K in the tissues, which, in turn, results in the production of VKD proteins that contain a decreased number (or a complete absence) of Gla. The Gla domain of these under-carboxylated blood proteins cannot adopt its natural conformation and, as a result, the VKD blood proteins have poor affinity for phospholipid surfaces. For this reason, the reactions that involve these proteins are significantly damped, and efficient clotting no longer occurs (9-11).

Gla is synthesized in mammals by post-translational modification of glutamate. The enzyme that catalyzes the conversion of glutamate to Gla is the vitamin K-dependent γ -glutamyl carboxylase (12). In addition to a glutamate-containing substrate, this enzyme requires carbon dioxide, reduced Vitamin K, and molecular oxygen as reactants. The

products of the carboxylase-catalyzed reaction are Gla, Vitamin K 2,3 epoxide, and water (13) (Fig. 2). The enzyme is found not only in liver (where the VKD blood proteins are produced and secreted), but also in a variety of other tissues, such as skin, lung, and kidney (13, 14). The widespread tissue distribution of carboxylase in humans suggests the presence of additional Gla-containing proteins of diverse function. To date, fourteen VKD proteins have been identified in humans, seven of which are the blood proteins mentioned above. Two other proteins (bone-gla and matrix-gla protein) are involved in bone metabolism (15), while another (Gas6) is involved in cell signaling (16, 17). Screenings of human nucleotide-based databases for sequences with homology to Gla domain sequences have identified four additional putative VKD proteins of unknown function: PRGP1, PRGP2, TMG3 and TMG4 (18, 19).

The Carboxylase Protein and Gene

The γ -glutamyl carboxylase is an integral membrane enzyme bound in the endoplasmic reticulum and Golgi (20-22) (Fig. 3). Although the enzyme initially proved refractory toward purification (23), it was purified to homogeneity from bovine liver in 1991 (24) and the human and bovine carboxylase cDNA's were subsequently isolated and cloned (25). The nucleotide sequence predicts a polypeptide 758 residues long; the hydrophobic amino-terminal half is predicted to have several (three to seven) transmembrane domains, while the carboxy-terminal half of the enzyme is relatively hydrophilic (25, 26). Recently, a study of the topology of the human carboxylase has been reported. Using *in vitro* translation and *in vivo* mapping techniques, it was demonstrated that the amino- and carboxy-termini of carboxylase are located on the cytoplasmic and luminal side of the endoplasmic reticulum, respectively, and that the carboxylase polypeptide spans the ER membrane at least five times (26) (Fig. 4). Given the limitations of these *in vitro* systems, it is impossible to say with certainty that there are five transmembrane regions; however, it was shown that five of the predicted transmembrane segments were capable of serving as stop transfer sequences *in vitro*. The human carboxylase is likely to be a glycoprotein because the bovine enzyme binds to lectin adsorbents (27, 28). Eight of nine predicted N-linked glycosylation sites reside in the carboxy-half of human carboxylase (25), and treatment of a carboxy-terminal fragment of purified bovine carboxylase with a glycosidase demonstrated that glycosylation occurs beyond residue 349 (29). In addition to an active site where glutamate and reduced Vitamin K bind, the presence of a high-affinity substrate recognition site (the propeptide binding site) on carboxylase is indicated by many structure-function studies. Both of these binding sites must, at least in part, face the lumen of the ER, where γ -carboxylation occurs (20, 22). The presence of other functionally important binding sites on carboxylase may be indicated by future studies of the enzyme.

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* This work was supported by National Institutes of Health grants HL48313 (D.W.S.)

Abbreviations: Gla, γ -carboxyglutamate; VKD, vitamin K-dependent protein; Glu, glutamate; KH₂, Vitamin K hydroquinone; BGP, bone-gla protein; MGP, matrix-gla protein; Gas6, growth arrest-specific protein 6; PRGP, proline rich-Gla protein; TMG, transmembrane Gla protein; NEM, N-ethylmaleimide; FIXQ/Sb – a 59 amino acid peptide that includes the human factor IX propeptide and Gla domain

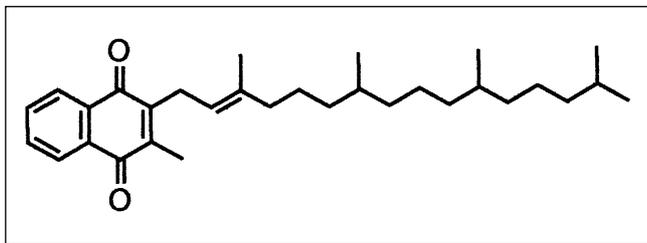
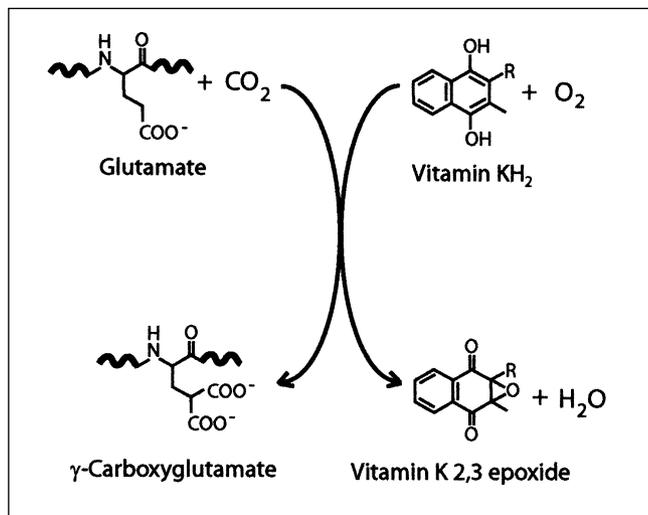
Fig. 1 Vitamin K₁

Fig. 2 The chemical reaction catalyzed by γ -glutamyl carboxylase. The reactants are a Glu on the peptide chain, carbon dioxide, reduced vitamin K, and oxygen. The products are Gla, vitamin K 2,3 epoxide, and water. The carboxylation half reaction is described by the left-most arrow, epoxidation by the right-most arrow

The organization of the gene for human γ -glutamyl carboxylase and the transcriptional activity in adults has been characterized. The gene is 13 kb in length, contains 15 exons, and has a single transcriptional start site 217 base pairs upstream of the start codon (30). Two major transcripts (differing in molecular weight) were identified in all human tissues examined, while the levels of carboxylase mRNA in the bovine tissues tested were found to be greatest in the liver. The gene maps to locus p12 of human chromosome 2 (31). The gene has also been characterized in the rat (32), and the temporal expression of this gene during rat embryo development has been studied by *in situ* hybridization

(33). Nerve, mesenchymal, and skeletal tissues were found to express carboxylase mRNA early during rat embryogenesis, while in hepatocytes expression was determined to occur much later in gestation. (33). Additionally, the generation of carboxylase knock-out mice has been reported (34). While heterozygous mice are phenotypically normal, pups homozygous for the knock-out gene died at birth of massive hemorrhage. Homozygous embryos were also found to have a developmental abnormality of the forebrain and mid-face that resembles the human syndrome of warfarin teratogenicity (34).

Carboxylase Catalysis

γ -Glutamyl carboxylase catalyzes two chemical reactions at its active site (35, 36). The physiologically important reaction is the addition of carbon dioxide to glutamate to form Gla. The other reaction is the oxygenation of vitamin K hydroquinone (KH₂) to Vitamin K 2,3 epoxide; this four electron oxidation of KH₂ occurs with concomitant reduction of molecular oxygen to H₂O (Fig. 2). Only the reduced form of Vitamin K can serve as a substrate in this reaction (37). In the presence of adequate amounts of substrates in *in vitro* reactions, carboxylase catalyzes Gla and Vitamin K epoxide formation with a 1:1 stoichiometry (38, 39). This apparent coupling of the epoxidation reaction to γ -carboxylation by the carboxylase suggests that there is a mechanism by which the enzyme transduces the free energy of Vitamin K oxidation to drive the γ -carboxylation reaction forward. A hypothesized molecular mechanism proposed by Dowd and co-workers to explain this coupling, although not proven, has gained general acceptance (40) (Fig. 5). In this mechanism, the enzyme-catalyzed pathway of KH₂ oxygenation to epoxide includes the formation of a reactive naphthoquinone intermediate, a Vitamin K alkoxide, that is a very strong base. This base is hypothesized to abstract a hydrogen ion from the γ -methylene group of glutamate (with a pK_a \approx 25-28) (41) to produce a reactive carbanion intermediate. Subsequent CO₂ addition to the glutamate carbanion would form the product, γ -carboxyglutamate. Support for a Vitamin K alkoxide as the coupling molecule comes from non-enzymatic model reactions (42) and from isotopic labeling studies (40, 43, 44). Additional evidence comes from the calorimetric measurement and calculations of the magnitude of heat released from the oxygenation of KH₂ analogs to epoxide (40, 45). These studies suggest that oxidation of KH₂, in conditions leading to the formation of the epoxide (instead of Vitamin K), can provide enough energy to generate a strong base species of Vitamin K (40).

The identification of catalytic residues or cofactors that participate in the γ -carboxylation and epoxidation reactions is proceeding in several

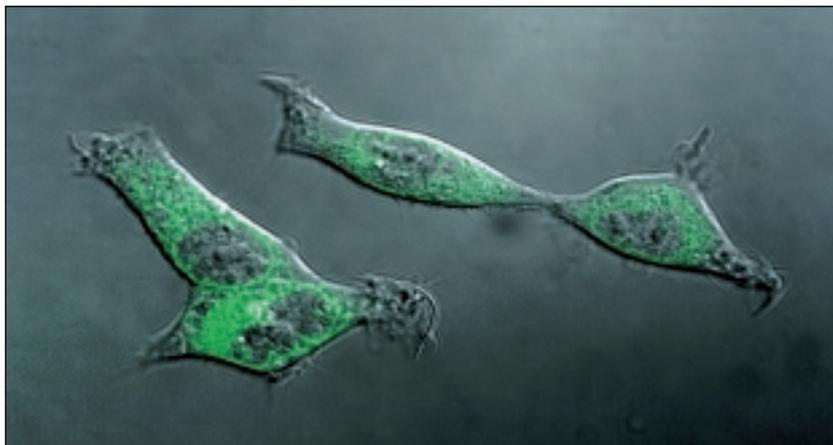


Fig. 3 Expression of recombinant human carboxylase with a fluorescent tag in *in vitro* cell culture. A construct coding for human carboxylase with a carboxy-terminal cyan fluorescent protein tag was stably transfected and expressed in HEK-293 cells. Cells were visualized with fluorescence microscopy (130). Note that carboxylase is localized outside of the nucleus or periphery of the cell

laboratories. These studies are facilitated by the availability of purified wild type or mutant recombinant carboxylase produced through mammalian or insect over-expression systems (46-48). Thus far, the presence of stoichiometrically bound prosthetic groups on carboxylase has not been reported. The apparent monooxygenase chemistry of carboxylase and inhibition of the enzyme by cyanide (49, 50), implicate heme as a possible catalytic prosthetic group on carboxylase. The lack of absorbance at 415 nm observed with purified recombinant human carboxylase, however, indicates $\leq 1\%$ of bound heme in these samples (51); this confirms earlier observations made with partially purified samples (50). Additionally, results from inductive-coupled plasma mass spectroscopy of purified recombinant carboxylase indicate $\leq 5\%$ bound iron, manganese, copper, or other transition metals on carboxylase (51). Early studies demonstrating that carboxylation activity is sensitive to thiol-reactive modifying reagents such as N-ethylmaleimide (NEM) or *p*-hydroxy-mercuribenzoate implicated cysteine residues as crucial for enzyme function (52-54). Further reports that KH_2 protects epoxidase activity alone (54), or epoxidase and carboxylation activity equally well (55), from these sulfhydryl modifiers imply that sensitive cysteines are located in the active site of carboxylase, and could act catalytically in these reactions. Based on the earlier protection data, Dowd and co-workers postulated the presence of two catalytic cysteines on carboxylase (40) (Fig. 5). One cysteine, in the form of a thiolate, acts as a weak base to catalyze deprotonation of KH_2 ; oxygen addition to the KH^- anion is predicted to drive the formation of the Vitamin K alkoxide coupling intermediate. Another cysteine thiol was postulated to coordinate the CO_2 , catalyzing the attack of this molecule by the glutamate carbanion to form Gla (Fig. 5). Two recent reports have demonstrated that isotopically-labeled NEM modifies either two (55) or two to three (56) cysteines residues of purified recombinant carboxylase when the incubation is carried out in the absence of co-substrates. Tryptic digestion of the labeled carboxylase followed by LC-MS analysis of the peptides identified Cys-99 and Cys-450 as two of the labeled cysteine residues. Because mutation of Cys-99 and Cys-450 also caused a severe loss of activity, the authors concluded that these were the active site residues (55); additional studies, however, will be needed to confirm that these residues are in fact located in the active site and act catalytically. While three additional cysteine mutations, C139S, C288S, and C311S were reported not to

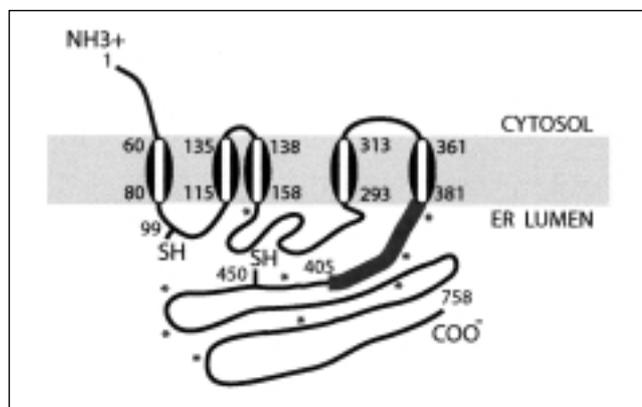


Fig. 4 Topology of the γ -glutamyl carboxylase, based on a recently reported study (26). The five predicted transmembrane domains in this study are indicated. Asterisks indicate the general locations of N-linked glycosylation sites (25). Two NEM-sensitive thiols (Cys-99 and Cys-450) (55) are also indicated. The location of the 25 residue sequence which is highly conserved between humans and *Drosophila* (112, 113) is indicated by a heavy line

affect carboxylation or epoxidation activity (55), the effects of mutation of other cysteines conserved between vertebrate carboxylases (C343 and C598) have not been reported.

Warfarin only weakly inhibits the γ -glutamyl carboxylase (36, 57). Warfarin and other coumarin-based vitamin K antagonists, however, potently inhibit the Vitamin K 2,3 epoxide reductase (57, 58), an enzyme required for reductive recycling of Vitamin K 2,3 epoxide back to the hydroquinone. The inhibition of the epoxide reductase by these drugs (59) depletes the levels of Vitamin K in the tissues, and, as a result, reduces the rate of γ -carboxylation activity (60). Despite the fact that epoxide reductase activity was first identified more than thirty years ago (61), very little biochemical characterization of this important enzyme has been accomplished, and the cDNA for the enzyme has not yet been identified. This reductase has proven to be extremely refractory toward purification, presenting an exciting but frustrating challenge for many investigators of the enzymology of the Vitamin K cycle.

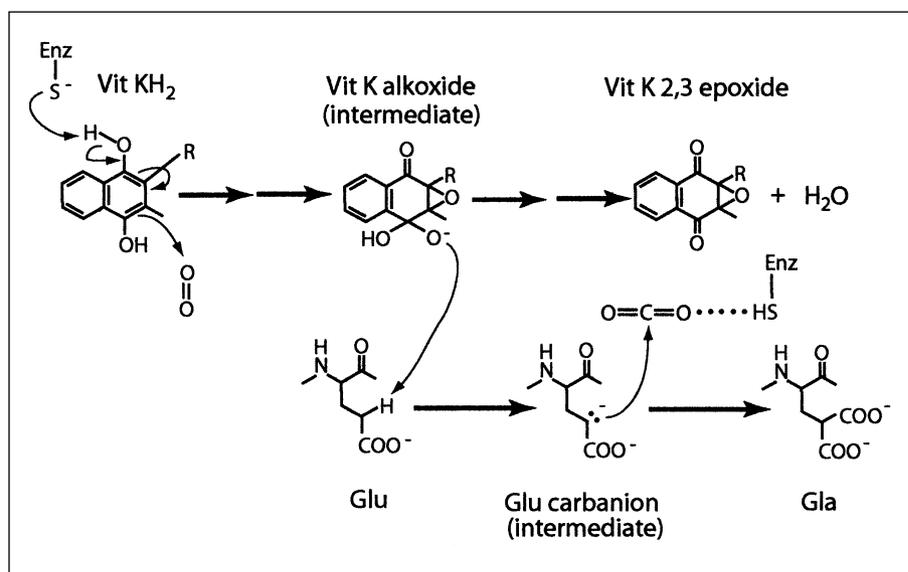


Fig. 5 Dowd's proposed mechanism for carboxylation. The top reaction sequence is a partial description of the hypothesized epoxidation reaction pathway, the bottom is for the γ -carboxylation reaction (40). The proposed catalytic action of two thiols is indicated. The Vitamin K alkoxide intermediate is the strong base that deprotonates Glu to form the reactive carbanion. Addition of CO_2 to the carbanion forms the product, Gla

Recognition of Vitamin-K Dependent Substrates by the Carboxylase

How does the carboxylase recognize proteins in the secretion pathway as substrates for γ -carboxylation? In the case of the VKD blood proteins, there is now overwhelming evidence that carboxylase binds to an ≈ 18 amino acid (62) "propeptide" found on the amino-terminus of these substrates (Fig. 6). The propeptide binds to the propeptide binding site, which is distinct from the carboxylase active site. This interaction is thought to provide the majority of the binding energy to anchor these substrates to the carboxylase (63), and allows the tethered Gla domain to be carboxylated multiple (up to 13) times by the active site of the enzyme (64, 65). All VKD blood proteins are synthesized in a precursor form, as a pre-pro-protein (66). The hydrophobic pre sequence, at the amino-terminal end of the precursor form, allows translocation of the nascent polypeptide through the ER membrane as it is synthesized. The pre sequence is cleaved off by a signal peptidase located in the lumen of the ER, and the remaining pro-protein is recognized by the carboxylase (Fig. 6) (67, 68). The first suggestion that the propeptide is required for γ -carboxylation was made by Pan and Price, who noticed substantial homology of the propeptide sequences of γ -carboxylated proteins (69). The requirement of a propeptide for γ -carboxylation of a VKD blood protein was confirmed through experiments utilizing mammalian cell culture techniques (67, 68). Additional studies have indicated that point mutations of the highly conserved hydrophobic residues at -16, -10, and -6 within the propeptide substantially reduce or eliminate carboxylation of that substrate in cell culture (68, 70). Mutation of less conserved residues at -15, -17 and -18 also lead to significant losses of carboxylation of substrate (71). These residues comprise, at least in part, the carboxylase recognition site in the propeptide (72). After the pro-blood protein is γ -carboxylated, it is transported to the Golgi apparatus, where the pro sequence is removed (22). A pair of highly conserved basic residues found at positions -4 and -1 on the propeptide serve as a recognition element for a pro-con-

vertase that catalyzes the cleavage of the propeptide (73). The blood protein is then secreted and circulates in the blood as a zymogen. The importance of the removal of the pro sequence for the normal function of a VKD blood protein is exemplified by the identification of Hemophilia B patients that have a point mutation at one of these conserved residues (e. g. R -4 N or R -1 S) in the factor IX propeptide (62, 74). In these patients, the mutated factor IX is secreted into the bloodstream with an attached propeptide (62, 74). The Gla domain cannot adopt its native conformation, and, as a result, the pro-factor IX is unable to bind tightly to acidic membranes, or to be activated by factor XI (75). These properties of the mutated factor IX likely cause the severe bleeding disorder observed in these patients.

The VKD bone proteins appear to undergo somewhat different mechanisms of carboxylation and processing than the VKD blood proteins. Matrix-gla protein, for example, is synthesized as a pre-protein that lacks a propeptide (76). A sequence that is homologous to the propeptides is located in the middle of the mature protein sequence, however, and it is likely that this substrate recognizes the propeptide binding site via this sequence (76, 77). Bone-gla protein is synthesized as a pre-pro-protein, but its propeptide has very poor affinity for the carboxylase (47). Decarboxylated bone-gla protein (without a propeptide), however, is a good substrate ($K_m \leq 3 \mu\text{M}$) for carboxylase (78), so it likely binds to the enzyme through an internal recognition sequence. Recent data also suggests that this substrate binds at a site on carboxylase that is not the propeptide binding site (79).

The propeptide is likely to be the major determinant of affinity of a VKD blood protein for the carboxylase, while the neighboring Gla domain appears to play a less significant role in this recognition. This hypothesis is supported through several lines of evidence. First, short peptides with sequences homologous or identical to highly conserved Gla domain sequences found in coagulation factors (e. g. FLEEV or FLEEL, based on residues +5 to +9 of prothrombin and factor VII, Fig. 6) are poor substrates ($K_m = 1-10 \text{ mM}$) for carboxylase in *in vitro* carboxylation reactions (80, 81). Longer peptides, however, which

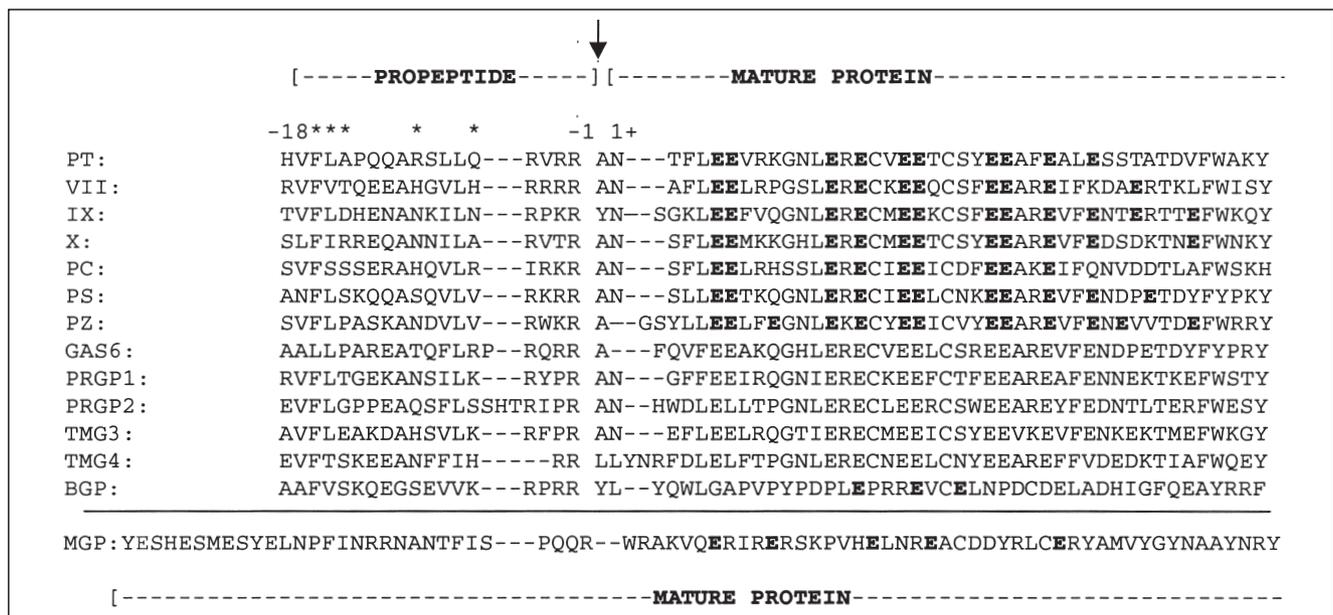


Fig. 6 Sequence alignment of the pro and mature peptide sequences, up to 45 residues, for the known and putative human VKD proteins. The VKD proteins known to contain Gla are prothrombin, factors VII, IX, and X, proteins C, S, and Z, BGP, MGP, and Gas 6. The locations of Gla residues are indicated by a bold E. Residues that are highly conserved are shaded. Asterisks indicate residues that are recognized by carboxylase for γ -carboxylation. An arrow indicates the cleavage site to produce the mature protein. Sequences of the known VKD proteins are from Prosite (except Gas 6, which was from Genbank), while the others are from Ref 19

include a propeptide attached to FLEEL, are much better substrates ($K_m \approx 3 \mu\text{M}$) (72, 82, 83). This ≈ 1000 -fold improvement in affinity is likely due to the tethering of weakly binding Glu's to the tighter binding propeptide. As a result, the local concentration of glutamate about the carboxylase active site would be effectively increased, lowering the K_m . It has been suggested that other sequences within the Gla domain of a VKD blood protein may make up a second recognition site for carboxylase, and a consensus sequence E¹⁶XXE²⁰XC²² has been identified (76) that is perfectly conserved on all human VKD protein sequences identified to date (Fig. 6). Indeed, expression of protein C with mutations either at E20 or C22 was found to produce a partially carboxylated product, compared to a control (84, 85). On the other hand, there is considerable evidence that sequences within the blood protein Gla domain do not play a significant role in recognition of these substrates by carboxylase. For example, a 59 amino acid peptide consisting of factor IX's propeptide and Gla domain (FIXQ/S) was found to be a good substrate for carboxylase ($K_m \approx 300 \text{ nM}$) (63, 86). By contrast, a peptide of the free Gla domain alone was found to be a poor substrate ($K_m \geq 140 \mu\text{M}$) (63). Furthermore, peptides containing either a prothrombin or factor IX propeptide attached to a glutamate-containing sequence with no homology to a Gla domain were found to be multiply carboxylated in tissue culture or *in vitro* (63, 87). This fact establishes that the propeptide is sufficient to direct multiple carboxylations of a substrate, and that domains outside the propeptide and Gla domain of a nascent blood protein may play an insignificant role in its recognition by carboxylase. Further support for the latter notion comes from the observation that an uncarboxylated factor IX substrate with its propeptide still attached has an apparent affinity for carboxylase similar to FIXQ/S (63).

The marked homology between propeptides led to the assumption that the propeptides of the different human VKD proteins have similar affinities for the carboxylase. This expectation proved incorrect: the affinities of the propeptides for carboxylase vary by more than a 100,000-fold. For the blood proteins, the factor X, factor VII, factor IX, and protein S propeptides have the highest affinities in *in vitro* reactions ($K_i = 2\text{-}50 \text{ nM}$) (47). In contrast, the prothrombin and protein C propeptides were found to have a 100-fold weaker affinity than the factor X propeptide. The physiological consequences of these affinity differences are still uncertain. The basis for the relatively poor affinities of protein C and prothrombin propeptides was determined, however, to be largely due to the identity of a single residue: -15 in protein C, and -9 in prothrombin. Changing these residues to those of amino acids found most frequently at the corresponding position in other propeptides increased their affinity for the carboxylase 100-fold or more (88). Curiously, the propeptide for bone-gla protein was found to have a substantially reduced affinity ($K_i > 500 \mu\text{M}$) (47). Changing only two residues (-6 and -10) of this propeptide to those of conserved sequence, however, increased its affinity (over 8,000-fold) to the level of the other tight binding propeptides. These studies demonstrate the importance of single amino acids in determining the affinity of a propeptide for carboxylase (88).

The docking of a VKD blood protein to an exosite on human carboxylase appears to be an evolutionary conserved method by which VKD substrates are recognized by their carboxylases. Currently, the only invertebrate organisms known to synthesize Gla are from the genus *Conus*, carnivorous marine snails that paralyze their prey with secreted venom. The venom from a single species contains up to 200 different kinds of toxic peptides, or conotoxins (89, 90). Several peptides in the conotoxin family have 2-5 Gla residues (91, 92). Most of these Gla containing-peptides are conantokins, so-called because they cause sleep when injected into young mice (90). The conantokins of

Conus are synthesized in a pre-pro-protein precursor form, as observed for VKD blood proteins in humans. Additionally, while unmodified peptides of the free conantokin Gla domain are poor substrates for *Conus* carboxylase, peptides containing a conantokin propeptide and Gla domain are good substrates, with affinity constants ($K_m \approx 5\text{-}30 \mu\text{M}$) approaching that seen for the FIXQ/S-human carboxylase interaction (93, 94). The *Conus* propeptides, however, have no obvious homology to human propeptides, and conantokin propeptide and Gla domain-containing peptides are very poor substrates for human carboxylase (93). The fact that these non-homologous propeptides from distantly related organisms (humans vs. *Conus*) have a similar method of recognition for their respective carboxylases indicates that the propeptide-carboxylase interaction may be defined by complimentary surfaces rather than evolutionarily conserved amino acid sequences (94).

Identification of Binding Sites on Carboxylase

Despite the fact that purified carboxylase has been available for ten years, remarkably little is known about the specific amino acids on the enzyme that play a part in functional binding sites. Several studies have utilized peptide-based affinity labels to identify portions of the carboxylase polypeptide that form functional binding sites. The results obtained from these studies, however, were often of low resolution. For example, a factor IX propeptide sequence with an internal benzoyl-phenylalanine (Bpa) moiety and a ¹²⁵I-tyrosine residue was photochemically cross-linked to the carboxylase to locate the propeptide binding site. The Bpa group allows light-catalyzed cross-linking of neighboring functional groups on the enzyme to the propeptide, while the radiolabel allows identification of labeled peptide(s) prepared by enzymatic digestion of the labeled enzyme. This peptide was concluded to label residues between 50 and 125 in one study (95), while a different group that utilized an identical peptide found that it bound to sequences between residues 438 and 507 (29). A point mutation study found roles for charged residues located at 234/235, 406/408, and 513/515 in propeptide binding (96). Disparate results were also received with attempts to identify the location of the active site with affinity agents (97-99). These studies suggest the possibility that non-contiguous regions of the enzyme may form the functionally important binding sites.

The molecular characterization of two patients with a rare inherited combined deficiency of all VKD blood proteins has revealed the presence of missense mutations in their carboxylase gene, which would result in a single amino acid substitution on the protein (100). The affect that one of these mutations (L394R) (101) has on the catalytic function of human carboxylase was determined using steady-state kinetic analysis of a purified recombinant mutated enzyme (102), and the characterization of another (W501S) (103) is in progress (104). The most striking effect of the L394R mutation was on the affinity of a small glutamate analog, as the K_i for this inhibitor was 110-fold greater for the mutant compared to the wild type enzyme. This result suggests that the primary defect in mutant L394R appears to be in its glutamate-binding site, and that residue L394 may be directly involved in glutamate binding or may stabilize the glutamate-binding site of wild-type carboxylase (102). Lesser effects on the apparent affinities of propeptide (7-fold difference) and KH_2 (5-fold difference), however, were also measured. In the earlier point mutation study, the mutation of charged residues at 513/515 also appeared to affect the binding of propeptide, glutamate, and KH_2 (96). The small effect on propeptide and KH_2 binding may indicate that L394 is involved in the allosteric linkage between the active and propeptide binding sites (105-107). Additional experiments would be required

to define the exact role of this amino acid in the structure of the carboxylase.

The cDNA's encoding the entire carboxylase polypeptide from a variety of vertebrate species, including human (25), cow (25, 108), rat (32), mouse (109), whale (110), and toadfish (110), have been cloned and sequenced. An alignment of these predicted polypeptide sequences shows, not surprisingly, a high amount of residue identity between the different vertebrate species (e. g. 86% between human and toadfish) (110). Vitamin K-dependent carboxylase activity has been identified in two invertebrate species, including *Conus* (111) and genus *Drosophila* (112). The *Drosophila* carboxylase cDNA has been recently cloned, and the predicted polypeptide sequence has 33% residue identity and 45% homology to the human carboxylase sequence (112, 113). An alignment of the human and *Drosophila* carboxylase sequences reveals that the polypeptides are very homologous between residues 381 and 405, with residue identity in 23 out of 25 positions (112). This region, which follows the last predicted transmembrane domain of the human carboxylase (Fig. 4), may contain sequences that form a functional binding site. The kinetic analysis of a carboxylase with a naturally occurring mutation in this conserved region (L394R) suggests a great effect on glutamate binding, so this region may comprise at least a part of the glutamate binding site (102).

Processivity and Regulation of Carboxylase Catalysis

Healthy adults are thought to have VKD blood proteins whose Gla domains are either completely or almost completely (114) carboxylated. This belief is based on the direct measurement (3, 115) of 9-13 Gla residues in this domain (depending on the identity of the VKD blood protein, Fig. 6), and also on the observation that a loss of as few as three carboxylations can severely impair the functionality of these proteins (10). Recent data from Nelsestuen and co-workers, however, suggests that a large fraction of VKD blood proteins *in vivo* are missing one to two carboxylations of the possible Gla sites in the Gla domain (114). The possibility that the carboxylase enzyme can carry out complete or nearly complete carboxylation of a protein substrate in a single binding event has been investigated. In an earlier study, mass spectrometry was used to determine the number of γ -carboxyl groups added to FIXQ/S by the carboxylase during an *in vitro* reaction under conditions where the substrate can only bind once to carboxylase before analysis (64). The carboxylated products were found to be comprised of species with 1 to 12 Gla's, and this population was dominated by fully (12 Gla's) or slightly undercarboxylated species. This result, along with the previous *in vivo* observation (114), is consistent with a stochastic model of substrate release from carboxylase with a rate constant significantly lower than the rate of carboxylation. This work was the first demonstration of the ability of carboxylase to carry out processive carboxylation (64), and confirmation of this mechanism comes from a recent kinetic study (65). Processive carboxylation of glutamates by the carboxylase is imaginable if they are tethered to a propeptide that binds very tightly to the enzyme, so that the rate of propeptide release is significantly slower than the rate of carboxylation (64). A recent comparison between the off rate of the factor IX propeptide and the rate of FLEEL turnover suggests that this occurs *in vitro*, as these rates were found to be 3000-fold different (107). A similar rate difference *in vivo* may ensure that a large fraction of VKD substrates are fully carboxylated in the normal physiological state. Other intracellular mechanisms, independent of carboxylase, may ensure that most of VKD blood proteins in the circulation remains fully carboxylated. For example, recent tissue culture studies suggest that undercarboxylated species of

protein C and prothrombin present in the endoplasmic reticulum are catabolized, perhaps due in large part to proteolytic degradation (116, 117). However, these mechanisms appear to be species-specific (116).

Carboxylase appears to utilize several regulatory mechanisms to increase the efficiency of carboxylation of VKD substrates. For example, several lines of evidence suggest that the structure of carboxylase changes when a protein substrate binds to the enzyme, and, that this change may regulate catalysis. First, the carboxylase enzyme contains a sequence (residues 495-518) that bears homology to a propeptide. It has been suggested that the presence of an internal propeptide may close the active site when a protein substrate is not bound, thus minimizing indiscriminate carboxylation (77, 118). Additionally, the epoxidation activity of carboxylase is stimulated by bound glutamate (48, 119). A recent report also suggests that carboxylase has no significant epoxidase activity in the absence of glutamate (48), a mechanism where epoxidation occurs only in the presence of glutamate would allow carboxylase to minimize the uncoupled (and unproductive) oxidation of the reduced vitamin when a substrate is not bound (48). Three glutamate residues within the carboxylase itself have been reported to be carboxylated to Gla (46), but the functional significance of this is unknown. It is hypothesized that, once the propeptide of the VKD substrate is bound to carboxylase, the enzyme carries out complete carboxylation followed by product release. There is considerable evidence that this complex mechanism has multiple levels of regulation. For example, there are several reports of allosteric linkage between the propeptide binding site and the active site of carboxylase (48, 105-107). *In vitro* studies indicate that the affinity of glutamate for its binding site increases 9-fold and the catalytic efficiency increases 18-fold upon propeptide binding (105, 120, 121). Additionally, the affinity of KH_2 for its binding site increases 7-fold or more when glutamate and a linked propeptide are bound to the carboxylase, compared to glutamate alone (79, 106). Propeptide binding also significantly increases the rate of formation of reaction intermediates and mobilizes enzyme cysteines; an 11-fold increase in the rate of glutamate carbanion formation (122) and an increased accessibility of NEM-sensitive cysteines (56) were observed in recent *in vitro* studies. We have recently suggested an attractive mechanism by which carboxylase can release the product after carboxylation is complete. In this study, the off-rate of a fluorescein-labeled factor IX propeptide from carboxylase was found to be significantly (9-fold) faster in the absence of co-substrates (FLEEL and KH_2), than in their presence (107). This data may suggest that when post-translational modification of a substrate is complete, the off-rate of the carboxylated product could be substantially increased from carboxylase if the enzyme's active site is unoccupied. This and earlier data suggest that the propeptide and active sites of the carboxylase are thermodynamically linked. This is a property by which carboxylase could enhance the catalytic efficiency of carboxylation, while at the same time retarding premature release of the substrate as it undergoes carboxylation. It should be emphasized, however, that many of these aforementioned studies utilized solubilized or partially purified carboxylase preparations at less than physiological temperatures. Whether these forms of regulation also exist for carboxylase under physiological conditions (e. g. bound in a membrane bilayer at 37° C) is presently unknown.

A Working Model of γ -Carboxylation

We have developed a model of γ -carboxylation to help rationalize the appearance of under-carboxylated VKD proteins in the circulation

of patients undergoing coumadin therapy. The essential feature of this model is the balance between the rate of carboxylation of a glutamate and the rate of product release from the carboxylase (64). The rate of carboxylation should be determined by the fractional occupancy of glutamate and the co-substrates KH_2 , CO_2 , and O_2 , at the carboxylase active site; these occupancies are determined by the substrate's local concentrations and affinities for the binding sites. We suggest that the rate of product release (off-rate) of a pro-blood protein from carboxylase is approximate to the off-rate of its propeptide; this hypothesis would assume that most of the binding energy of a blood-protein substrate to carboxylase resides in the propeptide (63). We have previously shown that the propeptides of different substrates have different affinities for the carboxylase (47) and, for this reason, we predict that the blood protein substrates will have somewhat different rates of release from carboxylase. For example, prothrombin's propeptide has a relatively poor affinity for carboxylase compared to factor X's (100-fold difference) (47); therefore, we predict that pro-prothrombin's off-rate from carboxylase will be significantly faster than that of pro-factor X.

We hypothesize that, in healthy adults, the off rate of the pro-substrate is dramatically slower than the rate of carboxylation, perhaps 3000-fold in the case of factor IX (107). This hypothesis is well supported by *in vitro* data for pro-factor IX catalysis (63, 64, 107) and we believe it also applies for the other VKD blood protein substrates as well. This rate difference would insure that after any VKD pro-blood protein binds to carboxylase through its propeptide, it will undergo complete or almost complete (114) carboxylation of the 9-13 Glu residues in its Gla domain (Fig. 6), before it is released from the enzyme. We hypothesize that in certain therapeutic or pathological settings, however, the difference between the rate of release of the substrate from the carboxylase and the rate of carboxylation diminishes, resulting in an increased production of under-carboxylated proteins. For example, the limited amounts of vitamin K hydroquinone in the tissues of patients on coumadin therapy would reduce the rate of carboxylation so that it no longer differs substantially from the range of off-rates of the pro-blood protein substrates. These substrates are released before carboxylation is completed, resulting in under-carboxylated VKD blood proteins in the circulation.

Our model also provides an explanation for the peculiar clinical manifestations of an individual with a "conditional" form of hemophilia B (123). In the absence of anticoagulants, this individual appears to have normal levels of carboxylated VKD blood proteins in his vasculature, and is healthy. When the individual was placed on warfarin therapy, however, his factor IX activity levels dropped to 1% of normal, while the activity of the other vitamin K-dependent coagulation factors were reduced to the expected 30-40% of normal (123). Inefficient γ -carboxylation of factor IX, relative to the other coagulation factors, only occurs during warfarin therapy. A molecular characterization of the individual's factor IX gene revealed a mutation at the highly conserved -10 position in the factor IX propeptide sequence (123-125). Furthermore, the mutated propeptide has 600-fold weaker affinity for carboxylase than the wild-type factor IX propeptide (88); this should substantially increase the off-rate of the mutant pro-factor IX from carboxylase. Our model provides an explanation for these clinical observations: During warfarin therapy, the rate of carboxylation of all VKD blood proteins is reduced in this patient. We hypothesize that the off-rates of the other pro-blood proteins (e.g. pro-factor VII, X, and pro-prothrombin) from carboxylase may still be significantly less than the rate of carboxylation to create some fully carboxylated forms (up to 30-40%) under these therapeutic conditions. The off-rate of the mutated pro-factor IX from carboxylase, however,

may be much closer to the rate of carboxylation. This could cause the nearly complete production of an incompletely carboxylated factor IX product during therapy, rendering the very low levels of factor IX activity.

Overexpression of VKD blood proteins in heterologous systems *in vitro* can also lead to their inefficient γ -carboxylation (23, 126, 127). For example, a large fraction of recombinant factor X expressed in mammalian cell culture is uncarboxylated, while a smaller fraction is fully carboxylated (e.g. 32% of the total protein produced) (128). We hypothesized that inefficient carboxylation of recombinant factor X was due to a combination of factors: 1) the very tight binding of factor X's propeptide to the carboxylase (47), and 2) its over-expression in tissue culture. These conditions could saturate the carboxylase sites with pro-factor X, making carboxylase limiting. In turn, this could result in much of the overexpressed pro-factor X substrate passing through the endoplasmic reticulum without ever binding to the carboxylase. We hypothesized that mutating the propeptide to a form that would bind more loosely (47) would increase the turnover rate of the recombinant pro-factor X from carboxylase, allowing more of the population in the endoplasmic reticulum to bind carboxylase and be γ -carboxylated. When a factor X chimera with an engineered prothrombin propeptide sequence was expressed, the fraction of fully carboxylated product was increased from ~32% to ~85% of the total protein produced (128); this observation supports our model.

Future Directions

Carboxylase has become more amenable to kinetic and biophysical characterization as larger quantities of enzyme have become available via over-expression of the recombinant protein. Many interesting questions remain about the mechanism of carboxylase catalysis that potentially could be answered through the use of transient kinetic, spectroscopic and calorimetric techniques. For example, recent data suggests that propeptide release is likely to be the rate-limiting step in factor IX turnover from carboxylase *in vitro*, suggesting that the turnover rate of factor IX by carboxylase is dominated by this rate (64, 107). Can this turnover model be generalized to other substrates? The propeptide of prothrombin, for example, binds 10-fold more loosely to carboxylase than factor IX's (47), while the gla domain may bind more tightly to the enzyme (129). If this is true, then the rate of propeptide release may affect turnover less for prothrombin than for factor IX. Additionally, the magnitude of the effect of substrate on propeptide off-rate for prothrombin may be different than that seen for factor IX. The precise location and electrostatic nature of the active and propeptide binding sites on carboxylase remain to be identified and characterized, and linkage residues between the two sites need to be identified. The thermodynamic basis (changes in enthalpy and entropy) for binding of propeptides to carboxylase can potentially be defined by calorimetry. The next decade should prove to be an exciting time for research on the mechanism of this intriguing enzyme.

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