Annu. Rev. Nutr. 2005. 25:127–49
doi: 10.1146/annurev.nutr.25.050304.092713

THE VITAMIN K–DEPENDENT CARBOXYLASE

Kathleen L. Berkner
Department of Molecular Cardiology, Cleveland Clinic Lerner College of Medicine at Case Western Reserve University, Lerner Research Institute, Cleveland, Ohio 44195; email: berknek@ccf.org

Key Words VKOR, vitamin K–dependent protein, hemostasis, carboxylation, vitamin K oxidoreductase

■ **Abstract** The vitamin K–dependent (VKD) carboxylase uses the oxygenation of vitamin K to convert glutamyl residues (Glus) to carboxylated Glus (Glas) in VKD proteins, rendering them active in a broad range of physiologies that include hemostasis, apoptosis, bone development, arterial calcification, signal transduction, and growth control. The carboxylase has a high-affinity site that selectively binds VKD proteins, usually through their propeptide, and also has a second low-affinity site of VKD protein interaction. Propeptide binding increases carboxylase affinity for the Glu substrate, and the coordinated binding of the VKD propeptide and Glu substrate increases carboxylase affinity for vitamin K and activity, possibly through a mechanism of substrate-assisted catalysis. Tethering of VKD proteins to the carboxylase allows clusters of Glus to be modified to Glas by a processive mechanism that becomes disrupted during warfarin therapy. Warfarin inhibits a vitamin K oxidoreductase that generates the reduced vitamin K cofactor required for continuous carboxylation and causes decreased carboxylase catalysis and increased dissociation of partially carboxylated, inactive VKD proteins. The availability of reduced vitamin K may also control carboxylation in r-VKD protein-expressing cells, where the amounts of reduced vitamin K are sufficient for full carboxylation of low, but not high, expression levels of VKD proteins, and where carboxylation is not improved by overexpression of r-carboxylase. This review discusses these recent advances in understanding the mechanism of carboxylation. Also covered is the identification of functional carboxylase residues, a brief description of the role of VKD proteins in mammalian and lower organisms, and the potential impact of quality control components on carboxylation, which occurs in the endoplasmic reticulum during the secretion of VKD proteins.

CONTENTS

INTRODUCTION 128
VKD PROTEIN FUNCTION 129
THE CARBOXYLASE REACTION 129
VKD PROTEIN-CARBOXYLASE INTERACTION 131
CARBOXYLATION OCCURS DURING THE SECRETION OF VKD PROTEINS 134
THE CARBOXYLASE IS A PROCESSIVE ENZYME 135

DISRUPTION OF PROCESSIVITY 136
QUALITY CONTROL AND CARBOXYLATION 138
CELL LINES AS MODEL SYSTEMS FOR ANALYZING CARBOXYLATION . . 138
IS CARBOXYLATION EFFICIENCY THE SAME IN ALL TISSUES? 140
STRUCTURE-FUNCTION ANALYSIS OF THE CARBOXYLASE 141
NONMAMMALIAN CARBOXYLASES 142
FUTURE PERSPECTIVES 143

INTRODUCTION

Vitamin K was discovered in the 1930s as a consequence of experiments showing that chicks fed a lipid-free diet were hemorrhagic (17). Major advances toward understanding vitamin K function were made in the 1970s by the discoveries that vitamin K–dependent (VKD) proteins contain glutamyl residues (Glus) that are gamma-carboxylated (i.e., Glas), and that the carboxylase generates Glas from Glus using reduced vitamin K as a cofactor (73). The carboxylase is an integral membrane enzyme localized in the endoplasmic reticulum (ER), where VKD proteins are carboxylated during their secretion. Multiple Glus in the Gla domains of VKD proteins are converted to Glas. Full carboxylation, which is required for VKD protein function, results in calcium coordination and consequent binding either to anionic phospholipids exposed on the surfaces of cells or to hydroxyapatite in the extracellular matrix. The expression of the human carboxylase was originally thought to be restricted to a limited number of tissues, based on activity assays; however, broader expression is indicated by RNA analysis made possible by the isolation of the carboxylase cDNA (39, 82). The expression analysis of human VKD proteins has actually been more informative with regard to carboxylase distribution, because a much larger number of tissues have been analyzed. These studies show a broad tissue distribution for several of the VKD proteins (e.g., the family of transmembrane Gla proteins, Gas 6, and matrix Gla protein) (20, 30, 31, 41). They also show that while the major site of hemostatic VKD protein synthesis is liver, at least some of these proteins are also synthesized elsewhere [e.g., protein S, which is expressed in several tissues (53)]. Thus, carboxylase expression is indicated in virtually all human tissues.

In humans, one carboxylase is responsible for modifying all VKD proteins and is the product of an autosomal gene (32). Carboxylase expression is developmentally regulated, occurring in skeletal and neural tissue during early rat embryogenesis and in hepatocytes at later stages (56), and the rat carboxylase gene has a novel promoter element that may regulate developmental expression (55). To date, carboxylase activity has only been detected in multicellular organisms, i.e., mammals, *Drosophila*, and the marine snail *Conus* (2, 16, 35, 78), which suggests the importance of VKD proteins to intercellular interactions. Recent homology searches have also revealed carboxylase orthologs in bacteria, which are presumed to have been acquired by horizontal transfer (60, 77), but whether they have activity is unknown.

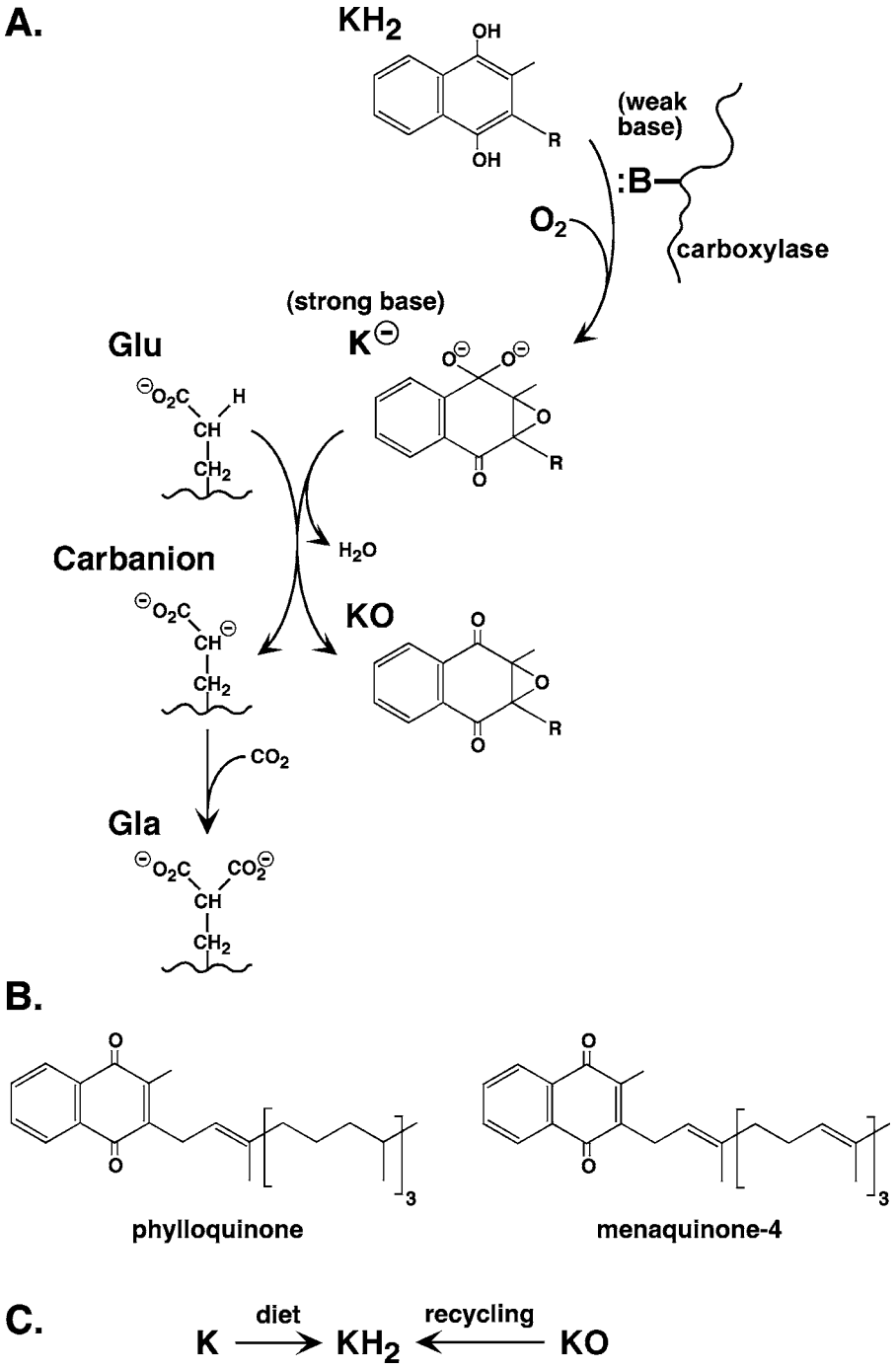
VKD PROTEIN FUNCTION

Our understanding of the biological relevance of VKD proteins to human physiology has undergone a substantial revision in the past two decades (reviewed in 7). Thus, until the 1980s, vitamin K was thought to be important only to hemostasis, with VKD protein identification being limited to hemostatic proteins (factors IX, VII, and X, prothrombin, and proteins C, S, and Z). However, vitamin K and carboxylation are now known to have a much broader physiological impact, and nonhemostatic proteins that have been identified include matrix Gla protein (48), an inhibitor of calcification, osteocalcin (46), a bone protein of unknown function, and Gas6 (41), which has several functions that include inhibition of apoptosis and proliferation. The transmembrane Gla proteins, a group of four VKD proteins, were discovered by virtue of their homology to known VKD proteins (30, 31), and their sequences predict functions in signal transduction. In addition, novel functions are being revealed for known hemostatic proteins, such as protein S, which facilitates phagocytosis as well as functioning as an anticoagulant (79). Finally, the carboxylase itself is also a VKD protein (6), and carboxylase carboxylation may be important in regulating the overall process of VKD protein carboxylation.

The only nonmammalian species in which the function of carboxylation is known is the marine snail *Conus* (2, 16), which uses small VKD peptides to paralyze prey by antagonizing neurotransmission. Carboxylase activity has also been observed in *Drosophila* (35, 78), but the function of carboxylation is unknown, as VKD proteins have not yet been identified in this organism.

THE CARBOXYLASE REACTION

The carboxylase uses the energy of vitamin K hydroquinone (KH_2) oxygenation to convert Glus to Glas in VKD proteins (Figure 1). During carboxylation, the KH_2 cofactor is oxidized to a vitamin K epoxide product, and the carboxylase is also an epoxidase. An explanation for how epoxidation drives carboxylation was indicated by chemical modeling studies in an enzyme-free system, which showed that KH_2 and O_2 react to form a strong vitamin K base intermediate that collapses to the vitamin K epoxide product upon protonation (19). The strong vitamin K base was of interest because studies with the carboxylase suggested that it converts the Glu substrate to a carbanion intermediate that then forms Gla by CO_2 addition (Figure 1); however, how the carbanion was generated was unknown because its formation would require a much stronger base (i.e., with a pKa of $\sim$25) than could be provided by carboxylase residues. Therefore, the strong vitamin K base could explain the role of vitamin K in carboxylation. Formation of the strong vitamin K base would first require KH_2 deprotonation for reaction with O_2, which led to the proposal of a "base amplification" model in which a weak carboxylase catalytic base deprotonates KH_2 to generate a strong vitamin K base that converts Glu to the carbanion intermediate and then to Gla (Figure 1) (19).



The base amplification model invoked a Cys as the carboxylase catalytic base that deprotonates KH_2; however, recent studies indicate that the base must instead be an activated amine (54). Cys was originally implicated by inhibitor studies with thiol-specific reagents like N-ethylmaleimide (NEM) (73), and biochemical mapping of native carboxylase revealed ^{14}C-NEM modification of Cys99 and Cys450 (51). These two residues also have been proposed to form a disulfide bond (75), but those studies did not use native protein and a double mutant lacking these two residues shows decreased ^{14}C-NEM reactivity (54) that indicates they are free thiols. However, the substitution of these two residues or even all of the ten Cys residues in the carboxylase by an amino acid (Ala) that cannot ionize to function as the catalytic base results in carboxylase mutants that are active (54). The decamutant shows unusual NEM reactivity that suggests the catalytic base is an activated amine, and one intriguing possibility is that Glu residues in the VKD substrate provide this activation (51, 54). Glu substrate causes an increase in carboxylase epoxidation (72), which may be due to a mechanism of substrate-assisted catalysis (Figure 2). Such a mechanism would regulate coupling between epoxidation and carboxylation, i.e., in the absence of substrate, the catalytic base would not be activated for KH_2 deprotonation, preventing the unfettered production of an undesirable highly reactive vitamin K base.

VKD PROTEIN-CARBOXYLASE INTERACTION

All mammalian VKD proteins contain a carboxylase recognition signal which is usually a high-affinity ~18 amino acid propeptide adjacent to the Gla domain (Figure 2) that is cleaved subsequent to carboxylation. Covalent attachment of the propeptide to small Glu-containing peptides lowers their K_m's ~1000-fold (to the low μM range) by increasing the local concentration of the Glu substrate. The mammalian VKD propeptides are homologous, and residues -16Phe, -10Ala, and -6Leu are the most highly conserved and have been shown to be functionally important

←

Figure 1 The carboxylase reaction. (*A*) The carboxylase uses vitamin K hydroquinone (KH_2) oxygenation to drive the conversion of glutamyl residues (Glu) to carboxylated glutamyl residues (Gla). The active site catalytic base (B:) is a weak base that deprotonates KH_2 to form a strong base (e.g., the dialkoxide shown, K^-), which then generates a Glu carbanion that reacts with CO_2 to form Gla while the K^- is converted to the vitamin K epoxide product (KO). (*B*) Vitamin K is a family of forms that includes phylloquinone and menaquinones. The menaquinones are more unsaturated and have a variable number of isoprenyl groups (e.g., one with four is shown). (*C*) Vitamin K in the diet exists in the quinone form (K), the form shown in *B*, and requires reduction to serve as a carboxylase cofactor. At least two pathways exist for reducing vitamin K quinone (discussed in the text), one of which also reduces the vitamin K epoxide product.

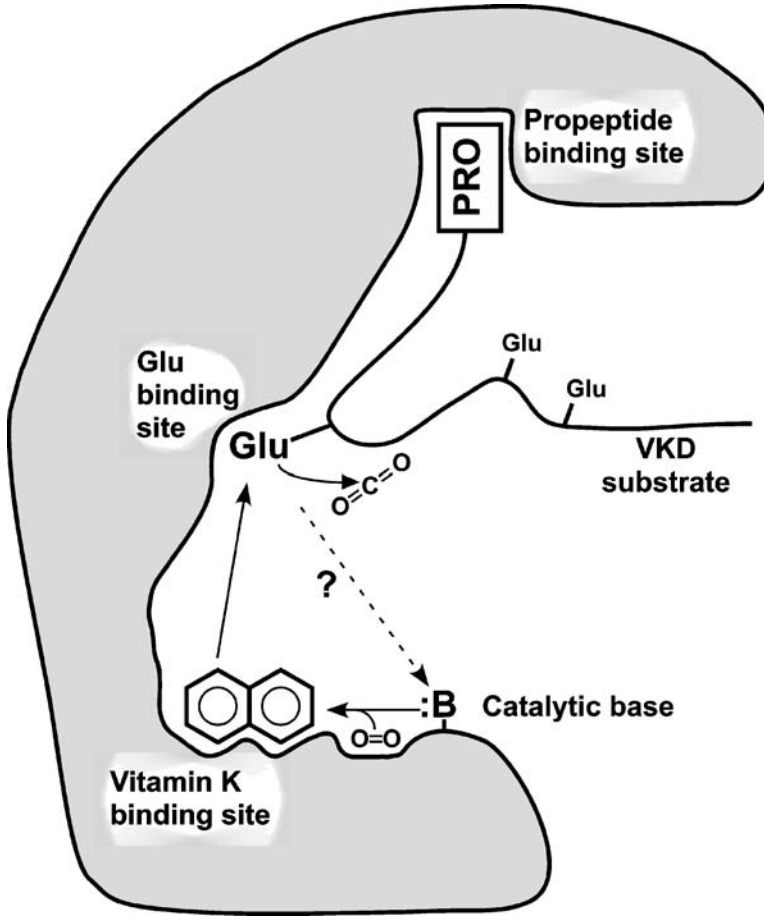


Figure 2 The carboxylase active site. The carboxylase binds vitamin K–dependent (VKD) proteins through their propeptide (PRO), a high-affinity domain in most VKD proteins that is cleaved after carboxylation. Propeptide binding increases the affinity of the carboxylase for the Glu substrate, and propeptide and Glu binding together increase the affinity of carboxylase for the vitamin K cofactor. Osteocalcin is unusual among VKD proteins in having a low-affinity propeptide and high-affinity sequence in the mature protein that binds a different site in the carboxylase (which is undefined and not indicated here). Carboxylation is initiated when the catalytic base deprotonates reduced vitamin K (described in Figure 1), and the Glu substrate may regulate carboxylation by activating the catalytic base, i.e., to increase its reactivity, as indicated by the dashed arrow. The Glu and vitamin K binding sites and catalytic base are drawn as spatially distinct entities for the purpose of illustration and almost certainly exist in a tightly coordinated complex during catalysis. Multiple Glus in the Gla domain of VKD substrates are carboxylated, which is accomplished by a processive mechanism.

by mutagenesis experiments (21). Nonetheless, the propeptides show substantial differences in carboxylase affinity when tested for their ability to compete with a truncated factor IX substrate that contains the propeptide and Gla domain (66). The osteocalcin propeptide shows the lowest affinity ($K_i > 500\ \mu M$), consistent with the divergence of two of the three highly conserved propeptide residues (at positions –10 and –6). However, even VKD propeptides that have all three of the conserved residues show up to a 100-fold difference in carboxylase affinity, with the factor X propeptide showing the highest and prothrombin and protein C propeptides the lowest affinities [K_i's of 3 nM or ~200 nM, respectively (66)]. Thus, other propeptide residues are also important to VKD protein-carboxylase interaction, and residues -18, -17, -15, and -9 have been functionally implicated (21).

Interestingly, the carboxylase has high affinity for mature osteocalcin, i.e., osteocalcin that does not contain the propeptide. Thus, the K_m for mature osteocalcin (~2 μM) is ~100- to 1000-fold lower than small Glu-containing peptides lacking the propeptide domain (26) and at least 70-fold lower than the factor IX Gla domain (68). Competition experiments indicate that mature osteocalcin does not bind the carboxylase through its propeptide binding site (26) nor is the high affinity simply due to osteocalcin Glu residue interaction with the carboxylase Glu binding site (27). Thus, the carboxylase has at least two sites for mediating high-affinity binding of VKD proteins: the propeptide binding site (Figure 2) that confers tight interaction with most of the VKD proteins and a second site that has not yet been identified that mediates high-affinity binding to mature osteocalcin.

Substrate binding causes allosteric changes that regulate carboxylation. Thus, free propeptide stimulates carboxylase activity on noncovalently attached Glu-containing substrates through a decrease in their K_m (14, 28). In addition, substrates in which the propeptide and Glu-containing sequences are covalently attached increase the affinity of the carboxylase for the vitamin K cofactor (63). Covalent attachment introduces a conformational constraint because the carboxylase Glu- and propeptide-binding sites are physically linked by the substrate (Figure 2), and this linkage may cause a structural change in the carboxylase that increases vitamin K binding. Alternatively, covalent attachment may generate a specific VKD structure that activates the carboxylase at a site distinct from Glu or propeptide binding, leading to increased vitamin K affinity. Regardless of the mechanism, the consequence of VKD protein binding regulating vitamin K affinity is increased efficiency of vitamin K utilization, which is important because the availability of vitamin K appears to control carboxylation in vivo (discussed below). Mutations in the carboxylase that decrease its affinity for the Glu or propeptide domain of VKD proteins might then be predicted to affect vitamin K binding, which is in fact the case: Patients with such mutations (described in more detail below) show at least partial correction for carboxylase function when administered supraphysiological doses of vitamin K (11, 64).

The vitamin K cofactor required by the carboxylase is the reduced form, KH_2 (Figure 1), which is generated largely by recycling of the vitamin K epoxide product (KO, Figure 1*C*). Recycling is accomplished by the combined actions of

VKOR (vitamin K oxidoreductase), whose gene was recently identified (34, 57), and a redox protein that supplies the electrons to VKOR (Figure 3), whose identity is not yet known. VKOR is an integral membrane enzyme in the ER, like the carboxylase, and is the target of anticoagulant therapy with coumarin inhibitors such as warfarin (73). VKOR exhibits broad tissue distribution (57), consistent with the widespread expression of carboxylase and VKD proteins. At least one tissue also has an alternative pathway for supporting carboxylation: Liver contains a high K_m quinone reductase that reduces the quinone (but not epoxide) form of vitamin K to KH_2 and therefore sustains carboxylation when dietary concentrations of vitamin K are high (Figure 1*C*).

The mechanism by which VKOR supplies KH_2 to the carboxylase is unknown. Each Glu to Gla conversion requires one molecule of KH_2 (Figure 1), and so the carboxylation of an individual VKD protein depends upon VKOR recycling of several vitamin K molecules (e.g., nine for protein C and the example shown in Figure 3). VKOR may provide KH_2 to the carboxylase through a direct interaction between the active sites of the two enzymes. Alternatively, carboxylase and VKOR may be physically distinct, with vitamin K shuttling between the enzymes by migration through the membrane, analogous to how the structurally similar ubiquinone functions in mitochondrial respiration.

A practical note on carboxylase interaction with cofactors and substrates is that a major advance in defining such interactions has been the production of active carboxylase in *Spodoptera frugiperda* cells, which do not express either endogenous carboxylase or VKD proteins (59). Older studies used tissue microsomes, where interpretation of results is complicated by the fact that the carboxylase is bound to VKD protein. Even carboxylase purified from tissue can have limited use because the preparations contain propeptide, used in the purification to separate carboxylase from VKD protein. A second area in which method development could significantly advance our understanding of VKD protein-carboxylase interaction is the generation and analysis of more natural VKD substrates. Studies have largely used small peptides derived from the Gla domain in combination with free or covalently attached propeptide, and a substrate that contains both propeptide and the entire Gla domain has been developed for only one VKD protein, factor IX (68). The natural (i.e., propeptide-containing, full-length) VKD substrates are difficult to prepare because they are large macromolecules. However, the development of these substrates will be valuable in addressing important questions, such as whether the carboxylase exhibits as wide a range in affinities for the propeptide-containing, full-length VKD substrates as it does for the propeptides.

CARBOXYLATION OCCURS DURING THE SECRETION OF VKD PROTEINS

VKD proteins engage the carboxylase in the ER during their secretion (Figure 3), and components of the quality control machinery therefore affect carboxylation. Assembly of VKD protein-carboxylase complexes is likely to be mediated by

chaperones, as demonstrated for other multisubunit complexes (24), but with the unusual twist that assembly forms a productive enzyme-substrate complex. The identity of the specific chaperones, and whether all VKD proteins use the same chaperones for assembly with the carboxylase, is not known. Also unknown is which cargo receptors, a class of proteins that mediate the exit of proteins out of the ER, facilitate the exit of VKD proteins released from the carboxylase (Figure 3). Translocation of the VKD proteins to the Golgi compartment results in additional posttranslational modifications that include proteolytic removal of the propeptide and, for various VKD proteins, N- and O-glycosylation, sulfation, phosphorylation, and aspartyl beta-hydroxylation.

THE CARBOXYLASE IS A PROCESSIVE ENZYME

All VKD proteins contain multiple Glus that undergo carboxylation (Figure 2), which is accomplished by a processive mechanism. Carboxylase processivity was initially suggested by the observation that a propeptide-Gla domain substrate is multiply carboxylated (42), and was unequivocally demonstrated by studies showing that factor IX-carboxylase complex incubated in the presence of "challenge" VKD protein results in full carboxylation of the factor IX before its release and replacement by the challenge protein (70). Thus, a single binding event between carboxylase and VKD protein can give rise to all of the Glu to Gla conversions in the VKD protein. Carboxylation is limited to the Glus residing within the Gla domain, which may be due to a minimum/maximum distance requirement (of ~5–40 amino acids) between the carboxylatable Glus and the propeptide and/or to a restriction imposed by a second site of VKD protein-carboxylase interaction (discussed below).

Processive carboxylation results from a slower rate of dissociation than catalysis (Figure 3), raising the question of how VKD proteins remain tethered to the carboxylase to maintain a slow rate of dissociation. For most VKD proteins, the high-affinity propeptide must be important to tethering, whereas in osteocalcin the high-affinity binding site in the mature protein (discussed above) may be more important. However, osteocalcin also contains a low-affinity propeptide that confers a second site of carboxylase interaction. In addition, the carboxylase contains a second, low-affinity site of interaction with VKD proteins like factor IX and prothrombin (50). Thus, all VKD proteins may bind carboxylase through two contact sites, one low-affinity and one high-affinity, with osteocalcin having reversed affinities to that of the other VKD proteins (Figure 4). Two sites of VKD protein-carboxylase interaction could increase the efficiency of carboxylation by clamping the Gla domain near the carboxylase active site to facilitate translocation of the multiple Glus and Glas, and studies do in fact suggest that propeptide-containing osteocalcin is more efficiently carboxylated than osteocalcin lacking the propeptide (4). A mechanism that involves two contact sites is also of interest with regard to whether there are differences in the efficiency of processivity among individual VKD proteins. As discussed above, the carboxylase affinity at one of the sites,

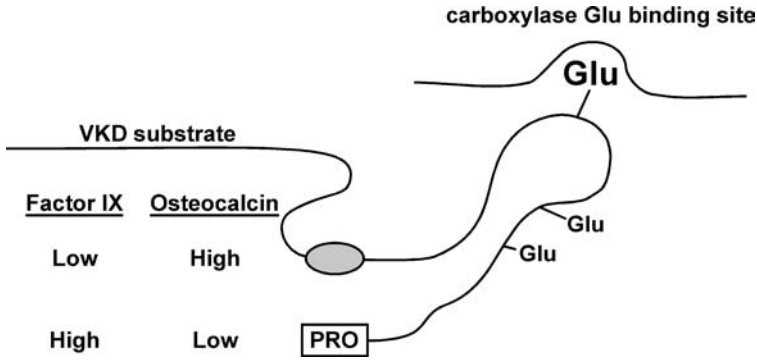


Figure 4 Two sites of vitamin K–dependent (VKD) protein-carboxylase interaction. Most VKD proteins (e.g., factor IX) have a high-affinity propeptide (PRO) and a second sequence (the shaded oval) of unknown identity that mediates low-affinity binding to the carboxylase. Osteocalcin has a low-affinity propeptide and a high-affinity domain of unknown identity. Whether the high-affinity osteocalcin and low-affinity factor IX sequences bind the same carboxylase site is also unknown. Two sites of VKD protein-carboxylase interaction can facilitate efficient carboxylation by juxtaposing the Gla domain near the carboxylase active site and can explain why carboxylation is restricted to the Gla domain.

i.e., the propeptide binding site, varies widely for different VKD proteins. However, the second site of VKD protein-carboxylase interaction could significantly impact the overall affinity, especially if there are differences in affinity at this second site.

Both partially carboxylated intermediates and fully carboxylated VKD product have the propeptide that mediates carboxylase interaction, raising the question of whether there is some change at the end of the reaction that effects preferential release of the fully carboxylated product. One change that marks when carboxylation is finished is the loss of Glu binding, and propeptide binding has been shown to be weaker in the absence of Glu substrate (47), which indicates that the effect of propeptide on Glu binding (discussed above) is reciprocated. However, loss of Glu binding is probably not the only factor that facilitates release because carboxylase isolated from r-carboxylase, factor IX–expressing cells remains associated with fully carboxylated factor IX (23), which indicates a dissociation rate far too slow to reconcile with normal in vivo turnover. This study also suggested that r-carboxylase overexpression titrates out a factor (e.g., excess VKD protein or a quality control component) that facilitates carboxylated VKD protein release.

DISRUPTION OF PROCESSIVITY

Carboxylase processivity is disrupted when vitamin K is limiting, for example during therapy with warfarin, which inhibits VKOR to cause a decreased rate of catalysis and premature dissociation of partially carboxylated VKD proteins

(Figure 3). The importance of the relative rates of catalysis versus dissociation to processivity was dramatically revealed by the phenotype of hemophiliacs who show an unusual sensitivity to warfarin (15, 45, 65). These patients exhibited normal hemostasis until they underwent warfarin therapy, which caused a selective decrease in factor IX activity, i.e., to ~1% of normal levels, which was well below the activities of other hemostatic VKD proteins (~30%–40%). Sequence analysis identified two independent mutations in the factor IX propeptide at either position –9 or at the highly conserved –10 position, and functional analysis showed substantial decreases in the affinity of these factor IX mutants for the carboxylase (15, 65). The weakened affinity of the mutant propeptides, then, caused an increased rate of dissociation that only became significant when the rate of catalysis was slowed by warfarin treatment.

Interestingly, the carboxylase affinity of the factor IX propeptide with the mutation at the –9 position is almost identical to that of the propeptides for protein C and prothrombin (66), yet neither of these VKD proteins shows a selective decrease in activity during warfarin anticoagulation (11, 38, 64). Thus, protein C and prothrombin, like factor X, are reduced to the 30%–40% activity level despite the fact that the carboxylase affinity for the factor X propeptide is about 100-fold higher than for that of the protein C and prothrombin propeptides (66). If the propeptide alone determines the rate of dissociation of VKD proteins from the carboxylase, then protein C and prothrombin would be predicted to behave like the factor IX mutant, i.e., showing a selectively larger decrease during warfarin therapy. The similarity in response of prothrombin, protein C, and factor X to warfarin therefore suggests that while there may be differences in carboxylase interaction and processivity among individual VKD proteins, they may not be as vast as suggested by the differences in propeptide affinities. Modulation of carboxylase interaction and processivity could be due to intrinsic effects, e.g., from the second site of VKD protein-carboxylase interaction, or extrinsic effects, i.e., from quality control components (discussed below).

The factor IX propeptide mutants were well carboxylated when vitamin K was available (i.e., in the absence of warfarin), which indicates that normally the rate of catalysis is so much faster than dissociation that processive carboxylation is not disrupted even by weakened affinity. This observation is of interest with respect to a separate study that analyzed a prothrombin mutant in which the Gla domain was deleted and the propeptide was fused to an unrelated Glu-rich portion of the molecule (22). Expression of this mutant in cultured cells produced a secreted protein that was multiply carboxylated. Thus, so long as vitamin K in the cultured cells supports a high rate of catalysis, propeptide tethering confers a rate of dissociation sufficiently slow to result in multiple carboxylations, even of an irrelevant sequence that may not have a second site of VKD protein-carboxylase interaction. It would be of interest to see whether the mutant prothrombin shows decreased processivity compared with normal prothrombin when tested at low vitamin K concentrations, where low rates of catalysis can reveal differences in the efficiency of carboxylation.

QUALITY CONTROL AND CARBOXYLATION

A full understanding of VKD protein carboxylation must consider how the secretory process affects carboxylation. One example is whether VKD proteins compete for carboxylase binding: Thus, multiple VKD proteins are expressed in some tissues [e.g., osteocalcin, Gas 6, protein S and matrix Gla protein in bone, and seven hemostatic VKD proteins in liver (7)] and these proteins may not bind equivalently to the carboxylase if there are large differences in affinities. However, as discussed above, the association of VKD proteins and carboxylase almost certainly is mediated by chaperones (Figure 3), and the increase in local concentration of VKD proteins and carboxylase due to the chaperones could override the differences in VKD protein affinities. Cargo receptors also have the potential to influence carboxylation by translocating VKD proteins to a different compartment, the Golgi, where the VKD proteins no longer have the opportunity to bind carboxylase. Consequently, under normal conditions, i.e., when reduced vitamin K is available to confer a high rate of catalysis, there may not be competition between VKD proteins. In contrast, during warfarin therapy the decreased rate of catalysis results in the accumulation of a precursor pool (because carboxylase turnover is slower but VKD protein synthesis still occurs) that disrupts the normal process. Precursor pool buildup may disrupt carboxylation directly, through competition between VKD proteins for the carboxylase, or indirectly, by saturating quality control components necessary for facilitating carboxylation. Studies in warfarin-treated tissue and cultured cells show a higher proportion of factor X than prothrombin complexed to carboxylase (18, 69), but it is unknown whether this result is due to differences in competition between prothrombin and factor X for initial binding to the carboxylase and/or to differences in the dissociation of prothrombin and factor X and/or to differences in quality control regulation. Thus, the multiple mechanisms involved in VKD protein carboxylation complicate interpretation of in vivo studies. Interpretation is even more difficult when the analysis is performed on secreted VKD proteins, e.g., those present in blood or secreted from cultured cells, because of an additional component of quality control: Undercarboxylated and uncarboxylated VKD proteins are preferentially degraded (i.e., versus fully carboxylated protein) by the proteasome pathway (76, 84). Consequently, this filtering can generate a secreted VKD protein subpopulation different from the original carboxylation product population. In summary, then, secreted VKD proteins represent the endpoint of a complex process that must be considered when extrapolating in vitro results to what actually occurs in vivo.

CELL LINES AS MODEL SYSTEMS FOR ANALYZING CARBOXYLATION

Many of the r-VKD proteins have been expressed in mammalian cells, where inefficient carboxylation often occurs. Thus, fully carboxylated VKD proteins are secreted when expression levels are low but uncarboxylated and undercarboxylated

forms are observed when the levels are high. Individual VKD proteins do not all respond the same; for example, secreted factor X is a mixture of uncarboxylated and fully carboxylated, but not partially carboxylated, protein, whereas secreted factor IX comprises all of these forms (5, 33). Variation among individual VKD proteins could be due to differences in their affinities for the carboxylase and/or how they are processed by quality control mechanisms (e.g., the efficiency of degradation of undercarboxylated forms by the proteosome pathway may not be the same for all VKD proteins). Overexpression of r-carboxylase does not improve the efficiency of VKD protein carboxylation even though the recombinant carboxylase is functional, i.e., showing an increase in activity proportional to the amount of overexpressed protein (23, 52). Inefficient carboxylation has confounded the functional analysis of r-VKD proteins, and has been a major impediment in producing large amounts of therapeutic VKD proteins for applications like treatment of hemophilia and sepsis.

Intracellular analyses of cell lines that express r-factor IX and either endogenous or r-carboxylase suggest that the reason r-carboxylase does not improve carboxylation efficiency is because it saturates the supply of reduced vitamin K. Thus, r-carboxylase overexpression results in a proportional increase in the number of intracellular factor IX-carboxylase complexes but not in the total rate of factor IX carboxylation (23). Pulse-chase analysis indicates that there is a threshold amount of reduced vitamin K sufficient for rapid catalysis of factor IX-carboxylase complexes in cells expressing endogenous carboxylase but not in cells overexpressing r-carboxylase (23). r-Carboxylase overexpression may saturate VKOR capacity in supplying reduced vitamin K (Figure 3), and studies that test the ability of r-VKOR to improve carboxylation will be of interest.

Although vitamin K availability can explain why r-carboxylase does not improve carboxylation, it cannot explain why high-level VKD protein expression causes the secretion of undercarboxylated protein. Thus, stably transfected clones isolated from the same progenitor cell line that express either high or low levels of an r-VKD protein have the same amount of endogenous carboxylase and reduced vitamin K, and therefore the rate of carboxylation of VKD protein-carboxylase complexes should be the same in both cell lines. The secretion of undercarboxylated protein must therefore be due to an increase in the rate of dissociation rather than a decrease in the rate of catalysis, which indicates that high-level VKD protein expression somehow perturbs the VKD protein-carboxylase complex. High-level expression leads to a large VKD precursor pool (Figure 3) that may perturb the complex by saturating the normal process of carboxylation facilitated by quality control components.

The events resulting from high-level expression are similar to those caused by warfarin, which also results in a large precursor pool (discussed above) and the secretion of undercarboxylated protein [shown for prothrombin (40)]. These similarities raise the question of whether the increased dissociation of partially carboxylated VKD proteins during warfarin therapy is actually due to two different effects: (*a*) a slow catalytic rate that increases the probability of

dissociation and (*b*) a large precursor pool that disrupts the complexes through an unknown mechanism. In reconsidering the factor IX mutants that show selective decreases in activity during warfarin therapy (discussed above), then, the preferential dissociation of the mutants may be due not only to a decrease in the rate of catalysis but also to precursor pool buildup that increases dissociation, with the mutants being more susceptible because of their weakened affinity for the carboxylase.

IS CARBOXYLATION EFFICIENCY THE SAME IN ALL TISSUES?

Vitamin K availability and consequent effect on the rate of catalysis impacts the efficiency of processive carboxylation, but whether this efficiency is the same in all tissues is unknown. Vitamin K is a family of forms comprising phylloquinone and menaquinones (Figure 1*B*), which share a common 2-methyl 1,4-naphthoquinone nucleus and differ by the polyisoprenoid at the 3-position (the "R" in Figure 1*A*). The main source of phylloquinone is green, leafy vegetables, whereas menaquinones, in the American diet, are obtained primarily from animal products. Phylloquinone and menaquinones show differences in tissue distribution and in lability (reviewed in 7, 9), which could impact their intracellular concentrations, and the functional contribution of these forms to in vivo carboxylation is not well understood. The quality of the diet therefore has the potential to vary the form and concentration of vitamin K in different tissues, which may impact the efficiency of processive carboxylation. This efficiency has important physiological consequences since full carboxylation is required for activity, as shown by the extensive characterization of hemostatic VKD proteins.

The carboxylation status in the general population has been assessed for only a subset of VKD proteins, i.e., hemostatic VKD proteins and osteocalcin. The hemostatic proteins are well carboxylated, and their main site of synthesis is liver, which is well vascularized to receive dietary vitamin K absorbed from the gut into blood. Whether dietary vitamin K is as equally available to other tissues is unknown. Undercarboxylated osteocalcin is frequently observed in the general population (8), which indicates that incomplete carboxylation occurs in bone where osteocalcin is synthesized. The extent of undercarboxylation is unknown because the analysis is indirect, i.e., on blood samples rather than bone tissue. Osteocalcin undercarboxylation could be due to decreased processivity that results from inefficient vitamin K delivery to bone and a consequent slow rate of catalysis, or could be due to a less processive mechanism because of the unique way osteocalcin interacts with the carboxylase (Figure 4). Evaluating other VKD proteins will clearly be of interest, given the number of additional physiologies also impacted by vitamin K.

STRUCTURE-FUNCTION ANALYSIS OF THE CARBOXYLASE

Identifying functional residues in the carboxylase has been difficult because it is an integral membrane enzyme without an available crystal structure or homology to other proteins that might provide clues to function. The general approaches used to identify functional carboxylase residues have included mutagenesis, biochemical mapping, interspecies homology comparison, and analysis of naturally occurring variants. The natural mutations are very rare: Only three have been reported so far, which is not surprising since carboxylation is required for a broad range of functions. The mutations are autosomal recessive and cause a combined defect in VKD hemostatic protein functions (11, 58, 64). The first carboxylase mutant identified, L394R (11), resides within a region (amino acids 374–418) that is evolutionarily highly conserved (2, 3, 16, 35, 78). L394R is impaired in both Glu and propeptide interaction (44), which is likely due to their reciprocal effects on carboxylase binding (discussed above) and which demonstrates the difficulties in designating residues as part of the Glu- or propeptide-binding site. Mutagenesis of neighboring residues revealed a mutant, Y395A, with normal propeptide but defective Glu binding (43), supporting the identification of this region as part of the Glu-binding site (Figure 5). L394R and Y395A show small decreases in vitamin K affinity (43), consistent with the observed effect of VKD protein binding on vitamin K affinity discussed above, and high concentrations of vitamin K partially correct hemostasis in the patients with the L394R mutation (11).

A second natural variant, W501S (64), appears to be important to propeptide interaction, as this mutant is associated with decreased propeptide binding but normal carboxylation of a small Glu substrate lacking the propeptide (62). W501 resides within a region (amino acids 495–518) that, interestingly, shares homology with VKD propeptides, and this similarity led to an initial proposal that this region is an autoregulatory domain that restricts carboxylation to propeptide-containing substrates (49). However, mutational analysis of W501S (62), and of mutants substituted at neighboring residues (36), suggests that this region may instead be the propeptide binding site (Figure 5). Like L394R, the W501S mutant shows a decrease in vitamin K affinity (62), and hemostasis can be partially corrected in patients by vitamin K supplementation (64). W501 is located in one of the two regions cross-linked by propeptide in vitro (83, 85; Figure 5) and is near R513, which also has been proposed to have a role in propeptide binding (71). R513 was identified by scanning mutagenesis, which also implicated the charged residue clusters R234/H235 and R406/H408 as important for propeptide interaction.

Other mutants that implicate functionally important residues are R485P, a third naturally occurring variant, which has not yet been characterized (58), K218A, which appears to be completely inactive (61), and C99A and C450A, which show decreases in activity that are propeptide dependent (54). Other relevant regions

include the N-terminus (i.e., amino acids 1–218; Figure 5), which has been functionally implicated by covalent modification with a suicide substrate (29), and the site of low-affinity VKD protein-carboxylase interaction (discussed above), which includes residues C343 and Y345 (50) and may also include R359 and H360 (61, 71). Sequences not yet identified include the catalytic base, the high-affinity osteocalcin binding site, and the vitamin K–binding domain.

One additional unknown is the membrane topology of the carboxylase. A key question regarding topology is how the carboxylase active site accesses both hydrophobic vitamin K and hydrophilic VKD proteins (that contain a basic propeptide and acidic Gla domain). The carboxylase sequence predicts seven hydrophobic regions (Figure 5), five of which act as signal anchor sequences in an in vitro assay (74), and the assumption that the hydrophobic regions are transmembrane in nature has led to a model in which the carboxylase resembles a seven-transmembrane receptor (Figure 6*A*). However, hydrophobic sequences can also form a different type of structural motif, observed in a new class of integral membrane enzymes whose crystal structures have recently been determined (reviewed in 10). These hydrophobic sequences run parallel to the lipid bilayer and insert into only one leaflet of the membrane (i.e., they are monotopic), thereby forming a tight interface between the membrane and aqueous environment (Figure 6*B*). A commonality of these enzymes, which are all structurally unrelated, is that they utilize hydrophobic substrates. Thus, the monotopic domains form an entrance that allows the hydrophobic substrates to move from the phospholipid bilayer into the active site, located in the cytoplasm or ER lumen. With the carboxylase, then, some of the hydrophobic sequences may form an entryway that allows vitamin K in the membrane to access the lumenal active site. At present, it is not possible to extrapolate broad generalities from this new class of enzymes to the carboxylase: Only a handful of crystal structures are known and they reveal significant diversity (e.g., some have both monotopic and transmembrane sequences while others are only monotopic). Their characterization demonstrates the uncertainty in predicting membrane topology; for example, the cytochrome P450 sequence predicts a transmembrane domain (shown in Figure 6*B*) (80) that is not supported by nuclear magnetic resonance analysis (10). The carboxylase must have at least one transmembrane sequence because it has a lumenal active site (13) but no leader sequence (82); however, the complexities described here indicate a need for further studies to define the actual topology. Given this limited understanding, designating residues as lumenal or cytoplasmic simply on the basis of the proposed topological structure (Figure 6*A*), as recently done (37), is premature.

NONMAMMALIAN CARBOXYLASES

The *Conus* carboxylase, like the mammalian ortholog, has epoxidase and carboxylase activities that are dependent upon vitamin K (2, 16). *Conus* VKD substrates have the same organization as that of mammals, i.e., with a propeptide adjacent to

the Glus undergoing carboxylation. However, there are differences: Carboxylation and subsequent propeptide cleavage in *Conus* results in small (10–20 amino acid) peptides that are not homologous to the larger mammalian VKD Gla domains (~40 amino acids) and that contain fewer Glas (2–5 in *Conus* versus up to 13 for the mammalian VKD proteins). The mammalian and *Conus* propeptides are not homologous, which likely reflects the fact that while the mammalian propeptide is restricted to carboxylated proteins, the *Conus* propeptide is also present in peptides that are not carboxylated. Thus, the *Conus* propeptide has an additional function unrelated to carboxylation, which is proposed to be directing the proper disulfide bond formation and folding of these highly disulfide-bonded and conformationally constrained peptides (81). In *Conus* peptides that are carboxylated, the propeptide is also a carboxylase recognition signal: The propeptide increases carboxylase affinity for the Glu substrate (1, 12), although the effect is much smaller than observed in the mammalian system (i.e., ~10-fold versus 1000-fold affinity increases). The specific residues in the *Conus* propeptide that mediate carboxylase binding have been difficult to identify. Early studies suggested the importance of hydrophobic residues (12) that were later ruled out, and basic residues have been proposed both to be (25) and to not be (1) important to carboxylase recognition. The *Conus* carboxylase can modify a mammalian propeptide-containing Glu substrate (16): Carboxylation is observed with *Conus* carboxylase isolated from *Spodoptera frugiperda* cells (16) but not from tissue (67), and the difference is probably due to the presence of interfering VKD substrates in the tissue.

Drosophila VKD proteins have not been isolated nor have orthologs been revealed by homology searches with mammalian proteins, and so the role of carboxylation in this organism is unknown. Carboxylase activity that depends on vitamin K has been demonstrated for the *Drosophila* carboxylase on small Glu-containing substrates (35, 78), and this ortholog contains the conserved sequences thought to be part of the Glu binding site (Figure 5) in mammalian carboxylases. Thus, the conversion of Glus to Glas is a likely function for the *Drosophila* carboxylase.

In contrast to *Conus* and *Drosophila*, bacterial orthologs of the carboxylase are divergent in the Glu binding region. As discussed above, these orthologs are thought to have been acquired by horizontal transfer of metazoan sequences, which may not have included transfer of VKD substrates. One intriguing possibility, then, is that the bacterial orthologs have adapted the ability to generate the strong vitamin K base (Figure 1) for some other metabolic use. Determining whether these orthologs even have epoxidase activity, then, will clearly be of interest.

FUTURE PERSPECTIVES

The carboxylase field is in an exciting phase, with recent significant advances having been made toward understanding the mechanism of carboxylation but with multiple areas yet to explore. Important goals include determining how processive carboxylation is accomplished, defining the carboxylase active site, and obtaining structural information. Determining how VKOR supplies reduced vitamin K to

the carboxylase and how quality control components affect carboxylation also will be critical to understanding the overall process. Finally, the function of the carboxylase in invertebrates and bacteria should be an interesting area of discovery.

ACKNOWLEDGMENTS

The author thanks Drs. Kurt Runge, Mark Rishavy, and John Suttie for their helpful comments on the review. This work is supported by NIH55666.

The *Annual Review of Nutrition* is online at http://nutr.annualreviews.org

LITERATURE CITED

1. Bandyopadhyay PK, Colledge CJ, Walker CS, Zhou LM, Hillyard DR, Olivera BM. 1998. Conantokin-G precursor and its role in gamma-carboxylation by a vitamin K-dependent carboxylase from a *Conus* snail. *J. Biol. Chem.* 273:5447–50
2. Bandyopadhyay PK, Garrett JE, Shetty RP, Keate T, Walker CS, Olivera BM. 2002. gamma-Glutamyl carboxylation: an extracellular posttranslational modification that antedates the divergence of molluscs, arthropods, and chordates. *Proc. Natl. Acad. Sci. USA* 99:1264–69
3. Begley GS, Furie BC, Czerwiec E, Taylor KL, Furie GL, et al. 2000. A conserved motif within the vitamin K-dependent carboxylase gene is widely distributed across animal phyla. *J. Biol. Chem.* 275:36245–49
4. Benton ME, Price PA, Suttie JW. 1995. Multi-site-specificity of the vitamin K-dependent carboxylase: in vitro carboxylation of des-gamma-carboxylated bone Gla protein and Des-gamma-carboxylated pro bone Gla protein. *Biochemistry* 34: 9541–51
5. Berkner KL. 1993. Expression of recombinant vitamin K-dependent proteins in mammalian cells: factors IX and VII. *Methods Enzymol*. 222:450–77
6. Berkner KL, Pudota BN. 1998. Vitamin K-dependent carboxylation of the carboxylase. *Proc. Natl. Acad. Sci. USA* 95:466–71
7. Berkner KL, Runge KW. 2004. The physiology of vitamin K nutriture and vitamin K-dependent protein function in atherosclerosis. *J. Thromb. Haemost.* 2:2118–32
8. Binkley NC, Krueger DC, Engelke JA, Foley AL, Suttie JW. 2000. Vitamin K supplementation reduces serum concentrations of under-γ-carboxylated osteocalcin in healthy young and elderly adults. *Am. J. Clin. Nutr.* 72:1523–28
9. Booth SL, Suttie JW. 1998. Dietary intake and adequacy of vitamin K. *J. Nutr.* 128:785–88
10. Bracey MH, Cravatt BF, Stevens RC. 2004. Structural commonalities among integral membrane enzymes. *FEBS Lett*. 567:159–65
11. Brenner B, Sanchez-Vega B, Wu SM, Lanir N, Stafford DW, Solera J. 1998. A missense mutation in gamma-glutamyl carboxylase gene causes combined deficiency of all vitamin K-dependent blood coagulation factors. *Blood* 92:4554–59
12. Bush KA, Stenflo J, Roth DA, Czerwiec E, Harrist A, et al. 1999. Hydrophobic amino acids define the carboxylation recognition site in the precursor of the gamma-carboxyglutamic-acid-containing conotoxin epsilon-TxIX from the marine cone snail *Conus* textile. *Biochemistry* 38:14660–66
13. Carlisle TL, Suttie JW. 1980. Vitamin K dependent carboxylase: subcellular

location of the carboxylase and enzymes involved in vitamin K metabolism in rat liver. *Biochemistry* 19:1161–67

14. Cheung A, Engelke JA, Sanders C, Suttie JW. 1989. Vitamin K-dependent carboxylase: influence of the "propeptide" region on enzyme activity. *Arch. Biochem. Biophys.* 274:574–81
15. Chu K, Wu SM, Stanley T, Stafford DW, High KA. 1996. A mutation in the propeptide of factor IX leads to warfarin sensitivity by a novel mechanism. *J. Clin. Invest.* 98:1619–25
16. Czerwiec E, Begley GS, Bronstein M, Stenflo J, Taylor K, et al. 2002. Expression and characterization of recombinant vitamin K-dependent gamma-glutamyl carboxylase from an invertebrate, *Conus* textile. *Eur. J. Biochem.* 269:6162–72
17. Dam H. 1935. The antihaemorrhagic vitamin of the chick. Occurrence and chemical nature. *Nature (Lond.)* 135:652–53
18. DeMetz M, Vermeer C, Soute BAM, Van Scharrenburg GJM, Slotboom AJ, Hemker HC. 1981. Partial purification of bovine liver vitamin K-dependent carboxylase by immunospecific adsorption onto antifactor X. *FEBS Lett.* 123:215–18
19. Dowd P, Hershline R, Ham SW, Naganathan S. 1995. Vitamin K and energy transduction: a base strength amplification mechanism. *Science* 269:1684–91
20. Fraser JD, Price PA. 1988. Lung, heart, and kidney express high levels of mRNA for the vitamin K-dependent matrix Gla protein. Implications for the possible functions of matrix Gla protein and for the tissue distribution of the gamma-carboxylase. *J. Biol. Chem.* 263:11033–36
21. Furie B, Bouchard BA, Furie BC. 1999. Vitamin K-dependent biosynthesis of gamma-carboxyglutamic acid. *Blood* 93:1798–808
22. Furie BC, Ratcliffe JV, Tward J, Jorgensen MJ, Blaszkowsky LS, et al. 1997. The gamma-carboxylation recognition site is sufficient to direct vitamin K-dependent carboxylation on an adjacent glutamate-rich region of thrombin in a propeptide-thrombin chimera. *J. Biol. Chem.* 272:28258–62
23. Hallgren KW, Hommema EL, McNally BA, Berkner KL. 2002. Carboxylase overexpression impairs factor IX secretion: implications for the release of vitamin K-dependent proteins. *Biochemistry* 41:15045–55
24. Hammond C, Helenius A. 1995. Quality control in the secretory pathway. *Curr. Opin. Cell. Biol.* 7:523–29
25. Hansson K, Ma X, Eliasson L, Czerwiec E, Furie B, et al. 2004. The first gamma-carboxyglutamic acid-containing contryphan. A selective L-type calcium ion channel blocker isolated from the venom of *Conus marmoreus*. *J. Biol. Chem.* 279:32453–63
26. Houben RJ, Jin D, Stafford DW, Proost P, Ebberink RH, et al. 1999. Osteocalcin binds tightly to the gamma-glutamylcarboxylase at a site distinct from that of the other known vitamin K-dependent proteins. *Biochem. J.* 341:265–69
27. Houben RJ, Rijkers DT, Stanley TB, Acher F, Azerad R, et al. 2002. Characteristics and composition of the vitamin K-dependent gamma-glutamyl carboxylase-binding domain on osteocalcin. *Biochem. J.* 364:323–28
28. Knobloch JE, Suttie JW. 1987. Vitamin K-dependent carboxylase. Control of enzyme activity by the "propeptide" region of factor X. *J. Biol. Chem.* 262:15334–37
29. Kuliopulos A, Nelson NP, Yamada M, Walsh CT, Furie B, et al. 1994. Localization of the affinity peptide-substrate inactivator site on recombinant vitamin K-dependent carboxylase. *J. Biol. Chem.* 269:21364–70
30. Kulman JD, Harris JE, Haldeman BA, Davie EW. 1997. Primary structure and tissue distribution of two novel proline-rich gamma-carboxyglutamic acid

proteins. *Proc. Natl. Acad. Sci. USA* 94:9058–62
31. Kulman JD, Harris JE, Xie L, Davie EW. 2001. Identification of two novel transmembrane gamma–carboxyglutamic acid proteins expressed broadly in fetal and adult tissues. *Proc. Natl. Acad. Sci. USA* 98:1370–75
32. Kuo WL, Stafford DW, Cruces J, Gray J, Solera J. 1995. Chromosomal localization of the gamma-glutamyl carboxylase gene at 2p12. *Genomics* 25:746–48
33. Larson PJ, Camire RM, Wong D, Fasano NC, Monroe DM, et al. 1998. Structure/function analyses of recombinant variants of human factor Xa: Factor Xa incorporation into prothrombinase on the thrombin-activated platelet surface is not mimicked by synthetic phospholipid vesicles. *Biochemistry* 37:5029–38
34. Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. 2004. Identification of the gene for vitamin K epoxide reductase. *Nature* 427:541–44
35. Li T, Yang CT, Jin D, Stafford DW. 2000. Identification of a *Drosophila* vitamin K-dependent gamma-glutamyl carboxylase. *J. Biol. Chem.* 275:18291–96
36. Lin PJ, Jin DY, Tie JK, Presnell SR, Straight DL, Stafford DW. 2002. The putative vitamin K-dependent gamma-glutamyl carboxylase internal propeptide appears to be the propeptide binding site. *J. Biol. Chem.* 277:28584–91
37. Lin PJ, Straight DL, Stafford DW. 2004. Binding of the factor IX gamma-carboxyglutamic acid domain to the vitamin K-dependent gamma-glutamyl carboxylase active site induces an allosteric effect that may ensure processive carboxylation and regulate the release of carboxylated product. *J. Biol. Chem.* 279:6560–66
38. Lind SE, Callas PW, Golden EA, Joyner KA Jr, Ortel TL. 1997. Plasma levels of factors II, VII and X and their relationship to the international normalized ratio during chronic warfarin therapy. *Blood Coagul. Fibrinolysis* 8:48–53
39. Liu Y, Nelson AN, Lipsky JJ. 1996. Vitamin K-dependent carboxylase: mRNA distribution and effects of vitamin K-deficiency and warfarin treatment. *Biochem. Biophys. Res. Commun.* 224: 549–54
40. Malhotra OP. 1987. Purification and characterization of dicoumarol-induced prothrombins. Evidence of 5-Gla variant with two (or more) polypeptide chains. *Thromb. Res.* 47:485–92
41. Manfioletti G, Brancolini C, Avanzi G, Schneider C. 1993. The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. *Mol. Cell. Biol.* 13:4976–85
42. Morris DP, Stevens RD, Wright DJ, Stafford DW. 1995. Processive post-translational modification. Vitamin K-dependent carboxylation of a peptide substrate. *J. Biol. Chem.* 270:30491–98
43. Mutucumarana VP, Acher F, Straight DL, Jin DY, Stafford DW. 2003. A conserved region of human vitamin K-dependent carboxylase between residues 393 and 404 is important for its interaction with the glutamate substrate. *J. Biol. Chem.* 278:46488–93
44. Mutucumarana VP, Stafford DW, Stanley TB, Jin DY, Solera J, et al. 2000. Expression and characterization of the naturally occurring mutation L394R in human gamma-glutamyl carboxylase. *J. Biol. Chem.* 275:32572–77
45. Oldenburg J, Quenzel EM, Harbrecht U, Fregin A, Kress W, et al. 1997. Missense mutations at ALA-10 in the factor IX propeptide: an insignificant variant in normal life but a decisive cause of bleeding during oral anticoagulant therapy. *Br. J. Haematol.* 98:240–44
46. Pan LC, Price PA. 1985. The propeptide of rat bone gamma-carboxyglutamic acid protein shares homology with other

vitamin K-dependent protein precursors. *Proc. Natl. Acad. Sci. USA* 82:6109–13

47. Presnell SR, Tripathy A, Lentz BR, Jin DY, Stafford DW. 2001. A novel fluorescence assay to study propeptide interaction with gamma-glutamyl carboxylase. *Biochemistry* 40:11723–33
48. Price PA, Fraser JD, Metz VG. 1987. Molecular cloning of matrix Gla protein: implications for substrate recognition by the vitamin K-dependent gamma-carboxylase. *Proc. Natl. Acad. Sci. USA* 84:8335–39
49. Price PA, Williamson MK. 1993. Substrate recognition by the vitamin K-dependent gamma-glutamyl carboxylase: identification of a sequence homology between the carboxylase and the carboxylase recognition site in the substrate. *Protein Sci*. 2:1987–88
50. Pudota BN, Hommema EL, Hallgren KW, McNally BA, Lee S, Berkner KL. 2001. Identification of sequences within the γ-carboxylase that represent a novel contact site with vitamin K-dependent proteins and that are required for activity. *J. Biol. Chem.* 276:46878–86
51. Pudota BN, Miyagi M, Hallgren KW, West KA, Crabb JW, et al. 2000. Identification of the vitamin K-dependent carboxylase active site: Cys-99 and Cys-450 are required for both epoxidation and carboxylation. *Proc. Natl. Acad. Sci. USA* 97:13033–38
52. Rehemtulla A, Roth DA, Wasley LC, Kuliopulos A, Walsh CT, et al. 1993. In vitro and in vivo functional characterization of bovine vitamin K-dependent gamma-carboxylase expressed in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* 90:4611–15
53. Rezende SM, Simmonds RE, Lane DA. 2003. Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex. *Blood* 103:1192–201
54. Rishavy MA, Pudota BN, Hallgren KW, Qian W, Yakubenko AV, et al. 2004. A new model for vitamin K-dependent carboxylation: The catalytic base that deprotonates vitamin K hydroquinone is not Cys but an activated amine. *Proc. Natl. Acad. Sci. USA* 101:13732–37
55. Romero EE, Marvi U, Niman ZE, Roth DA. 2003. The vitamin K-dependent gamma-glutamyl carboxylase gene contains a TATA-less promoter with a novel upstream regulatory element. *Blood* 102:1333–39
56. Romero EE, Velazquez-Estades LJ, Deo R, Schapiro B, Roth DA. 1998. Cloning of rat vitamin K-dependent gamma-glutamyl carboxylase and developmentally regulated gene expression in postimplantation embryos. *Exp. Cell Res.* 243: 334–46
57. Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, et al. 2004. Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 427:537–41
58. Rost S, Fregin A, Koch D, Compes M, Muller CR, Oldenburg J. 2004. Compound heterozygous mutations in the gamma-glutamyl carboxylase gene cause combined deficiency of all vitamin K-dependent blood coagulation factors. *Br. J. Haematol.* 126:546–49
59. Roth DA, Rehemtulla A, Kaufman RJ, Walsh CT, Furie B, Furie BC. 1993. Expression of bovine vitamin K-dependent carboxylase activity in baculovirus-infected insect cells. *Proc. Natl. Acad. Sci. USA* 90:8372–76
60. Schultz J. 2004. HTTM, a horizontally transferred transmembrane domain. *Trends Biochem. Sci*. 29:4–7
61. Shimizu A, Sugiura I, Matsushita T, Kojima T, Hirai M, Saito H. 1998. Identification of the five hydrophilic residues (Lys-217, Lys-218, Arg-359, His-360, and Arg-513) essential for the structure and activity of vitamin K-dependent carboxylase. *Biochem. Biophys. Res. Commun.* 251:22–26

62. Soute BA, Jin DY, Spronk HM, Mutucumarana VP, Lin PJ, et al. 2004. Characteristics of recombinant W501S mutated human gamma-glutamyl carboxylase. *J. Thromb. Haemost.* 2:597–604
63. Soute BA, Ulrich MM, Watson AD, Maddison JE, Ebberink RH, Vermeer C. 1992. Congenital deficiency of all vitamin K-dependent blood coagulation factors due to a defective vitamin K-dependent carboxylase in Devon Rex cats. *Thromb. Haemost.* 68:521–25
64. Spronk HM, Farah RA, Buchanan GR, Vermeer C, Soute BA. 2000. Novel mutation in the gamma-glutamyl carboxylase gene resulting in congenital combined deficiency of all vitamin K-dependent blood coagulation factors. *Blood* 96:3650–52
65. Stanley TB, Humphries J, High KA, Stafford DW. 1999. Amino acids responsible for reduced affinities of vitamin K-dependent propeptides for the carboxylase. *Biochemistry* 38:15681–87
66. Stanley TB, Jin DY, Lin PJ, Stafford DW. 1999. The propeptides of the vitamin K-dependent proteins possess different affinities for the vitamin K-dependent carboxylase. *J. Biol. Chem.* 274:16940–44
67. Stanley TB, Stafford DW, Olivera BM, Bandyopadhyay PK. 1997. Identification of a vitamin K-dependent carboxylase in the venom duct of a *Conus* snail. *FEBS Lett*. 407:85–88
68. Stanley TB, Wu SM, Houben RJ, Mutucumarana VP, Stafford DW. 1998. Role of the propeptide and gamma-glutamic acid domain of factor IX for in vitro carboxylation by the vitamin K-dependent carboxylase. *Biochemistry* 37:13262–68
69. Stanton C, Ross P, Hutson S, Wallin R. 1995. Evidence for competition between vitamin K-dependent clotting factors for intracellular processing by the vitamin K-dependent gamma-carboxylase. *Thromb. Res.* 80:63–73
70. Stenina O, Pudota BN, McNally BA, Hommema EL, Berkner KL. 2001. Tethered processivity of the vitamin K-dependent carboxylase: Factor IX is efficiently modified in a mechanism which distinguishes Gla's from Glu's and which accounts for comprehensive carboxylation in vivo. *Biochemistry* 40:10301–9
71. Sugiura I, Furie B, Walsh CT, Furie BC. 1996. Profactor IX propeptide and glutamate substrate binding sites on the vitamin K-dependent carboxylase identified by site-directed mutagenesis. *J. Biol. Chem.* 271:17837–44
72. Sugiura I, Furie B, Walsh CT, Furie BC. 1997. Propeptide and glutamate-containing substrates bound to the vitamin K-dependent carboxylase convert its vitamin K epoxidase function from an inactive to an active state. *Proc. Natl. Acad. Sci. USA* 94:9069–74
73. Suttie JW. 1985. Vitamin K-dependent carboxylase. *Annu. Rev. Biochem.* 54: 459–77
74. Tie J, Wu SM, Jin D, Nicchitta CV, Stafford DW. 2000. A topological study of the human gamma-glutamyl carboxylase. *Blood* 96:973–78
75. Tie JK, Mutucumarana VP, Straight DL, Carrick KL, Pope RM, Stafford DW. 2003. Determination of disulfide bond assignment of human vitamin K-dependent gamma-glutamyl carboxylase by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Biol. Chem.* 278:45468–75
76. Tokunaga F, Wakabayashi S, Koide T. 1995. Warfarin causes the degradation of protein C precursor in the endoplasmic reticulum. *Biochemistry* 34:1163–70
77. Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, et al. 2004. Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304:66–74
78. Walker CS, Shetty RP, Clark KA, Kazuko SG, Letsou A, et al. 2001. On a potential global role for vitamin K-dependent gamma-carboxylation in animal systems: evidence for a gamma-glutamyl

carboxylase in *Drosophila*. *J. Biol. Chem.* 276:7769–74

79. Webb JH, Blom AM, Dahlbäck B. 2002. Vitamin K-dependent protein S localizing complement regulator C4b-binding protein to the surface of apoptotic cells. *J. Immunol.* 169:2580–86
80. Williams PA, Cosme J, Sridhar V, Johnson EF, McRee DE. 2000. Mammalian microsomal cytochrome P450 monooxygenase: structural adaptations for membrane binding and functional diversity. *Mol. Cell* 5:121–31
81. Woodward SR, Cruz LJ, Olivera BM, Hillyard DR. 1990. Constant and hypervariable regions in conotoxin propeptides. *EMBO J*. 9:1015–20
82. Wu SM, Cheung WF, Frazier D, Stafford DW. 1991. Cloning and expression of the cDNA for human gamma-glutamyl carboxylase. *Science* 254:1634–36
83. Wu SM, Mutucumarana VP, Geromanos S, Stafford DW. 1997. The propeptide binding site of the bovine γ-glutamyl carboxylase. *J. Biol. Chem.* 272:11718–22
84. Wu W, Bancroft JD, Suttie JW. 1997. Structural features of the kringle domain determine the intracellular degradation of under-γ-carboxylated prothrombin: studies of chimeric rat/human prothrombin. *Proc. Natl. Acad. Sci. USA* 94:13654–60
85. Yamada M, Kuliopulos A, Nelson NP, Roth DA, Furie B, et al. 1995. Localization of the factor IX propeptide binding site on recombinant vitamin K-dependent carboxylase using benzoylphenylalanine photoaffinity peptide inactivators. *Biochemistry* 34:481–89

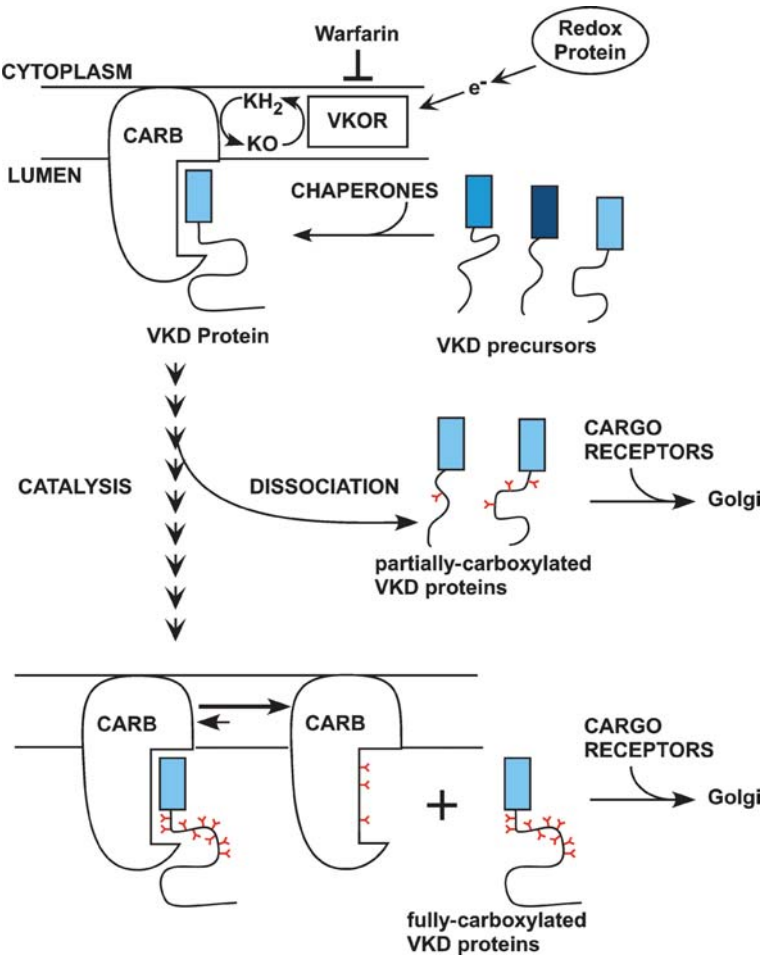


Figure 3 Vitamin K–dependent (VKD) protein carboxylation in the endoplasmic reticulum. VKD proteins contain propeptides with different affinities (indicated by the blue shading) for the carboxylase (CARB), and binding results in the conversion of multiple Glus to Glas (the red "Y's"; a VKD protein undergoing nine modifications is shown as an example). The carboxylase is also carboxylated, subsequent to the carboxylation of the VKD substrate. Each Glu to Gla conversion requires one reduced vitamin K (KH_2), which is recycled from the epoxide (KO) product of carboxylation by a vitamin K oxidoreductase (VKOR) and a redox protein of unknown identity. KH_2 availability supports a high rate of catalysis and low rate of dissociation that results in processive carboxylation, and warfarin disrupts normal carboxylation by blocking the supply of KH_2. Quality control components that affect carboxylation are chaperones, which mediate VKD protein-carboxylase assembly, and cargo receptors, which transport fully and partially carboxylated VKD proteins out of the endoplasmic reticulum. The quality control components specific to carboxylation have not yet been identified.

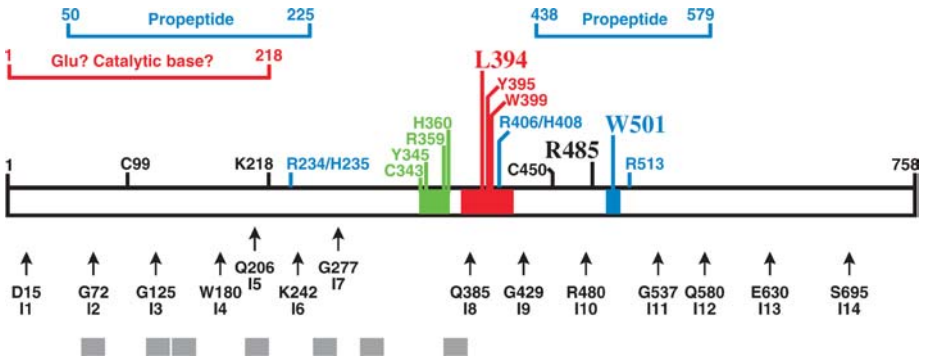


Figure 5 Functional human carboxylase residues. Residues proposed to be part of the propeptide- or Glu-binding site are shown in blue and red, respectively. The regions indicated above the carboxylase were identified by cross-linking with propeptide, or with a suicide substrate that could be mapping the Glu-binding site or the catalytic base. Residues in green are part of a region that binds vitamin K–dependent (VKD) proteins with low affinity. Some of the functional residues were identified in bovine carboxylase but are indicated here because these residues are identical in human and bovine carboxylases. Natural variants that impair carboxylation have been identified for residues L394, R485, and W501, shown in larger font. The arrows below the carboxylase indicate the exon/intron junctions and the intron number (I#), and the amino acids indicate the codons just before or interrupted by the intron. The gray boxes indicate hydrophobic domains in the carboxylase.

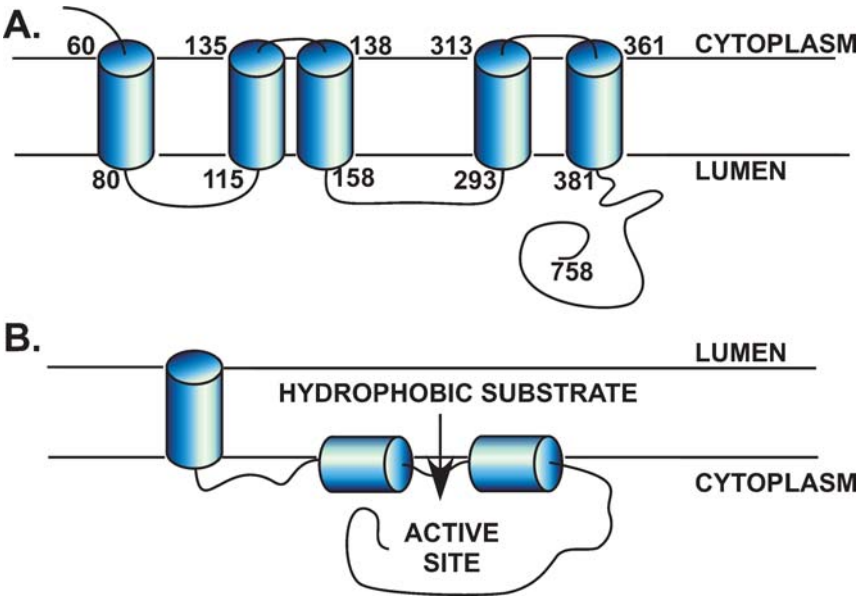


Figure 6 Uncertainties in carboxylase topology. The carboxylase gene predicts seven hydrophobic regions, and five act as signal anchor sequences in vitro, which has led to the proposed structure shown in *A*. Some of the hydrophobic sequences may instead be monotopic, i.e., inserting into only one leaflet of the membrane, as observed with other integral membrane enzymes with known crystal structures (e.g., cytochrome P450, shown in *B*). This topology allows hydrophobic substrates or cofactors (like vitamin K) to move from the membrane into the active site (which is cytoplasmic for the P450 shown and lumenal for the carboxylase).

Annual Review of Nutrition
Volume 25, 2005

CONTENTS

DIETARY FIBER: HOW DID WE GET WHERE WE ARE?, *Martin Eastwood and David Kritchevsky* 1

DEFECTIVE GLUCOSE HOMEOSTASIS DURING INFECTION, *Owen P. McGuinness* 9

HUMAN MILK GLYCANS PROTECT INFANTS AGAINST ENTERIC PATHOGENS, *David S. Newburg, Guillermo M. Ruiz-Palacios, and Ardythe L. Morrow* 37

NUTRITIONAL CONTROL OF GENE EXPRESSION: HOW MAMMALIAN CELLS RESPOND TO AMINO ACID LIMITATION, *M.S. Kilberg, Y.-X. Pan, H. Chen, and V. Leung-Pineda* 59

MECHANISMS OF DIGESTION AND ABSORPTION OF DIETARY VITAMIN A, *Earl H. Harrison* 87

REGULATION OF VITAMIN C TRANSPORT, *John X. Wilson* 105

THE VITAMIN K-DEPENDENT CARBOXYLASE, *Kathleen L. Berkner* 127

VITAMIN E, OXIDATIVE STRESS, AND INFLAMMATION, *U. Singh, S. Devaraj, and Ishwarlal Jialal* 151

UPTAKE, LOCALIZATION, AND NONCARBOXYLASE ROLES OF BIOTIN, *Janos Zempleni* 175

REGULATION OF PHOSPHORUS HOMEOSTASIS BY THE TYPE IIa Na/PHOSPHATE COTRANSPORTER, *Harriet S. Tenenhouse* 197

SELENOPROTEIN P: AN EXTRACELLULAR PROTEIN WITH UNIQUE PHYSICAL CHARACTERISTICS AND A ROLE IN SELENIUM HOMEOSTASIS, *Raymond F. Burk and Kristina E. Hill* 215

ENERGY INTAKE, MEAL FREQUENCY, AND HEALTH: A NEUROBIOLOGICAL PERSPECTIVE, *Mark P. Mattson* 237

REDOX REGULATION BY INTRINSIC SPECIES AND EXTRINSIC NUTRIENTS IN NORMAL AND CANCER CELLS, *Archana Jaiswal McEligot, Sun Yang, and Frank L. Meyskens, Jr.* 261

REGULATION OF GENE TRANSCRIPTION BY BOTANICALS: NOVEL REGULATORY MECHANISMS, *Neil F. Shay and William J. Banz* 297

POLYUNSATURATED FATTY ACID REGULATION OF GENES OF LIPID METABOLISM, *Harini Sampath and James M. Ntambi* 317

SINGLE NUCLEOTIDE POLYMORPHISMS THAT INFLUENCE LIPID METABOLISM: INTERACTION WITH DIETARY FACTORS, *Dolores Corella and Jose M. Ordovas* 341

THE INSULIN RESISTANCE SYNDROME: DEFINITION AND DIETARY APPROACHES TO TREATMENT, *Gerald M. Reaven* 391

DEVELOPMENTAL DETERMINANTS OF BLOOD PRESSURE IN ADULTS, *Linda Adair and Darren Dahly* 407

PEDIATRIC OBESITY AND INSULIN RESISTANCE: CHRONIC DISEASE RISK AND IMPLICATIONS FOR TREATMENT AND PREVENTION BEYOND BODY WEIGHT MODIFICATION, *M.L. Cruz, G.Q. Shaibi, M.J. Weigensberg, D. Spruijt-Metz, G.D.C. Ball, and M.I. Goran* 435

ANNUAL LIPID CYCLES IN HIBERNATORS: INTEGRATION OF PHYSIOLOGY AND BEHAVIOR, *John Dark* 469

DROSOPHILA NUTRIGENOMICS CAN PROVIDE CLUES TO HUMAN GENE–NUTRIENT INTERACTIONS, *Douglas M. Ruden, Maria De Luca, Mark D. Garfinkel, Kerry L. Bynum, and Xiangyi Lu* 499

THE COW AS A MODEL TO STUDY FOOD INTAKE REGULATION, *Michael S. Allen, Barry J. Bradford, and Kevin J. Harvatine* 523

THE ROLE OF ESSENTIAL FATTY ACIDS IN DEVELOPMENT, *William C. Heird and Alexandre Lapillonne* 549

INDEXES

Subject Index 573

Cumulative Index of Contributing Authors, Volumes 21–25 605

Cumulative Index of Chapter Titles, Volumes 21–25 608

ERRATA

An online log of corrections to *Annual Review of Nutrition* chapters may be found at http://nutr.annualreviews.org/