Molecular Basis of Vitamin K-Dependent \( \gamma \)-Carboxylation

By Bruce Furie and Barbara C. Furie

The vitamin K-dependent blood clotting and regulatory proteins, including prothrombin, factor IX, factor X, factor VII, protein C, and protein S, require a number of posttranslational modifications to yield the biologically active forms of these proteins that circulate in the blood. In addition to the typical processing characteristic of secreted proteins, including signal peptide cleavage, propeptide cleavage, disulfide bond formation, and glycosylation, these proteins undergo a unique modification involving the conversion of specific glutamic acids to \( \gamma \)-carboxyglutamic acid. This is the principle known biochemical reaction that is dependent on vitamin K. In the absence of vitamin K or in the presence of vitamin K antagonists, the synthesis of biologically active vitamin K-dependent proteins is impaired. These defects can have clinical sequelae, as in the bleeding associated with hemorrhagic disease of the newborn, vitamin K deficiency based on decreased food intake and concurrent antibiotic therapy, and complications of oral anticoagulant therapy with vitamin K antagonists.

The vitamin K-dependent blood clotting proteins play a special role in blood coagulation. Although these proteins are structurally related to the trypsin-chymotrypsin family, they are about twice the molecular size. Structural domains, distinct from the trypsin-homologous region, are responsible for membrane interaction or protein complex formation. The enzyme activity of the blood clotting proteases is greatly amplified by the presence of specific cofactors, such as factor VIII and factor V, in the presence of calcium ions and membrane surfaces. \( \gamma \)-Carboxyglutamic acid confers metal binding properties on the vitamin K-dependent proteins. In the presence of calcium ions, these proteins undergo a conformational change that leads to the expression of membrane binding properties. In the presence of membrane-bound cofactors, blood clotting enzymes, vitamin K, and membrane surfaces. \( \gamma \)-Carboxyglutamic acid confers metal binding properties on the vitamin K-dependent proteins. The cDNA sequences have been determined for the known vitamin K-dependent plasma proteins, including prothrombin, factor X, factor VII, factor IX, protein C, and protein S. These proteins demonstrate sequence homology about their \( \gamma \)-carboxyglutamic acid-rich domains. However, the mature uncarboxylated proteins do not serve as substrates for vitamin K-dependent carboxylation, but a slightly larger precursor form can be carboxylated in vitro.

In vitro studies using a recombinant expression system for the production of factor IX and prothrombin. The cDNA sequences have been determined for the known vitamin K-dependent plasma proteins, including prothrombin, factor X, factor VII, factor IX, protein C, and protein S. These proteins demonstrate sequence homology about their \( \gamma \)-carboxyglutamic acid-rich domains. However, the mature uncarboxylated proteins do not serve as substrates for vitamin K-dependent carboxylation, but a slightly larger precursor form can be carboxylated in vitro.

In addition, the vitamin K-dependent bone gla protein has little sequence homology to the plasma vitamin K-dependent proteins, yet the bone gla protein, like the vitamin K-dependent blood coagulation proteins, contains \( \gamma \)-carboxyglutamic acid. Analysis of the predicted translation products for the vitamin K-dependent proteins indicates that each of

**Vitamin K Action**

Vitamin K is an essential vitamin and is derived in the diet principally from green leafy vegetables (Fig 1). In the hepatocyte, vitamin K is reduced to the vitamin K hydroqui-
Warfarin action (hatched) is the vitamin K epoxide reductase. The vitamin K reductase. The reduced form of vitamin K, vitamin KH, is the substrate for the vitamin K-dependent carboxylase/vitamin K epoxidase. With the carboxylation of glutamic acid residues on the protein substrate, vitamin K epoxide is formed. CO₂ and molecular oxygen are also requisite substrates. The vitamin K epoxide is recovered by a reaction catalyzed by the vitamin K epoxide reductase, and vitamin K is generated. The major site of warfarin action (hatched) is the vitamin K epoxide reductase. Some vitamin K reductases are also inhibited.

These proteins are synthesized in a precursor form including a signal peptide and a propeptide. The propeptide intervenes between the signal sequence and the amino terminal end of the mature protein. Although the signal sequences, required for translocation of these polypeptides to the endoplasmic reticulum, vary in structure among these proteins, significant sequence homology exists among the propeptides (Fig 2). Phenylalanine -16; alanine -10; the hydrophobic amino acids at residues -17, -7, and -6; and the basic amino acids at residues -4, -3, -2, and -1 are well-conserved. Furthermore, another vitamin K-dependent bone protein, matrix gla protein, contains a sequence within these proteins is synthesized in a precursor form including a signal peptide and a propeptide. The propeptide intervenes between the signal sequence and the amino terminal end of the mature protein. Although the signal sequences, required for translocation of these polypeptides to the endoplasmic reticulum, vary in structure among these proteins, significant sequence homology exists among the propeptides (Fig 2). Phenylalanine -16; alanine -10; the hydrophobic amino acids at residues -17, -7, and -6; and the basic amino acids at residues -4, -3, -2, and -1 are well-conserved. Furthermore, another vitamin K-dependent bone protein, matrix gla protein, contains a sequence within the mature protein homologous to that of the propeptides of the vitamin K-dependent blood clotting proteins. The discovery of this structure, common to all proteins that contain γ-carboxyglutamic acid, led to the prediction that the propeptide or an equivalent sequence placed elsewhere in a protein might serve to designate specific precursor polypeptides for vitamin K-dependent γ-carboxylation in the liver or other organs.³⁰

Factor IX Cambridge is a mutant factor IX derived from a hemophilia B patient with a severe hemophilia phenotype.³¹ This protein contains an 18-residue extension on its amino terminus, representing an uncleaved propeptide. The substitution of arginine -1 by a serine precludes cleavage of the propeptide by an intracellular propeptidase with trypsin-like specificity. Factor IX Cambridge (Arg -1 → Ser) is incompletely carboxylated, providing direct evidence that a point mutation in the propeptide could impair vitamin K-dependent carboxylation.³² Similarly, factor IX San Dimas (Arg -4 → Gln) contains an uncleaved propeptide and is only partially carboxylated.³³ These observations provided additional impetus for determining the role of the propeptide in the carboxylation of glutamic acid residues in the vitamin K-dependent proteins.

To explore the precise role of the propeptide in vitamin K-dependent carboxylation, modifications were made in the factor IX cDNA coding for the propeptide by site-specific mutagenesis.³⁴ The various factor IX cDNA constructions, based on modifications of the wild type cDNA of factor IX, were expressed in Chinese hamster ovary cells.¹⁴ The amount of γ-carboxyglutamic acid formed in the various factor IX species was assessed using conformation-specific antibodies directed against the γ-carboxyglutamic acid-dependent, metal-stabilized structure and by direct amino acid analysis of an alkaline hydrolysate of the protein for γ-carboxyglutamic acid.³⁵ Deletion of the propeptide (residues -18 to -1) abolished carboxylation of factor IX. Substitution of alanine for phenylalanine at residue -16 within the propeptide or glutamic acid for alanine at residue -10 in the propeptide almost completely inhibited carboxylation. These mutations disrupted carboxylation, but had no effect on signal peptide cleavage or propeptide cleavage. The purified factor IX species that were derived from forms that contained mutations within the propeptide had molecular weights and N-terminal sequences that were identical to wild type recombinant factor IX and plasma-derived factor IX. Although mutations in the propeptide disrupted carboxylation, these mutations had no effect on another posttransla-

Fig 1. Vitamin K cycle. Vitamin K, ingested as part of the normal diet, is converted to the vitamin K hydroquinone by a vitamin K reductase. The reduced form of vitamin K, vitamin KH₂, is the substrate for the vitamin K-dependent carboxylase/vitamin K epoxidase. With the carboxylation of glutamic acid residues on the protein substrate, vitamin K epoxide is formed. CO₂ and molecular oxygen are also requisite substrates. The vitamin K epoxide is recovered by a reaction catalyzed by the vitamin K epoxide reductase, and vitamin K is generated. The major site of warfarin action (hatched) is the vitamin K epoxide reductase. Some vitamin K reductases are also inhibited.

Factor IX
Prothrombin
Factor X
Factor VII
Protein C
Protein S
Bone Gla protein

Thr Val Phe Leu Asp His Glu Asp Ala Arg Lys Ile Leu Asn Arg Pro Lys Tyr
Ser Leu Val His Ser Gln His Val Phe Leu Ala Pro Glu Asp Ala Ser Leu Gln Val Arg Val Arg Arg Ala
Leu Leu Leu Leu Gly Glu Ser Lys Ala Pro Leu Asp Ser Val Pro Ser Ser Ser Arg Glu Ala His Glu Val Ala Arg Ile Arg Gln Tyr
Thr Pro Ala Pro Leu Asp Ser Val Pro Ser Ser Glu Thr Glu Glu Ala His Glu Val Ala Arg Ile Arg Lys Tyr
Val Leu Pro Val Leu Glu Ala Asp Leu Pro Ser Arg Gin His Ala Ser Gin Val Ile Arg Arg Arg Arg Ala
Ser Gly Ala Glu Ser Ser Lys Ala Phe Val Ser Lys Gin Glu Gly Ser Glu Val Val Lys Arg Pro Arg Tyr

Fig 2. Sequence homology of the propeptides of the vitamin K-dependent proteins. The size of the propeptide has been established for factor IX³¹,³² and protein C.³³ Homologous regions of other vitamin K-dependent proteins are aligned. Residues that demonstrate sequence homology are boxed and shaded: regions with conservative amino acid substitutions are boxed. Copyright 1988 by Cell Press. Reprinted with permission.
that carboxylation was completely or almost completely independent of the mechanism for carboxylation. Hydroxylation requires neither vitamin K nor the γ-carboxylation recognition site and is mediated through a hydroxylation recognition site in the mature factor IX.\(^{36,37}\) Others have demonstrated that hydroxylation of aspartic acid or asparagine can occur in proteins that do not require vitamin K for their synthesis.\(^{38,39}\) Inhibition of hydroxylation of factor IX with inhibitors of 2-ketoglutarate-dependent dioxygenases, including dipiridyl, o-phenanthroline, and pyridine 2,4-dicarboxylate, does not impair in vitro coagulant activity.\(^{40}\) so the functional role of β-hydroxyaspartic acid remains unknown. The expression of propeptide deletion and point mutants allowed us to conclude that the amino-terminal portion of the factor IX propeptide comprises a carboxylation signal, termed the carboxylation recognition site, which designates an adjacent glutamic acid-rich domain for γ-carboxylation. A similar series of experiments involving deletion of various segments of the propeptide within the precursor form of protein C also demonstrated the requirement of the protein C propeptide for carboxylation.\(^{41}\)

Similar studies have been performed to evaluate the effect of mutation within the propeptide of prothrombin on the carboxylation of prothrombin.\(^{42}\) In these experiments, an expression system that yields fully carboxylated prothrombin was used.\(^{43}\) Mutation of histidine to glycine at residue -18, valine to serine at residue -17, leucine to glycine or aspartic acid at residue -15, and alanine to aspartic acid at residue -10 results in partial inhibition of carboxylation of recombinant prothrombin expressed in Chinese hamster ovary cells.\(^{44}\) In contrast, mutation of alanine to serine at residue -14 or serine to valine at residue -8 does not affect carboxylation. Based on the structural prerequisites of the propeptide for both factor IX and prothrombin, these results would suggest that residues -18, -17, -16, and -10 define aspects of the carboxylation recognition site, whereas residues at -14 and -8 do not.

Circular dichroism studies have been used to evaluate the secondary structure of the propeptide. Eighteen residue synthetic peptides based on the primary structure of the propeptide of either human factor IX or prothrombin were found to have little organized structure in aqueous solution. However, most peptides of this size have minimal stable three-dimensional structure. The peptide may have stable structure or assume a stable structure in the context of the native protein or when associated with membrane surfaces on which the carboxylation reaction likely proceeds. If a peptide has a tendency to assume a stable secondary structure, this structure may often be induced by the inclusion of solvents such as trifluoroethanol. In the case of the peptides, 35% trifluoroethanol in water induces significant α-helical structure.\(^{45}\) A molecular model, which is constrained by the requirement for α helical structure, shows the potential for a helix in the propeptide. It is most likely that the carboxylation recognition site, on the N-terminal portion of the propeptide, is adjacent to the helical region. Residues -18, -17, -16, -15, and -10 form the carboxylation recognition site (see cover figure). Phenylalanine -16 (red) is critical to γ-carboxylation. Histidine -18, valine -17, leucine -15, and alanine -10 (all in brown) also are important for γ-carboxylation. In contrast, alanine -14 and serine -8, arginine -4, and arginine -1 (all shown in yellow) are not part of the carboxylation recognition site. Amino acids that were not tested are shown in white. The red-brown surface defines amino acids that are components of the carboxylation recognition site. Determination of the three-dimensional structure of the propeptide, including the carboxylation recognition site, by two-dimensional nuclear magnetic resonance (NMR) techniques is currently in progress and provides preliminary support for the presence of α-helix (D. Sanford, W. Bachovchin, J. Sudmeir, B. Furie, B.C. Furie, unpublished results).

In Vitro Studies of the Role of the Propeptide: Studies With Synthetic Peptides

The study of the carboxylation reaction in vitro has been hampered by the absence of compounds that sufficiently mimic the natural substrates. The discovery of a pentapeptide, FLEEL (Phe-Leu-Glu-Glu-Leu), which is carboxylated by crude microsomal preparations containing the carboxylase, provided an assay substrate for development of purification strategies for the vitamin K-dependent carboxylase.\(^{46}\) However, only the first glutamic acid in FLEEL is carboxylated, and only small amounts of substrate are converted to product.\(^{46,47}\) Most importantly, FLEEL binds poorly to the carboxylase; this reaction is characterized by a Km of about 5 to 10 mmol/L. If the propeptide contained a critical carboxylation signal in the naturally occurring substrate, we predicted that a synthetic peptide incorporating the propeptide and an adjacent region containing carboxylatable glutamic acid residues would serve as a suitable substrate for the carboxylase. To develop such a substrate for the in vitro assay of the bovine liver vitamin K-dependent γ-carboxylase and to understand the substrate recognition requirements of the carboxylase in vitro, we have prepared synthetic peptides incorporating the propeptide structures of prothrombin and factor IX\(^ {47}\) (Fig 3). A 28-residue peptide, proPT28, based on the sequence from residues -18 to +10 in prothrombin, includes the complete propeptide and the first 10 amino acids of carboxyprothrombin. Carboxylation of this peptide in the in vitro carboxylation assay in which \(^{14}\)CO\(_2\) is incorporated into the glutamic acids on the synthetic peptide was characterized by a Km of 3.6 μmol/L. In contrast, FLEEL was carboxylated with a Km of about 2,200 μmol/L in this system. A 10-residue peptide (prothrombin, from residues +1 to +10) and a 20-residue peptide (prothrombin, from residues -10 to +10), both lacking the intact propeptide, were poor substrates. The Km of the latter peptide was 850 μmol/L. Because mutation of phenylalanine to alanine at -16 almost completely disrupted carboxylation of factor IX in the in vivo expression system,\(^{48}\) a peptide substrate was synthesized in which phenylalanine was substituted by alanine at the -16 position in proPT28. This substrate,
proPT28/FA-16, had a Km of 200 μmol/L, significantly altered from proPT28. A synthetic propeptide (prothrombin, from residues −18 to −1) was a competitive inhibitor of carboxylation, with a Ki of 3.5 μmol/L. These results indicate that an intact carboxylation recognition site is required for efficient in vitro carboxylation and that this carboxylation recognition site binds tightly to the carboxylase. Furthermore, the same residues shown to be part of the carboxylation recognition site for in vivo carboxylation of recombinant proteins are required for the in vitro carboxylation of synthetic peptides.

Knobloch and Suttie, using an in vitro carboxylation system, have demonstrated that the factor X propeptide stimulates the carboxylation of small substrates containing glutamic acid. They have interpreted this phenomenon as possible allosteric regulation in which, distinct from the docking function of the propeptide, the carboxylase activity is regulated. Although this observation has been confirmed, we have preferred to interpret this stimulation as a perturbation of an extended substrate binding site of the carboxylase by the propeptide, altering the kinetics of the action of the carboxylase on glutamic acid substrates. Because there is presumably no free propeptide available to the carboxylase under physiologic conditions and since the propeptide is a competitive inhibitor of the carboxylation of propeptide containing substrates, the effect of the free propeptide on carboxylase activity is of interest only for exploring the relative contributions of the carboxylation recognition site and the glutamic acid to carboxylase structure and function. Direct evidence for a second, distant allosteric binding site for the propeptide that is separate from the active site is lacking. A regulatory function for the propeptide in the physiologic control of the carboxylase appears unlikely based on current data.

**Synthetic substrates as a probe of the carboxylation reaction.** Synthetic peptides that contain the γ-carboxylation recognition site and acidic amino acid side chains were compared as substrates for in vitro vitamin K-dependent γ-carboxylation (Fig 3). The 28-residue synthetic peptides proPT28 and proFIX28, based on profactor IX from −18 to +10, were efficiently carboxylated with a Km of about 3 μmol/L. However, the Vmax for carboxylation of proPT28 was about two times greater than that for proFIX28. To determine whether the rates of carboxylation of the proPT28 and proFIX28 substrates were intrinsically related to the differences in their γ-carboxylation recognition sites, pep-
tides were synthesized in which the propeptide of factor IX was substituted for that of prothrombin (proFIX/PT28), and the propeptide of prothrombin was substituted for that of factor IX (proPT/FIX28). The carboxylation of these peptides were compared with proPT28 and proFIX28. ProPT/FIX28, a 28-residue peptide based on residues -18 to -1 in prothrombin and residues +1 to +10 in factor IX, was carboxylated at a rate similar to proPT28. The Vmax for this substrate was 118% of that of proPT28. ProFIX/PT28, a peptide based on residues -18 to -1 in factor IX and residues +1 to +10 in prothrombin, was carboxylated with a Vmax comparable with that of proFIX28. Although the γ-carboxylation recognition sites on profactor IX and proprothrombin both bind to the carboxylase with an affinity of about 2 to 3 μmol/L, these results indicate that the substrate containing the prothrombin γ-carboxylation signal is slightly more efficiently carboxylated. This in vitro experiment parallels the results obtained in the expression of prothrombin and factor IX in heterologous cells. Despite the use of identical expression vectors and identical host cells, while prothrombin was expressed in a fully carboxylated form, similar or lower expression levels of factor IX were routinely seen. Do these proteins have a defect in their carboxylation recognition sites located within the propeptide? We compared the binding of a series of peptides that all contained the propeptide of prothrombin and varying amounts of the Gla domain. Demonstration that the propeptide (residues -18 to -1), proPT28 (residues -18 to +10), and proPT54 (-18 to +36) bind equivalently to the carboxylase argues strongly that residues within the Gla domain do not contribute significantly to substrate recognition and that the carboxylation recognition site is located only within the propeptide. A summary of the properties of the carboxylation recognition site in the propeptide is presented in Table 1.

Table 1. Characteristics of the γ-Carboxylation Recognition Site on the Propeptide of the Vitamin K-Dependent Proteins

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Description</th>
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<tbody>
<tr>
<td>1. Located adjacent to the glutamic acid residues</td>
<td>that are the carboxylase substrates</td>
</tr>
<tr>
<td>2. Probably binds directly to the vitamin K-dependent</td>
<td>carboxylase.</td>
</tr>
<tr>
<td>3. Includes α-helical structure.</td>
<td></td>
</tr>
<tr>
<td>4. Residues -18, -17, -16, -15, and -10 of the propeptide</td>
<td>part of this carboxylation signal; residues</td>
</tr>
<tr>
<td></td>
<td>-14, -8, -4, and -1 are not part of this site.</td>
</tr>
<tr>
<td>5. The sequence of the prothrombin propeptide permits</td>
<td>slightly more efficient carboxylation than the sequence of the factor IX</td>
</tr>
<tr>
<td></td>
<td>propeptide.</td>
</tr>
<tr>
<td>6. Before secretion, the propeptide is cleaved from the</td>
<td>vitamin K-dependent blood clotting and regulatory proteins.</td>
</tr>
<tr>
<td></td>
<td>7. Required for carboxylation. Whether this signal is sufficient for</td>
</tr>
<tr>
<td></td>
<td>carboxylation is unknown.</td>
</tr>
<tr>
<td>8. The carboxylation recognition site is located within the propeptide of the vitamin K-dependent blood clotting proteins and does not appear to have components in the Gla domain.</td>
<td></td>
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Table 2. Characteristics of the Carboxylase Substrates

1. Glutamic acid is the only amino acid that is carboxylated at a reasonable rate. Aspartic acid and carboxymethylcysteine carboxylation is minimal.

2. Target glutamic acids are within 45 to 50 residues of the carboxylation recognition site.

3. Peptides missing or containing truncated forms of the carboxylation recognition site are poor substrates.

4. The context of the glutamic acid (the amino acid sequence surrounding the glutamic acid) does not appear to influence the rate of carboxylation.

5. Aspartic acid does not inhibit the carboxylation of adjacent glutamic acids.

cysteine are poor substrates for the carboxylase, the distance between the position of the extractable hydrogen in the substrate and the enzyme active site apparently being critical. A summary of the characteristics of carboxylase substrates appears in Table 2.

Purification of the Vitamin K-Dependent Carboxylase

The vitamin K-dependent carboxylase is an integral membrane protein, which in crude form is very unstable. Although this protein has been detergent-solubilized and partially purified in a number of laboratories, these characteristics have interfered with the isolation of the enzyme by conventional methods. Based on the interaction of this enzyme with the γ-carboxylation recognition site on the propeptide of the precursor forms of the vitamin K-dependent blood coagulation proteins, an affinity purification strategy was recently devised. This method uses an affinity chromatography matrix that has as the active moiety a peptide ligand containing the carboxylation recognition site. The carboxylase from bovine liver microsomes was solubilized with CHAPS, a nonionic detergent known to extract this protein from the membrane fraction without inhibiting the enzymatic activity. The solubilized protein was subjected to ammonium sulfate fractionation and the carboxylase isolated by affinity chromatography using synthetic peptides based on the structure of the prothrombin propeptide. We evaluated two peptides that included N-terminal linker segments for their utility as ligands for affinity chromatography. One peptide, KKKGGGIGGKAANHVFAPQQARSLQRVRR, incorporated a 14-residue linker suitable for coupling to CNBr-activated agarose and containing prothrombin propeptide. This peptide, designed to include a trilysine N-terminus for coupling, an 11-residue spacer arm, and the propeptide, inhibited the carboxylase assay in its free form and when coupled to agarose, indicating its potential use for affinity purification. The other peptide, KGGHVFLAPQQARSLQRVRR, contains a single lysine N-terminus for coupling, a 2-residue spacer arm, and the prothrombin propeptide. This peptide also inhibited the carboxylase assay in its free form and in its agarose-coupled form. The partially purified carboxylase, solubilized and fractionated with ammonium sulfate, could be applied to either of these columns and eluted with 10 mmol/L prothrombin propeptide. A major band on sodium dodecyl sulfate gel electrophoresis was observed with a molecular weight of 77,000 in the presence or absence of β-mercaptoethanol. However, in the presence of the high concentrations of propeptide, only minimal carboxylase activity was measurable even with substrates devoid of a propeptide sequence. Rabbit polyclonal antibodies raised to the protein isolated from the affinity matrix inhibited the carboxylase activity in crude enzyme preparations when either FLEEL or proPT28 were used as substrate.

To obtain enzymatically active carboxylase, an alternate affinity purification strategy was used. The propeptide analog, CGGHVFLAPQQARSLQRVRR, was coupled through the NH₂-terminal cysteine to an activated thiolagarose column permitting elution of the propeptide:enzyme complex by reductive cleavage of the disulfide bond. This is a feasible approach because it is well-established that the carboxylase activity is stable to reducing agents. The propeptide-carboxylase complex was eluted with dithiothreitol. The eluted protein, dominated by a component with a molecular weight of 77,000, was electrophoretically identical to the protein eluted from the matrices described above with
Posttranslational carboxylation of vitamin K-dependent proteins during protein synthesis. The signal recognition particle binds to the signal peptide, leading to the formation of a ribosome-particle-messenger RNA complex on the endoplasmic reticulum. The signal peptide is translocated to the luminal aspect of the rough endoplasmic reticulum. After signal peptide cleavage, the propeptide is expressed on the nascent polypeptide chain. The propeptide, containing the γ-carboxylation recognition site, binds to the vitamin K-dependent carboxylase associated within the endoplasmic reticulum. Specific glutamic acids are converted to γ-carboxyglutamic acids, then the protein is transported to the trans-Golgi where the propeptide is cleaved. The fully processed protein is secreted into the circulation.

Our working model of the enzyme envisions that the vitamin K-dependent carboxylase is an integral membrane protein associated with the endoplasmic reticulum or an early compartment between the rough endoplasmic reticulum and the trans-Golgi. The presence of the carboxylation signal (the γ-CRS) on the propeptide of the precursor form of the vitamin K-dependent proteins is responsible for the interaction of these proteins with a complementary surface on the carboxylase itself (Fig 4). On binding of the substrate, the carboxylase modifies specific glutamic acid residues within reach of the enzyme active site while the substrate remains anchored to the extended substrate binding site of the enzyme. After carboxylation of the glutamic acid residues, the modified protein dissociates from the carboxylase. The propeptide of the vitamin K-dependent blood coagulation proteins is subsequently cleaved. While there are experimental data to support some aspects of the model, much remains to be tested and additional questions remain. For example, it has not been established whether glutamic acid residues are randomly carboxylated or whether the process is directional and coordinated. Borowski et al.\textsuperscript{90,96}, who have localized the γ-carboxyglutamic acid residues within naturally occurring partially carboxylated prothrombins, have observed one variant prothrombin in which Gla 16 is missing, but glutamic acid residues before and after this residue are carboxylated. In contrast, Liska and Suttie\textsuperscript{41} have concluded that bovine prothrombin synthesized in the presence of warfarin is carboxylated preferentially from the most amino-terminal γ-carboxyglutamic acid residues. In the absence of further experiments, the directionality of the carboxylation reaction must await future clarification. Vitamin K plays an essential role in the carboxylation reaction as a cofactor, but the mechanism of the reaction remains unknown. With the availability of carboxylase of higher purity, detailed mechanistic analysis should lead to a better understanding of this unique enzymatic process.

Posttranslational Vitamin K-Dependent Carboxylation

Mammalian proteins destined for secretion are synthesized with a signal peptide. During the initial events of translation, the signal recognition particle binds to the elongating polypeptide chain that contains the N-terminal signal peptide. A receptor on the endoplasmic reticulum...
binds the signal recognition particle, leading to the formation of a ribosome-signal recognition particle-nascent polypeptide chain complex on the outer aspect of the endoplasmic reticulum (Fig 5). The signal peptide leads to translocation of the nascent polypeptide chain to the endoplasmic reticulum, and the signal peptide is cleaved from the growing polypeptide chain within the lumen of the endoplasmic reticulum by a signal peptidase. The next recognition element to be expressed in the polypeptide chain of vitamin K-dependent proteins is the γ-carboxylation recognition site within the propeptide. In a process that likely takes place posttranslationally but may also take place co-translationally, the newly expressed propeptide binds to the carboxylase located within the membrane. The carboxylase modifies glutamic acid residues adjacent to the propeptide containing the carboxylation signal. After modification, the substrate protein is released and is transported to the trans-Golgi for final processing and secretion. Although no experimental evidence is currently available for the vitamin K-dependent proteins, extrapolation from other systems would suggest that the propeptide is likely cleaved by a propeptidase within the trans-Golgi. The mature protein is then released into the blood as an inactive zymogen.

Although many proteins contain propeptides, until recently the function of these propeptides has been unknown. Demonstration that the propeptide of factor IX directs γ-carboxylation represents an example of a specific biologic function for a propeptide during posttranslational processing and protein trafficking.33 The large propeptide of von Willebrand factor (vWF) is intimately related to the formation of the multimeric structure of vWF dimers; this event is critical for the formation of vWF of high biologic activity. The propeptide of albumin appears to be required for efficient transport through the secretory pathway involving the endoplasmic reticulum.63 Other proteins contain recognition elements that target the protein for localization to specific organelles. The COOH-terminal retention sequence on membrane proteins of the endoplasmic reticulum,64 the retention sequences on proteins localized to the luminal aspect of the endoplasmic reticulum,65 and N-terminal sequences that direct proteins to the mitochondria are critical recognition elements within polypeptides involved in protein sorting. Within this context, the carboxylation signal on the vitamin K-dependent proteins represents an additional recognition element that directs a specific posttranslational processing event.

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Molecular basis of vitamin K-dependent gamma-carboxylation

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