Molecular Basis of Vitamin K-Dependent γ-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 γ-carboxyglutamic acid. This is the principal 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. γ-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, γ-carboxyglutamic acid confers metal binding properties on the vitamin K-dependent proteins. 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, factor IX, and protein C, and protein S. These proteins demonstrate sequence homology about their γ-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 γ-carboxyglutamic acid. Analysis of the predicted translation products for the vitamin K-dependent proteins indicates that each of these proteins contains "vitamin KH, by vitamin K reductase activities, one of which is warfarin-sensitive. This reduced form of vitamin K is a cofactor in a reaction, catalyzed by a vitamin K-dependent carboxylase, in which glutamic acid residues are modified to γ-carboxyglutamic acid. Besides the reduced form of vitamin K, this reaction requires molecular oxygen, carbon dioxide, and the precursor form of a vitamin K-dependent protein as the substrate. During this reaction, γ-carboxyglutamic acid residues are generated and vitamin KH, is converted to vitamin K epoxide. The vitamin K epoxide is cycled back to vitamin K by the vitamin K epoxide reductase, an enzyme sensitive to inhibition by sodium warfarin.

Role of the Propeptide of Vitamin K-Dependent Protein Precursors

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, factor IX, and protein C, and protein S. These proteins demonstrate sequence homology about their γ-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 γ-carboxyglutamic acid. Analysis of the predicted translation products for the vitamin K-dependent proteins indicates that each of these proteins contains "vitamin KH, by vitamin K reductase activities, one of which is warfarin-sensitive. This reduced form of vitamin K is a cofactor in a reaction, catalyzed by a vitamin K-dependent carboxylase, in which glutamic acid residues are modified to γ-carboxyglutamic acid. Besides the reduced form of vitamin K, this reaction requires molecular oxygen, carbon dioxide, and the precursor form of a vitamin K-dependent protein as the substrate. During this reaction, γ-carboxyglutamic acid residues are generated and vitamin KH, is converted to vitamin K epoxide. The vitamin K epoxide is cycled back to vitamin K by the vitamin K epoxide reductase, an enzyme sensitive to inhibition by sodium warfarin.

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 mature protein. Although the signal sequences, re- vitamin K reductase. The reduced form of vitamin K, vitamin KH,, 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 the vitamin K-dependent blood clotting proteins. The discov ery 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.31 Similarly, factor IX San Dmas (Arg –4 → Gln) contains an uncleaved propeptide and is only partially carboxylated.32 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.33 The various factor IX cDNA constructions, based on modifications of the wild type cDNA of factor IX, were expressed in Chinese hamster ovary cells.34 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 structure35 and by direct amino acid analysis of an alkaline hydrolysate of the protein for γ-carboxy glutamic acid.36 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-

![Figure 1](image-url)

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 the vitamin K-dependent blood clotting proteins. The discov ery 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

![Figure 2](image-url)

Fig 2. Sequence homology of the prepeptides of the vitamin K-dependent proteins. The size of the propeptide has been established for factor IX31,32 and protein C.3 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.
tional process, β-hydroxylation of aspartic acid 64. Direct
determination of the γ-carboxyglutamic acid and β-hydroxy-
aspartic acid compositions of these factor IX species showed
that carboxylation was completely or almost completely
inhibited while β-hydroxylation was normal. Thus, β-
hydroxylation of aspartic acid 64 is a process that is
independent of the mechanism for carboxylation. Hydroxy-
lation requires neither vitamin K nor the γ-carboxylation
recognition site and is mediated through a hydroxyla-
tion recognition site in the mature factor IX. 36,37 Others have
demonstrated that hydroxylation of aspartic acid or aspar-
agine 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 dipyridyl, 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 carboxyla-
tion 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 require-
ment 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,
valeine 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 recombi-
nant prothrombin expressed in Chinese hamster ovary
cells. 42 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, −15, 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
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 propeptides, 35%
trifluoroethanol in water induces significant α helical
structure. 44 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

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 pentapep-
tide, FLEEL (Phe-Leu-Glu-Glu-Leu), which is carboxylated
by crude microsomal preparations containing the carbox-
ylase, provided an assay substrate for development of purifi-
cation strategies for the vitamin K-dependent carboxylase. 45
However, only the first glutamic acid in FLEEL is carboxyl-
ylated, and only small amounts of substrate are converted to
product. 5–6 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 prop-
epptide and an adjacent region containing carboxylatable glu-
tamic 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 acarboxyprothrombin. Carboxylation of this peptide in the
in vitro carboxylation assay in which 14CO2 is incorporated
into the glutamic acids on the synthetic peptide was charac-
terized 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, 49 a peptide substrate was
synthesized in which phenylalanine was substituted by alan-
ine at the −16 position in proPT28. This substrate,
### Table

<table>
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<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Km (pmol/L)</th>
<th>Relative Ki</th>
<th>Velocity (μmol/L)</th>
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<td>Thr Val Phe Leu Asp His Glu Asn Ala Asn Lys Ile Leu Asn Arg Pro Lys Arg</td>
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<td>40</td>
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</tr>
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</table>

### Figure 3

**Synthetic peptides as substrates for the vitamin K-dependent carboxylase.** In these experiments in vitro carboxylation of peptides containing the γ-carboxylation recognition site was evaluated by the incorporation of $^{14}$CO$_2$. The prothrombin propeptide is shown in gray shaded boxes and the factor IX propeptide is shown in red shaded boxes. The amino terminus of the mature sequences of prothrombin and factor IX are shown in black and red, respectively. The Km and Ki values are in micromoles per liter. The velocities of the reaction are given relative to proPT28.

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 carboxylation activity is of interest only for exploring the relative contributions of the carboxylation recognition site and the glutamic acids 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 proFIX28.48 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.34,43 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 characterized by production of partially carboxylated factor IX.

Factor IX Cambridge31 and factor IX San Dimas32 are partially carboxylated mutant factor IX forms that circulate in the blood with the propeptide attached to the amino terminus of the factor IX molecule. The reason for their impaired carboxylation and their lack of coagulant activity remains unclear. Do these proteins have a defect in their carboxylation recognition site or are some of the glutamic acids inaccessible to the carboxylase in these mutants? Synthetic peptides were prepared, including proFIX28/RQ-1, based on factor IX Cambridge (Arg - 1 → Ser) and proFIX28/RQ-4, based on factor IX Oxford30 and Factor IX San Dimas (Arg - 4 → Gin).32 These peptides were compared with proFIX28 as carboxylase substrates (Fig 3). ProFIX28/RS-1, proFIX28/RQ-4, and proFIX28 had equivalent Km and Vmax values. Furthermore, in vitro carboxylation of 59-residue peptides based on the sequence of factor IX (residue 18 to residue 41) were carboxylated with a Km of about 1 μmol/L, regardless of the presence or absence of the mutation at residues -4 and -1.51 Thus, the propeptides in the mutant factor IXs contain an intact γ-carboxylation recognition site; the γ-carboxylation recognition site does not include residues -4 and -1 of factor IX. Yet, factor IX modified at position -4 and expressed in mammalian cells is only partially carboxylated,25 as are the natural hemophilic proteins, factor IX Cambridge and factor IX San Dimas. The molecular basis for impaired carboxylation of these mutant proteins is then likely due to structural perturbations of the glutamic acid-rich region imposed by mutation in a linking segment between the γ-carboxylation recognition site and the glutamic acid substrates.

Price et al32 identified a consensus sequence within the vitamin K-dependent blood coagulation proteins, Glu-X-X-Glu-Cys (located at residues 16 to 22 in human prothrombin) and suggested that the carboxylation recognition site might include this region of the Gla domain in addition to 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.

Aspartic acid has been previously shown to undergo carboxylation within small synthetic peptides that bind poorly to the carboxylase.44 However, β-carboxyaspartic acid has not been observed in the vitamin K-dependent proteins. Because the propeptide-containing peptide substrates more closely resemble the natural substrates of the carboxylase than do the smaller peptide substrates, we explored whether propeptide-containing peptides that included aspartic acid might serve as alternative substrates (Fig 3). A peptide containing aspartic acids at the equivalent of positions 6 and 7 in prothrombin was minimally carboxylated, with the upper limit of the rate of aspartic acid carboxylation about 1% of the rate of glutamic acid carboxylation.49 This peptide was a competitive inhibitor of carboxylation (Ki 5 μmol/L). However, peptides containing Asp 6-Glu 7 and Glu 6-Asp 7 were carboxylated at equivalent rates, 68% and 55%, respectively, of proPT28 containing Glu 6 and Glu 7. Thus, although aspartic acid is not a substrate for the carboxylase, it does not inhibit the carboxylation of adjacent glutamic acids. Analogs of proPT28 containing Ala 6-Glu 7 or Glu 6-Ala 7 were carboxylated at equivalent rates, 70% and 82%, respectively, of proPT28. These results indicate that both Glu 6 and Glu 7 can be carboxylated in these synthetic peptides. Neither cysteine nor carboxymethylcysteine, an analog of homoglutamic acid, was carboxylated when substituted for Glu 6 in a peptide containing Asp at position 7. In contrast to glutamic acid, aspartic acid and carboxymethyl-

<table>
<thead>
<tr>
<th>Table 1. Characteristics of the γ-Carboxylation Recognition Site on the Propeptide of the Vitamin K-Dependent Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Located adjacent to the glutamic acid residues that are the carboxylase substrates.</td>
</tr>
<tr>
<td>2. Probably binds directly to the vitamin K-dependent carboxylase.</td>
</tr>
<tr>
<td>3. Includes α-helical structure.</td>
</tr>
<tr>
<td>4. Residues -18, -17, -16, -15, and -10 of the propeptide are part of this carboxylation signal; residues -14, -8, -4, and 1 are not part of this site.</td>
</tr>
<tr>
<td>5. The sequence of the prothrombin propeptide permits slightly more efficient carboxylation than the sequence of the factor IX propeptide.</td>
</tr>
<tr>
<td>6. Before secretion, the propeptide is cleaved from the vitamin K-dependent blood clotting and regulatory proteins.</td>
</tr>
<tr>
<td>7. Required for carboxylation. Whether this signal is sufficient for 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>
</tr>
</tbody>
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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.

### 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 carboxylmethylcysteine 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, KKKGGGIGGKAAAAHVFLAPQQARSLLQRVRR, 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, KGGHVFLAPQQARSLLQRVRR, 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, CGGHVFLAPQQARSLLQRVRR, was coupled through the NH2-terminal cysteine to an activated thiol-agarose 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
VITAMIN K-DEPENDENT \(\gamma\)-CARBOXYLATION

Transfer RNA growing peptide chain

Messenger RNA

CYTOPLASM

ENDOPLASMIC RETICULUM

Signal peptide cleavage

Propeptide

Carboxylase

Propeptide cleavage

Trans golgi

\(\gamma\)-Carboxylation

PLAZMA

Fig 5. 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 \(\gamma\)-carboxylation recognition site, binds to the vitamin K-dependent carboxylase associated within the endoplasmic reticulum. Specific glutamic acids are converted to \(\gamma\)-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.

propeptide. The preparation eluted by reductive cleavage had a specific carboxylase activity that was 10,000-fold enriched over the crude microsome preparation. The vitamin K-dependent carboxylase exhibits vitamin K epoxidase activity, as originally suggested by Wallin and Suttie, indicating that these two activities are indeed properties of a single enzyme (Fig 1).

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 \(\gamma\)-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., who have localized the \(\gamma\)-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 have concluded that bovine prothrombin synthesized in the presence of warfarin is carboxylated preferentially from the most amino-terminal \(\gamma\)-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 polyepitope chain complex on the outer aspect of the endoplasmic reticulum (Fig 5). The signal peptide leads to translocation of the nascent polyepitope chain to the endoplasmic reticulum, and the signal peptide is cleaved from the growing polyepitope chain within the lumen of the endoplasmic reticulum by a signal peptidase. The next recognition element to be expressed in the polyepitope 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 action: Significance of vitamin K epoxide reductase inhibition. Biochemistry 17:1371, 1978

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Molecular basis of vitamin K-dependent gamma-carboxylation

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