The Propeptides of the Vitamin K-dependent Proteins Possess Different Affinities for the Vitamin K-dependent Carboxylase*

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The vitamin K-dependent γ-glutamyl carboxylase catalyzes the modification of specific glutamates in a number of proteins required for blood coagulation and associated with bone and calcium homeostasis. All known vitamin K-dependent proteins possess a conserved eighteen-amino acid propeptide sequence that is the primary binding site for the carboxylase. We compared the relative affinities of synthetic propeptides of nine human vitamin K-dependent proteins by determining the inhibition constants (K_i) toward a factor IX propeptide/γ-carboxyglutamic acid domain substrate. The K_i values for six of the propeptides (factor X, matrix Gla protein, factor VII, factor IX, PRGP1, and protein S) were between 2–35 nM, with the factor X propeptide having the tightest affinity. In contrast, the inhibition constants for the propeptides of prothrombin and protein C are ~100-fold weaker than the factor X propeptide. This study demonstrates that the affinities of the propeptides of the vitamin K-dependent proteins vary over a considerable range; this may have important physiological consequences in the levels of vitamin K-dependent proteins and the biochemical mechanism by which these substrates are modified by the carboxylase.

The vitamin K-dependent carboxylase catalyzes the post-translational modification of specific glutamates to γ-carboxyglutamate (Gla) in a number of proteins. Most vitamin K-dependent proteins are involved in the hemostatic process (prothrombin, factors VII, IX, and X, and proteins C, S, and Z), whereas two others (bone Gla protein and matrix Gla protein) are associated with bone (1–4). Two new putative vitamin K-dependent proteins of unassigned function, proline-rich Gla proteins (PRGP1 and PRGP2), were identified by sequence homology searches and are believed to be membrane proteins (5).

A conserved eighteen-amino acid sequence essential for substrate recognition is found in all vitamin K-dependent proteins and was first identified by Pan and Price (6) based on sequence comparisons of the blood and bone vitamin K-dependent proteins. The conserved region is present as a propeptide sequence amino-terminal to the highly conserved Gla domains of the vitamin K-dependent blood proteins and is proteolytically removed to form the mature protein. With bone Gla protein, this sequence is also present as a propeptide amino-terminal to the mature form of the protein, whereas with matrix Gla protein the vitamin K-dependent propeptide-like sequence is part of the mature form of the protein (7). Confirmation of the importance of the propeptide sequence in carboxylation is demonstrated by experiments where deletion of the propeptide abrogates carboxylation of factor IX or protein C expressed in cell culture (8, 9). In addition, mutagenesis studies have identified a number of highly conserved amino acids (e.g. Phe–16, Ala–10, Leu–6) as well as less conserved positions (–17 and –15) whose mutation affects carboxylation (8, 10–12). Glutamate-containing peptides with covalently linked propeptides have 1000-fold lower K_m values than similar peptides without the propeptide sequence and are competitively inhibited by free propeptide (13). A peptide containing the propeptide and Gla domain of factor IX (FIXproGLA) has a sub-micromolar K_m for the carboxylase and can be fully carboxylated in a processive manner (14, 15).

There is significant evidence that the vitamin K-dependent propeptide sequence is the primary binding site for the carboxylase. The decarboxylated mature forms of the vitamin K-dependent blood proteins (i.e. without the propeptide sequence) (16, 17) or the Gla domains themselves (18) are poor substrates for the carboxylase. In addition, the propeptide attached to normally uncarboxylated glutamate-containing peptides are multiply carboxylated both in vivo and in vitro (18, 19). Peptides containing the factor IX propeptide followed by factor IX Gla domain, the rest of the factor IX sequence, or a random glutamate containing sequence have similar K_m values for the carboxylase (18). Therefore, the propeptide of the vitamin K-dependent proteins appears to confer the perceived affinity of the carboxylase for its substrate with little or no contribution from other domains. An exception to this may be with the vitamin K-dependent bone Gla protein in which an attached propeptide is not necessary for efficient binding to the carboxylase (18, 20–22).

Since the importance of the propeptide sequence in vitamin K-dependent carboxylation was first identified based on the presence of highly conserved residues at specific positions, it has been assumed that the propeptides form similar structures and therefore bind the carboxylase with similar affinities. A previous study has questioned this assumption (23), but a systematic comparison of the relative affinities has not been reported. Therefore, we determined the relative affinities of the propeptides of nine vitamin K-dependent proteins by competitive inhibition of a factor IX propeptide/Gla domain substrate.
Previously, these comparisons could not be performed because of the presence of endogenous substrates in mammalian micromolar preparations of the enzyme or contamination by free propeptide used to elute the affinity purified carboxylase. Therefore, these studies were performed using carboxylase purified by a metal ion-dependent antibody from recombinant insect cells which do not contain vitamin K-dependent proteins and are free of endogenous substrates (24). We find that the inhibition constants of most of the propeptides vary over a 100-fold range and the propeptide of bone Gla protein has severely reduced carboxylase binding affinity. Because variations in amino acid sequences of the highly conserved propeptide sequences must confer the varied affinities, such studies should provide insights into which amino acids within the propeptide are important for carboxylase recognition. In addition, the variation in affinities of the propeptides may have important physiological consequences in affecting the levels of vitamin K-dependent proteins in vivo and the biochemical mechanism by which these proteins are modified by the carboxylase.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were reagent grade. Peptides based on the propeptide sequences (Fig. 2) of the human vitamin K-dependent proteins were chemically synthesized, purified by reverse phase high performance liquid chromatography and verified to be correct by ion spray mass spectrometry by Chiron Technologies (Clayton Victoria, Australia). The concentrations of all peptides were determined by amino acid analysis. The FIXproGLA (R-4Q, R-18) peptide was prepared as described previously (14). The insect cell expression vector (pVL1392) was purchased from Pharmingen (San Diego, CA), and the baculovirus viral DNA (BacVector 3000) was purchased from Novagen (Madison, WI). The Sf9 cells (Spodoptera frugiperda) were obtained from the Lineburger Cancer Center at the University of North Carolina-Chapel Hill, and the High Five cells (Trichoplusia ni) were a gift from Dr. Thomas Kost of Glaxo-Wellcome Inc. (Research Triangle Park, NC). The HPC4 antibody affinity resin was provided by Dr. Charles T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). The FLAG antibody and FLAG peptide standards were purchased from Sigma.

**Expression of r-Carboxylase in High Five Cells**—The cDNA encoding the human vitamin K-dependent carboxylase was sub-cloned into the EcoRI site of the pVL1392 vector. The sequence coding for the FLAG antibody epitope (DYKDDDDK) was inserted at the amino-terminal end of the carboxylase, and a HPC4 antibody tag (EDQVDPRLIDQ) (26) was added at the carboxyl-terminal end. The engineered vector was cotransfected with baculovirus BacVector 3000 triple-cut virus DNA into Sf9 cells. Recombinant virus was isolated by plaque purification, amplified, and titered by plaque assay according to the instructions of the manufacturer (27). Expression of carboxylase was done by infection of ~2 × 10^9/ml High Five cells with the recombinant virus at a multiplicity of infection of ~1. Cells were collected after 48 h by centrifugation and stored at ~80 °C.

**Preparation of Microsomes from High Five Cells**—A total of 1.8 × 10^9 cells from 1.2 liters of culture expressing the recombinant human carboxylase was washed twice with Buffer A (20 mM phosphate (pH 7.4), 150 mM NaCl, 1× protease inhibitor mixture (28) and 10% glycerol) and resuspended in 50 ml of Buffer A. The sample was homogenized by 30 strokes with a Dounce homogenizer and then sonicated with four 5-s pulses using a Ultrasonic Cell Disrupter. Cellular debris was removed by centrifugation at 4000 × g for 15 min, and the supernatant was centrifuged at 105,000 × g for 1 h. The microsomal pellet was resuspended in 20 mM phosphate (pH 7.4), 500 mM NaCl, 1× protease inhibitor mixture, and 10% glycerol and stored at ~80 °C.

**Purification of r-Carboxylase Using HPC4 Antibody Resin**—Microsomes were diluted to a final protein concentration of 12 mg/ml and solubilized by the addition of an equal volume of solubilization buffer (50 mM Tris (pH 7.4), 0.15 mM NaCl, 1% CHAPS and 0.2% phosphatidylcholine, 10% glycerol, and 1× PIC mixture) at 4 °C for 1 h. The solubilized microsomes were centrifuged at 105,000 × g for 1 h, and the pellet was discarded. A total of 10 ml of HPC4 resin was equilibrated with wash buffer (20 mM Tris (pH 7.4), 0.15 mM NaCl, 0.5% CHAPS and 0.2% phosphatidylcholine, 1× protease inhibitor mixture) and added to the solubilization supernatant along with a final concentration of 5 mM CaCl2 and incubated overnight with gentle stirring. The resin was centrifuged and poured into a column, washed with 50 ml of wash buffer plus 5 mM CaCl2, and eluted with wash buffer plus 10 mM EDTA. Carboxylase samples were collected, aliquoted, and stored at −70 °C. Concentration of enzyme was estimated from dot blots of carboxylase using the anti-FLAG antibody and known concentrations of FLAG peptide standards.

**Carboxylase Assays**—The inhibition constants (K_i) for the various propeptides were determined from the ability of the propeptides to inhibit carboxylation of the FIXproGLA substrate. Purified recombinant carboxylase (~40 nM final concentration) was mixed on ice with a final concentration of 25 mM MOPS (pH 7.4), 0.5 mM NaCl, 0.28% CHAPS, 0.12% phosphatidylcholine, 222 µM vitamin K hydroquinone, 6 mM dithiothreitol, and 5 µCi of NaH14CO3 (specific activity, 54 mCi/mmol; ICN Corp). Aliquots of this mixture were added to an indicated final concentration of propeptide and FIXproGLA peptide. Reactions were transferred to a 20 °C water bath and incubated for 1 h. Reactions were stopped by the addition of 75 µl of 1 N NaOH, and the total amount of CO2 incorporation was determined as described previously (23). The background 14CO2 incorporation in the absence of the substrate was subtracted from each assay point. This background averaged <1 nM/min compared with 0.6 nM/min observed in the absence of vitamin K and was unaffected by the propeptide concentrations used in our assays except as noted with the factor VII propeptide. Data for inhibition of varied concentrations of the FIXproGLA substrate by set concentrations of the factor IX, factor X, and prothrombin propeptides were fit by numerical integration using the program Dynafit2 (29) to determine the most likely inhibition mechanism and estimates of the kinetic parameters. Values for the inhibition constant (K_i) for each propeptide were determined by fitting the data for inhibition of a set concentration of the FIXproGLA substrate (0.5 µM) with each propeptide by nonlinear regression to the equation for tight binding competitive inhibition (Equation 1) where K_i is the apparent inhibition constant, I is the total propeptide concentration, E is the total carboxylase concentration, and K_m is the Michaelis constant for the FIXproGLA peptide (30).

\[
v = \frac{k_{cat}[FITXproGLA]}{2K_m + [FITXproGLA]} \left( \frac{I + K_i - E}{I + K_i} \right) + \frac{K_m}{1 + \left( \frac{[FITXproGLA]}{K_m} \right)} \tag{1}
\]

All K_i values are reported as the average of at least three independent determinations ± S.D. For the factor VII propeptide, an equation (Equation 2) was derived using the rapid equilibrium assumption based on the mechanism in Scheme 1 which accounts for carboxylation of the propeptide itself.

\[
v = \frac{V_{max}[FIX - 18 to 41]}{K_{cat}} + \frac{V_{max}[D]}{K_{cat}} \left( 1 + \frac{I}{K_m + rac{[FITXproGLA]}{K_m}} \right) \tag{2}
\]

**RESULTS AND DISCUSSION**

The purpose of this study was to compare the relative affinities of the propeptides of nine vitamin K-dependent proteins for the vitamin K-dependent γ-glutamyl-carboxylase. These studies were conducted with recombinant human vitamin K-dependent carboxylase expressed in insect cells and purified by a metal ion-dependent antibody to an engineered epitope tag. Therefore this carboxylase preparation is free of endogenous

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2 Available on the World Wide Web at http://www.biokim.com.
Affinities of Vitamin K-dependent Propeptides

As can be seen in Figs. 2 and 3, the relative affinities of the vitamin K-dependent propeptides vary over a significant range. The propeptide of factor X has the tightest affinity ($K_i = 2.6 \text{ nM}$) followed by matrix Gla protein ($K_i = 5.8 \text{ nM}$). The propeptides of protein S ($K_i = 12.3 \text{ nM}$) and PRGP1 ($K_i = 12.8$) are in the same range followed by factor IX ($K_i = 33.6 \text{ nM}$). PRGP1 is a putative vitamin K-dependent protein that was identified by homology searches based on the highly conserved Gla domain sequence motifs of the vitamin K-dependent proteins (5). However, it is not known whether this protein is actually carboxylated. Because the $K_i$ of the PRGP1 propeptide is within the range observed for the known carboxylated vitamin K-dependent proteins and the propeptide is all that is required to direct carboxylation of an adjacent glutamate containing region (18, 19), the PRGP1 precursor likely is able to bind and be modified by the vitamin K-dependent carboxylase.

An unusual phenomenon is observed with the factor VII propeptide when it is used as an inhibitor in the competition assays. As seen in Fig. 4A, increasing concentrations of the factor VII propeptide reduce the carboxylase activity to a point, but at higher concentrations the activity increases. When the inhibitor (factor VII propeptide) is varied in the absence of the FIXproGLA substrate, a concentration-dependent increase in carboxylation is observed (Fig. 4A). Small glutamate-containing peptides without covalently linked propeptides are commonly used as substrates for the carboxylase and are carboxylated at much higher rates than substrates with attached propeptides (31). The factor VII propeptide, unlike the other vitamin K-dependent propeptides, contains a di-glutamate pair and appears to be acting as a glutamate substrate for the carboxylase. Apparent carboxylation of the factor VII IX propeptide, which contains a single glutamate, is also observed, albeit at a significantly lower level than that of the factor VII propeptide. However, at the concentrations of propeptides used in our assays, incorporation of radioactive CO$_2$ into these propeptides did not affect our $K_i$ determinations (Fig. 4A, inset). There is no apparent incorporation of radioactive CO$_2$ into the propeptides.
The effect of the various propeptides on the carboxylation of 0.5 μM factor IX propeptide/ 
Gla domain peptide is shown. Data were normalized to the carboxylation of the FIXproGLA peptide in the absence of propeptide. Propeptides used are as follows: factor X (■), matrix Gla protein (○), protein S (△), PRGP1 (▲), factor IX (□), prothrombin (▲), protein C (△), and bone Gla protein (○).

![Inhibition of FIXproGLA carboxylation by propeptides](image)

**FIG. 3.** Inhibition of FIXproGLA carboxylation by propeptides of various vitamin K-dependent propeptides. The effect of the various propeptides on the carboxylation of 0.5 μM factor IX propeptide/ 
Gla domain peptide is shown. Data were normalized to the carboxylation of the FIXproGLA peptide in the absence of propeptide. Propeptides used are as follows: factor X (■), matrix Gla protein (○), protein S (△), PRGP1 (▲), factor IX (□), prothrombin (▲), protein C (△), and bone Gla protein (○).

**FIG. 4.** Inhibition of FIXproGLA carboxylation by factor VII propeptide. A, the effect of varying concentrations of factor VII propeptide (▲) or matrix Gla protein propeptide (△) on the carboxylation of 0.5 μM FIX proGLA is shown. The line for factor VII propeptide in the presence of FIXproGLA was drawn according to Equation 2 using

\[ V_{max} = 11 ± 2.3 \text{ nmol/min}, V_{max, control} = 733 ± 47 \text{ nmol/min}, K_m = 60 \text{ nm}, K_p = 2,920 \text{ μM}, \text{ and } K_f = 11 ± 2.8 \text{ nm}. \]

The carboxylation of the factor VII propeptide (△) in the absence of the FIXproGLA peptide is also shown. Inset, background carboxylation with varying concentrations of propeptides of matrix Gla protein (○), prothrombin (△), factor IX (▲), and factor VII (●) in the absence of FIXproGLA substrate. B, inhibition of various concentrations of FIXproGLA by 0 nm (●), 20 nm (▲), and 80 nm (□) factor VII propeptide. Lines were drawn using Equation 1 with

\[ k_{cat} = 0.29 ± 0.01 \text{ min}^{-1}, V_{max} = 38 \text{ nm}, K_m = 60 ± 7.6 \text{ nm}, \text{ and } K_i = 5.7 ± 0.8 \text{ nm}. \]

of prothrombin and matrix Gla protein, which do not contain glutamates (Fig. 4A, inset). These observations suggest that amino acids surrounding a particular glutamate may greatly influence its affinity for the carboxylase active site and that glutamate pairs in particular have higher affinity for the carboxylase active site as has been noted previously (32). Because these results indicate that the propeptide binding site and glutamate active site of the carboxylase can be occupied by separate factor VII propeptide molecules, we analyzed the data for inhibition by the factor VII propeptide using an equation

\[ K_i = 11.1 ± 0.8 \text{ nm}. \]

To further validate the estimate of the \( K_i \) for the factor VII propeptide, we also analyzed the effect of the factor VII propeptide on varying concentrations of the FXproGLA propeptide (Fig. 4B). The concentrations of the factor VII propeptide used in this experiment are well below the concentrations at which it demonstrates background activity. The value of the \( K_i \) from this experiment is 5.7 ± 0.8 nm, similar to the value obtained by analysis of varying propeptide at one substrate concentration (Fig. 4A). Although it is possible that the factor VII propeptide is carboxylated in vivo, this observation is likely a consequence of the high concentrations of propeptide used in these inhibition assays. Nevertheless, this phenomena does demonstrate the concept that amino acids surrounding a particular glutamate can significantly affect its carboxylation.

In contrast to the other propeptides, we were unable to measure significant inhibition using the bone Gla protein propeptide. We observe less than 10% inhibition by 400 μM bone Gla protein propeptide, therefore it must have significantly weaker affinity (\( K_i > 500 \text{ μM} \)) than that observed for the other propeptides. The bone Gla propeptide is the only known vitamin K-dependent propeptide sequence that contains a glnate at the highly conserved position −10. A previous study has shown that substitution of glnate in place of alanine at position −10 in a factor IX propeptide/Gla domain peptide increases its \( K_m \) for the carboxylase 30-fold (23). Therefore this alteration may be at least partially responsible for the severely reduced affinity of the bone Gla propeptide. Studies have shown that decarboxylated bone Gla protein without a covalently attached propeptide is an excellent substrate for the carboxylase (18, 20–22) although a covalently attached propeptide is required for efficient carboxylation of the vitamin K-dependent blood clotting proteins (8, 9, 16, 17). Therefore, the bone Gla protein substrate has a very weak propeptide but appears to have a strong binding site within its Gla domain, whereas the vitamin K-dependent blood proteins have strong propeptides but weak binding sites within their Gla domains. This suggests the possibility that bone Gla protein and the blood coagulation proteins may have separate mechanisms for substrate recognition. Interestingly, the \( K_i \) for the propeptide-like sequence in the other known vitamin K-dependent bone protein, matrix Gla protein, appears to be in the same range as the blood clotting proteins. Therefore the weak affinity of the bone Gla protein propeptide is not characteristic of the vitamin K-dependent bone proteins. Whether the propeptide plays a role in the carboxylation of bone Gla protein will require further investigation.

The comparisons of the relative affinities of the vitamin K-dependent proteins in this study show that the affinities of most of the vitamin K-dependent propeptides vary over a 10-fold range. Two propeptides (prothrombin and protein C) have significantly weaker affinities, and the propeptide of bone Gla protein is at least 200,000-fold weaker than the factor X propeptide. The physiological importance and consequences of the broad range of propeptide affinities are unclear. For the vitamin K-dependent blood coagulation proteins, the propeptide is essential for substrate recognition and binding of the carboxylase. Vitamin K-dependent carboxylation proceeds through a processive mechanism in which multiple glutamic acid residues can be modified in a single binding event (15). Therefore vitamin K-dependent carboxylation proceeds...
through a multi-step pathway in which multiple binding interactions and/or catalytic steps may contribute to the perceived affinity of the substrate for the carboxylase; nevertheless, the critical role of the propeptide in substrate recognition suggests that the binding affinity of the propeptide for the carboxylase should significantly affect the binding of the entire substrate.

Two in vivo studies provide evidence for variation in the affinities of the vitamin K-dependent blood coagulation proteins for the carboxylase. De Metz et al. (34) have found that liver microsomes from warfarin-treated cows accumulate uncarboxylated precursors of prothrombin, factor IX, and factor X. Although the prothrombin precursor would be expected to be present at higher concentrations, 69% of the carboxylase was complexed with factor X precursors, whereas 21% was associated with prothrombin and 8% with factor IX (34). Further evidence for the high affinity of the propeptide of factor X for the carboxylase is that warfarin treatment of HepG2 cells increases the level of factor X associated with the carboxylase with a concomitant decrease in the amount of prothrombin associated with the carboxylase (25). In a cell line that does not express factor X, no such competition was observed, and the authors ascribe this phenomena to a reduced affinity of the prothrombin precursor compared with that of factor X precursors. Both of these studies are consistent with our observation that factor X propeptide has a significantly higher affinity for the carboxylase than the prothrombin propeptide, and therefore the observed variations in affinities of the propeptides can impact the binding of the entire vitamin K-dependent protein substrates.

Patients with mutations in the factor IX propeptide demonstrate marked clinical effects, which demonstrates the physiological importance of propeptide affinity. Propeptide mutations in factor IX that increase the $K_m$ of the substrate for the carboxylase cause an unusual sensitivity to warfarin in several patients (23, 33). Without warfarin treatment, these patients demonstrate normal or near-normal factor IX activity, well below the range observed for the other vitamin K-dependent factors. This indicates that reduced propeptide binding affects the level of a particular vitamin K-dependent protein. Regulation of the levels of vitamin K-dependent proteins should be subordinate to a number of factors such as transcription levels and relative protein stability. Nevertheless, the observed competition of the factor X and prothrombin substrates for the carboxylase and the clinical consequences of factor IX propeptide mutations suggest that the significant variations in the affinities of the vitamin K-dependent propeptides may also have a significant role in determining the levels of these proteins.

This work provides the first extensive study of the relative affinities of the vitamin K-dependent propeptides for the carboxylase. Despite the sequence similarities observed for all the propeptides, there is a wide variation in the observed affinities of the propeptides for the carboxylase. Further work will be required to ascertain the structural determinants of these variable affinities and the physiological consequences of these observations.

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