Role of the Propeptide and γ-Glutamic Acid Domain of Factor IX for *in Vitro* Carboxylation by the Vitamin K-Dependent Carboxylase†

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ABSTRACT: The vitamin K-dependent γ-glutamyl carboxylase catalyzes the processive carboxylation of specific glutamates in a number of proteins related to blood coagulation and bone. To address the independent importance of the propeptide, γ-carboxyglutamic acid (Gla) domain and elements beyond the Gla domain of factor IX in vitamin K-dependent carboxylation, we have examined the kinetics of carboxylation of peptides containing (1) propeptide and Gla domain, (2) the Gla domain alone, (3) uncarboxylated bone Gla protein, (4) propeptide followed by the entire uncarboxylated factor IX molecule, and (5) the factor IX propeptide followed by a non-Gla domain sequence. Our studies indicate that peptides with a covalently linked propeptide have K_m values similar to the physiological substrate of the carboxylase. In contrast, the Gla domain of factor IX has a ≥230-fold higher K_m for the carboxylase than the corresponding peptide with a covalently linked propeptide. This contrasts with bone Gla protein, another vitamin K-dependent protein, which appears not to require a covalently linked propeptide for high-affinity binding to the carboxylase. Analysis of the carboxylation products of a propeptide/non-Gla domain substrate indicate that it is carboxylated multiple times in a processive manner. These studies show that the perceived binding affinity of the carboxylase substrate and processivity is conferred by the propeptide without requiring the conserved Gla domain sequences and that factor IX and bone Gla protein may have distinct mechanisms of interacting with the carboxylase.

The vitamin K-dependent γ-glutamyl carboxylase is an integral membrane protein that is found in the rough endoplasmic reticulum and has been purified from bovine liver (*1*), cloned, and sequenced (*2*, *3*). In addition to its carboxylase activity, the purified vitamin K-dependent carboxylase also possesses the vitamin K epoxidase activity (*4*). The vitamin K-dependent carboxylase catalyzes the post-translational modification of specific glutamic acid residues to γ-carboxyglutamic acid (Gla)[1] in a number of proteins essential for blood clotting as well as for the bone-related proteins: bone Gla protein (osteocalcin) and matrix Gla protein (*5*−*8*). Vitamin K-dependent proteins may be even more prevalent than previously thought as evidenced by the discovery of growth-arrest protein gas-6 (*9*) and the very recent identification of two putative vitamin K-dependent membrane proteins PRGP1 and PRGP2 (*10*).

The amino-terminal domain of the mature vitamin K-dependent blood proteins where multiple glutamic acid residues are converted to γ-carboxyglutamic acid is referred to as the Gla domain. The Gla domains of the known vitamin K-dependent blood proteins share extensive sequence identity. In addition, all of the known vitamin K-dependent proteins contain a highly conserved propeptide region that is proteolytically removed to form the mature protein (except for the matrix Gla protein where the propeptide-like sequence is found within the mature form of the protein) (*11*). Expression of vitamin K-dependent proteins in mammalian cell culture demonstrates that deletion of the propeptide region from factor IX (*12*) or protein C (*13*) abolishes carboxylation, while specific amino acid substitutions within the 18 amino acid propeptide sequence affect carboxylation (*12*, *14*, *15*). Furthermore, naturally occurring mutations in the factor IX propeptide region have been found in warfarin-sensitive hemophilia B patients (*16*, *17*). Synthetic peptides spanning the propeptide and first 10 residues of the Gla domain of factor IX or prothrombin have an apparent affinity for the carboxylase that is 3 orders of magnitude lower than

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; FLEEL, pentapeptide Phe-Leu-Glu-Glu-Leu; proFIX19, AVFLDHENANKILNRPKRY; proFIX, full-length uncarboxylated human factor IX with the propeptide still attached; proFIX(GLA), full-length carboxylated human factor IX with the propeptide still attached; FIXproGLA41, 59 amino acid peptide containing the human factor IX propeptide and first 41 residues of factor IX Gla domain (sequence −18 to 41); FIXproGLA46, 64 amino acid peptide containing the human factor IX propeptide and the entire Gla domain and hydrophobic stack sequence (sequence −18 to 46); FIXproNonGLA, 52 amino acid peptide containing the factor IX propeptide followed by a 34 amino acid non-Gla domain sequence.

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the small glutamate substrate FLEEL (*18, 19*). These studies indicate that the propeptide plays an essential role in the carboxylase's ability to recognize its substrate.

There is also ample evidence that the interaction of the carboxylase with the propeptide activates the enzyme. Evidence for propeptide activation of the enzyme is seen in studies which suggest that the propeptide lowers the apparent K_m for glutamate (*20*), lowers the apparent K_m for vitamin K (*21*), increases formation of the carbanion intermediate (*22*), and activates the epoxidase activity of the carboxylase (*23*). Therefore the propeptide has a dual role as a primary recognition element for the carboxylase as well as an allosteric activator of enzyme activity.

Most studies of the carboxylase have utilized small glutamic acid-containing substrates, such as the pentapeptide FLEEL or peptides containing the propeptide and two potential carboxylation sites. Although these studies have provided important information about the carboxylase, elucidation of carboxylase kinetics and mechanism requires the use of peptides that are multiply carboxylated and that more closely resemble the native substrates of the enzyme. Previous work from our laboratory has shown that the FIXproGLA41 peptide, which contains the factor IX propeptide followed by the first 41 amino acids of the Gla domain of factor IX, possesses a number of qualities expected of *in vivo* substrates of the vitamin K-dependent carboxylase. Specifically this peptide contains all 12 glutamates normally carboxylated in factor IX, has a physiologically reasonable affinity for the carboxylase, can be fully carboxylated (*24*), and is processively carboxylated (*25*). Therefore, the propeptide and the Gla domain alone are sufficient to direct processive carboxylation. In contrast to numerous studies which indicate that an amino-terminal propeptide extension is important for recognition by the carboxylase, studies with thermally decarboxylated bone Gla protein indicate that a covalently attached propeptide is not necessary for binding of the carboxylase (*26*−*28*). Bone Gla protein is a 49 amino acid vitamin K-dependent protein that contains three Gla residues and is expressed with an 18 amino acid propeptide extension analogous to the blood vitamin K-dependent proteins. The mature bone Gla protein shares little sequence identity with the Gla domains of the blood vitamin K-dependent proteins, except for a conserved sequence identified by Price and Fraser (*11*). Therefore, for bone Gla protein, essential elements other than the propeptide appear to be involved in substrate recognition.

In this paper the relative contribution of the propeptide and Gla domain of factor IX to vitamin K-dependent carboxylation has been examined by *in vitro* kinetic studies with purified components. These studies show that the perceived binding affinity of the carboxylase substrate and processivity is conferred by the propeptide without requiring the conserved Gla domain sequences and that factor IX and bone Gla protein may have distinct mechanisms of interacting with the carboxylase.

EXPERIMENTAL PROCEDURES

Reagents. All chemicals were reagent grade. Vitamin K_1 (phylloquinone) was from Abbott Laboratories; FLEEL was purchased from Bachem; phosphatidylcholine, Type X-E, from dried egg yolk is from Sigma. $NaH^{14}CO_3$ with specific activity of 56.4 mCi/mmol was from ICN Pharmaceuticals.

Proteins. Bovine carboxylase was purified as previously described and concentrated by chromatography on SP-Sepharose (Pharmacia), and its concentration was estimated by using the specific activity of the purified protein according to Wu et al. (*1*). The proteins proFIX and proFIX(GLA) expressed in Chinese hamster ovary cells were kindly provided by Mary Switzer of the Purification Process Development Department at Genetics Institute Inc. (Andover, MA). Human plasma factor IX was purchased from Haematological Technologies (Essex Junction, VT). The factor IX propeptide (proFIX19) was synthesized by Dr. Frank Church (University of North Carolina at Chapel Hill). The chemically synthesized mature form of human bone Gla protein (amino acids 1−49) was provided by Dr. Berry Soute (Maastricht University, The Netherlands). Oligonucleotides for cloning and mutagenesis were from Oligos Etc., Inc. (Guilford, CT).

Construction of Expression Vectors. The multifunctional expression vector phagemid pMc292 described previously (*24*) was used for both mutagenesis and expression of foreign peptides in *Escherichia coli*. This phagemid contains an insert that codes for the propeptide and first 41 amino acids of the Gla domain of human factor IX. The methionine at amino acid 19 is mutated to isoleucine as found in mouse factor IX. Arginines at −4 and −1 are mutated to glutamine and serine, respectively. Initially a vector was constructed that contained the gene10 sequence folowed by the factor IX propeptide/Gla domain/egf1/egf2 domains in the pMc292 phagemid. This was done using oligonucleotides 5′-T GAG AGA GAA TGC ATA GAA AAG TGT-3′ and 5′-GGA GGA TCC TAT GCT GGT TCA CAG GAC T-3′ to amplify a fragment from the human factor IX cDNA that engineers a *Nsi*I site at the 5′ end, a *Bam*HI site at the 3′ end, and codes for amino acid residues 20−127 of human factor IX. This fragment was digested with *Nsi*I/*Bam*HI and ligated into the *Nsi*I/*Bam*HI-digested pMc292 phagemid. The resulting vector is referred to as pMc292(IX−18−127) and was used for further mutagenesis and cloning.

In Vitro Mutagenesis. All *in vitro* mutagenesis were performed by the uracil method (*29*) using single-stranded DNA prepared using M13KO7 (*30*). The FIXproGLA46 construct was generated by inserting a stop codon after amino acid 46 using oligonucleotide 5′-CAG TAT GTT TAA GGA TCC CAG TGT GAG-3′. The FIXGLA46 construct was created by using oligonucleotide 5′ATT ATC GCT AAG TAC GCC ATG TAT AAT TCA GGT AAA CTC GAG-3′ and the original pMc292 phagemid to loop out from alanine at −18 to the serine at −1. The FIXproNon-GLA construct was generated by using oligonucleotide 5′-TCT TGA ATC AGC CAA AGA GCG GAA CCT TGA GAG AGA ATG TAG AGG AGA AAA GTG TA-3′, which loops out from tyrosine at position 1 to the middle of the codon at position 12, which codes for glycine. The oligonucleotide also replaces a stop codon that would occur eight amino acids after the end of the propeptide with a glutamate and changes a lysine at postion 9 to glutamate. This introduces a frameshift that results in a construct that codes for the factor IX propeptide attached to a 45 amino acid non-Gla domain sequence.

Expression, Purification, and Characterization of Peptides. The various peptides were prepared as described previously (*24*) with the following modifications. The inclusion bodies

were isolated from either a 4 L culture or a 5 L culture prepared with a BioFlo IIc fermentor (New Brunswick Scientific). Peptides that contained a tryptophan residue (FIXproGLA46 and FIXGLA46) were digested with cyanogen bromide in 72% trifluoroacetic acid in place of trichloroacetic acid to minimize oxidation of the tryptophan residues. After cyanogen bromide cleavage, the dried-down peptides were resuspended in 50 mL of 8 M guanidine hydrochloride. The peptides were sulfonated by the addition of 0.15 g of sodium tetrathionate, 0.3 g of sodium sulfite, and adjustment of the pH to 9.0 with ammonium hydroxide and incubating at room temperature for 16 h. The sulfonated peptides were then dialyzed against three changes of 25 mM MOPS, pH 8.0, and 10 mM NaCl to precipitate the gene 10 fragments while the target peptides remained in solution. The gene 10 fragments were removed by centrifugation at 12000*g* for 15 min. The supernatant was loaded on a DEAE-Sepharose CL-6B (Sigma) column and the target peptide was eluted by the addition of 25 mM MOPS, pH 8.0/250 mM NaCl. Sulfonation was reversed by incubating the eluted peptides at room temperature for 3 h with 100 mM dithiothreitol. The peptides were further purified by reversed-phase HPLC on a Rainin 0.46 × 50 mm C18 column (83-203-F5). Peptides were eluted with a linear 10 min gradient (1 mL/min) of 0% buffer A (0.1% TFA) to 100% buffer B (90% acetonitrile/0.1% TFA). The peptides were dried down by rotary evaporation.

Confirmation of Peptides by Electrospray Ionization Mass Spectroscopy. The molecular mass of each peptide was determined by using a Fisons-VG Quattro BQ triple-quadropole mass spectrometer equipped with a pneumatically assisted electrostatic ion source operating at atmospheric pressure. Samples were reconstituted in 50% aqueous acetonitrile containing formic acid (1% v/v) and were introduced by loop injection into a stream of 50% aqueous acetonitrile flowing at 6 μL/min. Mass spectra were acquired in the multichannel analyzer (MCA) mode from *m*/*e* 700−1400 with a scan time of 10 s. The mass scale was calibrated with horse heart myoglobin (M_r 16 951.48) with a resolution corresponding to a peak width at half-height of 1.0 Da for *m*/*e* 893. The mass spectra were transformed to a molecular mass scale with software supplied by the manufacturer.

Kinetics of Carboxylation of Propeptide-Containing Peptides. Reactions (125 μL) were conducted at 17 °C for 8 h with indicated amount of purified bovine carboxylase and 25 mM MOPS, pH 7.4, 0.5 M NaCl, 0.16% CHAPS, 0.16% phosphatidylcholine, 222 μM vitamin K hydroquinone, 6 mM DTT, and 5 μCi of $NaH^{14}CO_3$ (specific activity 54 mCi/mmol; ICN Corp.). A final concentration of 60 nM free factor IX propeptide from the purified carboxylase is also present. Reactions were quenched by the addition of 75 μL of 1 N NaOH and transferred to vials containing 1 mL of 5% trichloroacetic acid. Samples were gently boiled to remove unincorporated $^{14}CO_2$ and total incorporation was determined by scintillation counting. Previous studies on the kinetics of $^{14}CO_2$ incorporation into representative peptides of the vitamin K-dependent substrates have employed short incubation times (<1 h) to measure carboxylation. A previous study along with this work indicate that the time course of carboxylation of peptides with an attached propeptide demonstrates a biphasic burst with an initial rapid pre-steady-state phase followed by a slower linear steady-state phase (*25*). Therefore, to measure the steady-state kinetics of $^{14}CO_2$ incorporation, we used prolonged incubation times such that we were measuring the steady-state rather than the pre-steady-state rate. Inhibition experiments were done under the same reaction conditions and incubation times as above. For inhibition studies with proFIX(GLA), control experiments were done in the absence of substrate to account for traces of uncarboxylated or partially carboxylated proFIX in the proFIX(GLA) preparation. The $^{14}CO_2$ incorporation in the absence of substrate was small (<8%) compared to FIXproGLA46 carboxylation and was subtracted from the activity in the presence of substrate.

Determination of the Extent of Carboxylation of FIXproNonGLA by Mass Spectrometry. The number of carboxylations of the FIXproNonGLA peptide was determined by a method similar to that used previously to demonstrate processivity in FIXproGLA41 carboxylation (*25*). Briefly, a 5 mL carboxylation reaction containing 20 nM purified bovine carboxylase, 25 mM MOPS, pH 7.4, 0.15 M NaCl, 0.16% CHAPS, 0.16% phosphatidylcholine, 120 μM vitamin K hydroquinone, 200 μCi of $NaH^{14}CO_3$ (specific activity 54 mCi/mmol; ICN Corp.) and 2.5 μM FIXproNon-GLA peptide were incubated at 17 °C and were supplemented with an additional 120 μM vitamin K hydroquinone after 12 h. After 17 h the reaction was quenched by acidification with acetic acid to remove free $^{14}CO_2$ and neutralized with sodium hydroxide. The reaction was diluted 20-fold, buffered to pH 5.0, and bound to 1 mL of SP-Sepharose and eluted with 0.5 M NaCl. The labeled material was loaded on a 0.46 × 50 mm Rainin C18 HPLC column and eluted at a flow rate of 1 mL/min with a linear gradient from 0% acetonitrile/0.1% trifluoroacetic acid to 100% acetonitrile/0.1% trifluoroacetic acid. Fractions along the elution gradient were collected and those containing $^{14}CO_2$ incorporation above background were pooled and dried down by rotary evaporation. The dried-down sample was resuspended in 20 μL of 10 mM ammonium hydroxide. Samples were then analyzed by on-line mass spectrometry as described previously (*25*).

Kinetics of Carboxylation of Gla Domains. Reactions were performed at 17 °C for 1 h with 4 nM purified bovine carboxylase, 25 mM MOPS, pH 7.4, 0.5 M NaCl, 0.16% CHAPS, 0.16% phosphatidylcholine, 0.6 M ammonium sulfate, 20 μM proFIX19, 222 μM vitamin K hydroquinone, 6 mM DTT, and 5 μCi of $NaH^{14}CO_3$ (specific activity 54 mCi/mmol; ICN Corp.). Unlike the time course of propeptide-containing peptides, the time course of carboxylation of the Gla domains did not demonstrate a burst but rather was linear during the 1 h reaction course.

RESULTS

Expression, Purification, and Identification of Peptides. All peptides were expressed in *E. coli*, purified by high-performance liquid chromatography, and verified by ion spray mass spectrometry. FIXproNonGLA is expressed as a 63 amino acid peptide, but cleavage by cyanogen bromide removes 11 amino acids from its C-terminus. The resulting peptide is 52 amino acids with an approximately 1:1 mixture of the homoserine or homoserine lactone residue at the C-terminus (Table 1). The experimental molecular mass for each of the peptides is as follows: FIXproGLA46 is 7645.7

Table 1: Sequences of Factor IX and Bone Gla Protein-Based Peptides[a]

FIXproGLA46	AVFLDHENANKILNQPKS-YNSGKLEEFVQGNLERECIEEKCSFEEAREVFENTERTTEFWKQYV
FIXGLA46	YNSGKLEEFVQGNLERECIEEKCSFEEAREVFENTERTTEFWKQYV
Bone Gla Protein	YLYQWLGAPVPYPDPLEPRREVCELNPDCDELADHIGFQEAYRRFYGPV
FIXproNonGLA	AVFLDHENANKILNQPKS-GTLRENVEEKSVVLKKHEKFLKTLKEQLNFGSShS

[a] The propeptide sequence is underlined. Known carboxylated glutamates in factor IX and bone Gla protein and potential carboxylation sites in FIXproNonGLA are indicated in boldface type. hS stands for homoserine/homoserine lactone.

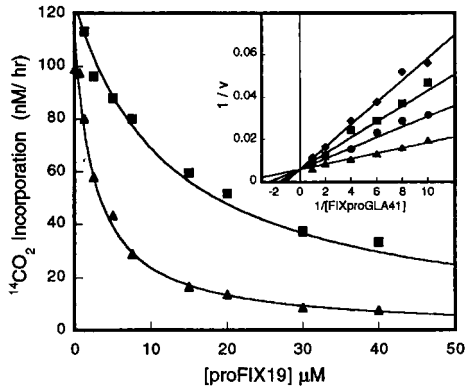

FIGURE 1: Free propeptide is a competitive inhibitor of FIXproGLA41 carboxylation. The effect of free propeptide (proFIX19) on carboxylation was examined with 1 μM (▲) and 5 μM (■) FIXproGLA41 at 17 °C for 8 h with ~10 nM purified bovine carboxylase. Data were fit by nonlinear regression and lines were drawn according to the equation for competitive inhibition (*35*) using $V_{max} = 124 \pm 15$ nM/h, $K_m = 0.10 \pm 0.05$ μM, and $K_i = 0.22 \pm 0.14$ μM. Inset: Lineweaver−Burk plot of the effect of 0.068 μM (▲), 0.32 μM (●), 0.57 μM (■), and 1.1 μM (◆) proFIX19 on carboxylation of FIXproGLA41. Lines were drawn using the equation for linear competitive inhibition with $V_{max} = 171 \pm 16$ nM/h, $K_m = 0.16 \pm 0.05$ μM, and $K_i = 0.19 \pm 0.04$ μM.

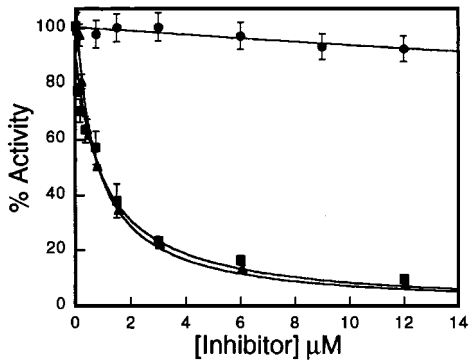

FIGURE 2: Fully carboxylated factor IX with the propeptide still attached inhibits FIXproGLA46 carboxylation. The effect of free propeptide (▲), proFIX(GLA) (■), and carboxylated FIX (●) on FIXproGLA46 carboxylation was examined at 17 °C for 8 h with 10 nM purified bovine carboxylase. Indicated data points are the average of at least two independent experiments and the error bars indicate the standard deviation. Lines were drawn with the equation for competitive inhibition with $K_m = 0.21 \pm 0.14$ and $K_i = 0.29$ μM $\pm$ 0.03 (propeptide) or 0.40 μM $\pm$ 0.07 [proFIX(GLA)].

Table 2: Kinetic Constants for $^{14}CO_2$ Incorporation for Peptide Substrates with Purified Bovine Carboxylase

substrate[a]	$K_{m_{app}} \pm$ SD[b] (μM)	$V_{max} \pm$ SD[b] (nM/h)
FIXproGLA41	0.19 ± 0.10	173 ± 45.3
FIXproGLA46	0.27 ± 0.09	191 ± 28.5
proFIX	0.36 ± 0.04	169 ± 15.0
FIXproNonGLA	0.46 ± 0.18	62.6 ± 27.6

[a] Reactions were performed as detailed under Experimental Procedures using at least 10 concentrations of each substrate. Kinetic parameters were determined by fitting the data to the Michaelis−Menten equation by nonlinear regression. [b] Values are ± standard deviation, obtained from at least three independent determinations.

± 0.4 Da (predicted, 7646.4), FIXGLA46 is 5625.31 ± 1.96 Da (predicted, 5626.1) and FIXproNonGLA is 5938.7 ± 0.29 Da (predicted, 5938.6) for the homoserine form and 5920.49 ± 0.57 Da (predicted 5920.6) for the homoserine lactone form.

Inhibition of Carboxylation by Free Propeptide and proFIX(GLA). To ascertain the importance of the propeptide in substrate recognition by the carboxylase, we examined the effect of free propeptide (proFIX19) on carboxylation of a propeptide/Gla domain (FIXproGLA41) peptide. As seen in Figure 1, proFIX19 acts as a competitive inhibitor of FIXproGLA41 carboxylation with a $K_i = 0.22 \pm 0.10$ μM. Competitive inhibition indicates that binding of free propeptide to the propeptide binding site of the carboxylase prevents association of the FIXproGLA41 substrate with the carboxylase. This is consistent with an initial interaction of the carboxylase with its substrate through the propeptide sequence rather than the Gla domain or glutamic acid residues themselves. In addition, we find that carboxylated factor IX with the propeptide still attached [proFIX(GLA)] is a product inhibitor of a propeptide/Gla domain peptide carboxylation with a K_i of 0.40 ± 0.07 μM, similar to that observed for free propeptide. In contrast, plasma factor IX, which does not contain the amino-terminal propeptide extension, is unable to inhibit carboxylation at the concentrations tested (Figure 2). Therefore, product inhibition by proFIX(GLA) is conferred by the propeptide and is a result of preventing access of the substrate to the propeptide binding site.

Kinetics of Factor IX-Based Peptides with Purified Bovine Carboxylase. As shown in Table 2, peptides with an attached propeptide: FIXproGLA41, FIXproGLA46, and proFIX, demonstrate similar apparent K_m values (0.2−0.4 μM) that are ~6000-fold lower than the K_m observed for the pentapeptide substrate FLEEL. The proFIX peptide is the full-length uncarboxylated factor IX with the propeptide still attached and should be analogous to the native *in vivo* substrate of the carboxylase. Therefore, the similar K_m values and carboxylation rates of the propeptide/Gla domain peptides to the full-length factor IX molecule suggest that in our *in vitro* system these peptides are good models for the physiological substrate of the carboxylase and elements beyond the propeptide and Gla domain are not required for carboxylation.

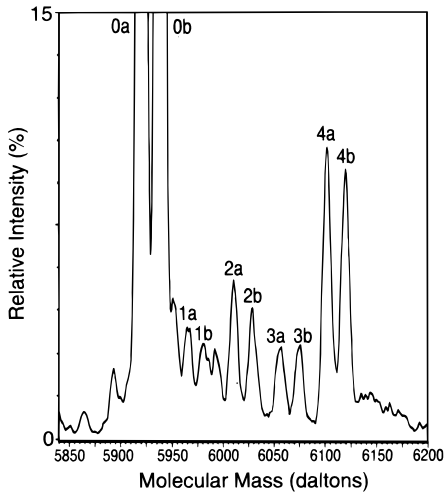

FIGURE 3: Mass spectroscopic analysis of preparative FIXproNonGLA carboxylation reaction. Labeled fractions from preparative reaction and purified by reverse-phase HPLC were analyzed by on-line mass spectrometry as described under Experimental Procedures. The numbers above each peak indicate the number of carboxylations required to yield the observed molecular mass of the homoserine lactone (a) and homoserine form (b). The relative intensity for the uncarboxylated homoserine lactone form (0a) was 89% and that for the homoserine form (0b) was 100%.

To examine whether the propeptide alone is adequate to direct carboxylation of a glutamate-containing substrate, we expressed in *E. coli* a peptide that contains the FIX propeptide sequence followed by a non-Gla domain sequence. This sequence (Table 1), FIXproNonGLA, although containing five glutamic acid residues, lacks the highly conserved sequences characteristic of the Gla domains of the vitamin K-dependent blood proteins. As can be seen in Table 2, FIXproNonGLA is carboxylated with a K_m of 0.46 $\pm$ 0.18 μM, which is similar to the range observed for the factor IX propeptide/Gla domain peptides. The observed V_{max} for the FIXproNonGLA peptide is approximately 3-fold lower than that observed for the FIX propeptide/Gla domain peptides.

Extent of Carboxylation of the FIXproNonGLA Peptide. To ascertain if the propeptide alone is able to direct multiple carboxylations of an attached substrate containing glutamates, we determined the extent of carboxylation of the FIXproNonGLA peptide. FIXproNonGLA was carboxylated in a preparative reaction with a large substrate excess over the enzyme, extracted by batch absorption, and eluted from SP-Sepharose. The peptide was isolated by reverse-phase HPLC and all the fractions containing ^{14}C label were pooled. On-line mass spectrometry of the labeled fraction is presented in Figure 3 and shows a distribution of carboxylation states from 0 to 4 carboxylations. The uncarboxylated FIXproNonGLA is present as two roughly equal mass forms corresponding to the homoserine lactone at the C-terminal residue (5920.6 Da) or the free homoserine form (5938.5 Da). Each carboxylation increases the mass 45.4 $\pm$ 0.2 Da, as demonstrated previously (*25*). Doublets corresponding to each carboxylation state of both the homoserine lactone and homoserine isomer are observed. We were unable to identify peak(s) conforming to the expected mass of the 5 carboxylation state. The major product of the reaction is the uncarboxylated form consisting of >75% of the total peptide. The 1, 2, and 3 carboxylation states are present at $\sim$5% each and the 4 carboxylation states comprises 10% of the total peptide.

Table 3: Kinetic Constants for $^{14}CO_2$ Incorporation for Gla Domain Peptide Substrates with Purified Bovine Carboxylase

substrate[a]	$K_{m_{app}} \pm$ SD[b] (μM)	$V_{max} \pm$ SD[b] (nM/h)
FLEEL	1190 $\pm$ 300	14 700 $\pm$ 1810
bone Gla protein	0.608 $\pm$ 0.07	3610 $\pm$ 82.6
FIXGLA46	$\geq$140 $\pm$ 32	3030 $\pm$ 202

[a] Reactions were performed as detailed under Experimental Procedures using at least 8 concentrations of each substrate. Kinetic parameters were determined by fitting the data to the Michaelis−Menten equation by nonlinear regression. The highest concentration of substrate used for FIXGLA46 was 250 μM, so the value obtained is a lower estimate; therefore the real value may be higher. [b] Values are $\pm$ standard deviation, obtained from at least three independent determinations.

The observed distribution of carboxylation states is consistent with a processive mechanism in which multiple carboxylations are catalyzed in a single binding event. The majority of the FIXproNonGLA peptide is uncarboxylated, while the fraction of peptide that is carboxylated appears to be multiply carboxylated with the 4 carboxylation state being the major form. Although other less likely formal mechanisms could give rise to the carboxylation profile we observe, the FIXproNonGLA carboxylation profile is consistent with a processive mechanism. This profile is also consistent with that shown in a previous study, which demonstrated that the FIXproGLA41 peptide is processively carboxylated (*25*). Therefore, the propeptide alone is able to confer processive carboxylation of an attached substrate that lacks the highly conserved Gla domain sequences found in the vitamin K-dependent blood coagulation proteins.

Kinetics of Carboxylation of Gla Domains of Vitamin K-Dependent Proteins. In contrast to the peptides with an attached propeptide, we find that the Gla domain of factor IX (FIXGLA46) alone is a poor substrate for the carboxylase. For example, in the presence of 20 μM factor IX free propeptide, we estimate its K_m to be 140 $\pm$ 32 μM (Table 3) as compared to 0.2 μM for FIXproGLA46. Because of solubility problems, the highest concentration of substrate that could be achieved was 250 μM, which is 2-fold greater than its apparent K_m. Therefore, the actual K_m may be somewhat higher. Nevertheless, the apparent affinity of the FIXGLA46 peptide is clearly weaker ($\sim$500-fold) than that observed for the corresponding peptide with an attached propeptide (FIXproGLA46). We also compared the carboxylation of the FIXGLA46 peptide to that of the pentapeptide substrate FLEEL and the uncarboxylated mature form of bone Gla protein. The apparent K_m for the small glutamate substrate FLEEL is 1190 $\pm$ 300 μM, similar to previously reported values using purified carboxylase (*4*). The uncarboxylated bone Gla protein has a $K_m = 0.61 \pm 0.07$ μM (Table 3), nearly identical to the value obtained by Benton et al. (*28*). Therefore the apparent K_m for the FIXGLA46 peptide is at least 230-fold higher than observed for uncarboxylated bone Gla protein and is closer to the range observed for small glutamate substrates.

DISCUSSION

Our studies indicate that the propeptide is the initial binding site, confers substrate affinity, and is sufficient to direct multiple carboxylations in a single binding event (i.e.,

processive). In contrast to bone Gla protein, an attached propeptide is required for efficient association of the Gla domain of factor IX with the carboxylase. Evidence that the initial interaction between the enzyme and substrate is mediated by the propeptide is provided by inhibition studies, which indicate that free propeptide is a competitive inhibitor of FIXproGLA41 carboxylation. In addition, carboxylated full-length factor IX with the propeptide still attached inhibits carboxylation of FIXproGLA41 with a K_i similar to that of free propeptide. Therefore the carboxylation state of the Gla domain does not appear to affect binding of the substrate to the carboxylase through the propeptide. Further evidence for the importance of the propeptide is provided by experiments which show that substrates with a covalently linked propeptide have similar apparent affinities for the carboxylase. The K_m values are in the 0.2−0.5 μM range whether the propeptide is followed by just the Gla domain (FIXproGLA41), the Gla domain and hydrophobic stack domain (FIXproGLA46), the rest of the factor IX molecule (proFIX), or a non-Gla domain sequence containing glutamic acid residues (FIXproNonGLA). Therefore, regions beyond the propeptide do not affect the apparent binding affinity of the carboxylase for its substrate.

An amino-terminal propeptide alone is also able to direct processive carboxylations of an attached glutamate-containing region without the requirement for the highly conserved sequences present in the Gla domains of the vitamin K-dependent blood proteins. This is demonstrated by the carboxylation studies of the FIXproNonGLA peptide, which contains the factor IX propeptide but not the highly conserved Gla domain sequences characteristic of the vitamin K-dependent proteins. The pattern of carboxylation of FIXproNonGLA peptide is consistent with the processive mechanism previously observed for the factor IX propeptide/Gla domain peptide (*25*). *In vivo*, processivity would be expected to be highly efficient, with molecules undergoing carboxylation being fully carboxylated. With FIXproNonGLA, we find that 40% of the peptide that undergoes carboxylation is fully carboxylated, similar to the fraction fully carboxylated with the FIXproGLA41 (*25*). Thus, the FIXproNonGLA peptide is processively carboxylated in a manner similar to the factor IX propeptide Gla domain peptide, indicating that the Gla domain sequences are not required for processivity. Therefore, the binding affinity of the propeptide and the propeptide's ability to activate the carboxylase are sufficient to allow multiple carboxylations of factor IX during one binding event.

The reduced rate of carboxylation of FIXproNonGLA is also likely a consequence of a processive mechanism of carboxylation. For processivity to occur, the rate of release of the substrate must be slower than the rate of carboxylations. For example, if propeptide dissociation were rate-limiting, the observed rate of carboxylation would be proportional to the number of glutamates that can be carboxylated in a single enzyme turnover. Thus, a substrate with fewer than 12 carboxylation sites would undergo fewer carboxylations in a single enzyme turnover and would appear to be carboxylated at a proportionally slower rate. This is consistent with our observations of the ~3-fold lower V_{max} for FIXproNonGLA peptide compared to FIXproGLA41. Recently, it has been shown that a chimera containing the prothrombin propeptide and a normally uncarboxylated glutamate-rich region of thrombin is efficiently carboxylated in mammalian cell culture (*31*). This finding, along with our studies, indicates that, both *in vitro* and *in vivo*, the propeptide alone can direct multiple carboxylations in an attached region containing glutamates.

The sequences of the Gla domains of the vitamin K-dependent proteins are highly conserved; therefore, it seems plausible that they play an important role in carboxylation. For example, a conserved sequence (Glu-X-X-X-Glu-X-Cys) has been identified within the Gla domains of all known vitamin K-dependent proteins (*11*). In addition, the widely used substrate FLEEL was derived from a subset of Gla domain sequences. Although decarboxylated prothrombin was reported to be a poor substrate for the carboxylase (*32*), several studies have shown that bone Gla protein without an attached propeptide is an excellent substrate (*26*, *27*). Moreover, in the presence of free factor X propeptide, bone Gla protein with a covalently linked propeptide has only a modestly lower K_m than bone Gla protein without an attached propeptide (*28*). Therefore we examined whether the Gla domain of factor IX has a similar recognition site for the carboxylase.

Our results indicate that the Gla domain alone of factor IX has a poor apparent affinity for carboxylase and that an attached propeptide is required for high-affinity binding of factor IX. The K_m for the FIXproGLA46 is 500-fold lower than that observed for the Gla domain alone (FIXGLA46), whereas the K_m for bone Gla protein without an attached propeptide is in the same range as those for covalently linked propeptide substrates. The propeptide of bone Gla protein contains a glycine at the highly conserved −10 position, which is alanine in all other known vitamin K-dependent proteins. It has been shown that substitution of glycine for alanine at the −10 position increases the K_m of the FIXproGLA41 peptide 30-fold (*16*); therefore the bone Gla protein propeptide may possess a significantly weaker affinity for the carboxylase than the factor IX propeptide. Consequently, bone Gla protein and factor IX may have evolved different binding sites that confer substrate recognition and may be carboxylated by distinct mechanisms. Further studies will be required to verify this distinction and understand its physiological significance.

Binding of physiologically relevant substrates for the vitamin K-dependent carboxylase proceeds through a multistep pathway. With factor IX, the initial binding step is through the propeptide, which localizes the glutamates-to-be-carboxylated in the vicinity of the active site. Subsequent interactions are then required between the glutamates-to-be-carboxylated and the active site. Our studies with FIXproNonGLA indicate that the position of the glutamates or the Gla domain structure is not required for multiple processive carboxylations, but we cannot rule out the possibility that the characteristic Gla domain structure may direct the specificity or enhance the efficiency of carboxylation. The Gla domain sequences may play a role in determining which glutamates are carboxylated. For example, residues immediately surrounding a glutamate-to-be-carboxylated may influence its affinity for the active site once it is localized there by binding at the propeptide. Evidence for this is seen in the effect of adjacent amino acids on the activities of small glutamate-containing peptides (*33*) and with partially or uncarboxylated glutamates in bone Gla

protein and matrix Gla protein (*34*). The domain structures of all the vitamin K-dependent blood proteins are highly similar; therefore, even in the uncarboxylated partially unfolded state, these domains may possess secondary structure that localizes all the glutamates-to-be-carboxylated proximal to the active site. Spatial orientation and physical distance of glutamates-to-be-carboxylated from the active site may also play a pivotal role in whether a particular glutamate is modified. With FIXproNonGLA, only four of the five glutamates are carboxylated; therefore, absence of structure that localizes one of the glutamates proximal to the active site or adjacent amino acids that influence a particular glutamate's affinity for the active site may prevent the fifth modification. The glutamates in the Gla domains of the vitamin K-dependent proteins may be ideally positioned for modification, or carboxylation could induce a structural change that enhances subsequent modifications (i.e., cooperativity). Alternatively, native substrates may have an active release mechanism in which the accumulation of negative charge or carboxylation itself could cause a structural change in the Gla domain that signals that carboxylation is complete and directs product release. In such circumstances, the carboxylated product would be expected to have a weaker affinity for the enzyme than free propeptide or the uncarboxylated substrate. This is not observed with proFIX(GLA) in our *in vitro* system, which suggests that completion of carboxylation itself is not a release mechanism. We cannot rule out the possibility that other accessory proteins absent from our *in vitro* assays may be critical for signaling the completion of carboxylation *in vivo*.

In conclusion, this work demonstrates that, with an *in vitro* system using purified components, peptides containing the propeptide and Gla domain are good models for the physiological substrate in carboxylation by the vitamin K-dependent carboxylase. The initial interaction of the vitamin K-dependent carboxylase with its substrate is through the propeptide, which confers the perceived affinity of the enzyme for its substrate without apparent influence of the Gla domain or regions beyond the Gla domain. Binding through the propeptide is also adequate to direct multiple carboxylations in a single binding event without the requirement for the highly conserved sequences in the Gla domains of the vitamin K-dependent proteins. Further studies will be required to understand steps beyond the initial interaction of the propeptide with the carboxylase, such as the interaction of the glutamates with the active site and product release and dissociation steps in the mechanism of efficient and processive carboxylation by the vitamin K-dependent carboxylase.

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