Tethered Processivity of the Vitamin K-Dependent Carboxylase: Factor IX Is Efficiently Modified in a Mechanism Which Distinguishes Gla's from Glu's and Which Accounts for Comprehensive Carboxylation in Vivo†

Olga Stenina, B. Nirmala Pudota, Beth A. McNally, Eric L. Hommema, and Kathleen L. Berkner*

Department of Molecular Cardiology, Lerner Research Institute, and Jacobs Center for Thrombosis and Vascular Biology, Cleveland Clinic Foundation, 9500 Euclid Avenue, NB50, Cleveland, Ohio 44195

Received April 6, 2001; Revised Manuscript Received June 14, 2001

ABSTRACT: The vitamin K-dependent (VKD) carboxylase binds VKD proteins via their propeptide and converts Glu's to γ-carboxylated Glu's, or Gla's, in the Gla domain. Multiple carboxylation is required for activity, which could be achieved if the carboxylase is processive. In the only previous study to test for this capability, an indirect assay was used which suggested processivity; however, the efficiency was poor and raised questions regarding how full carboxylation is accomplished. To unequivocally determine if the carboxylase is processive and if it can account for comprehensive carboxylation in vivo, as well as to elucidate the enzyme mechanism, we developed a direct test for processivity. The in vitro carboxylation of a complex containing carboxylase and full-length factor IX (fIX) was challenged with an excess amount of a distinguishable fIX variant. Remarkably, carboxylation of fIX in the complex was completely unaffected by the challenge protein, and comprehensive carboxylation was achieved, showing conclusively that the carboxylase is processive and highly efficient. These studies also showed that carboxylation of individual fIX/carboxylase complexes was nonsynchronous and implicated a driving force for the reaction which requires the carboxylase to distinguish Glu's from Gla's. We found that the Gla domain is tightly associated with the carboxylase during carboxylation, blocking the access of a small peptide substrate (EEL). The studies describe the first analysis of preformed complexes, and the rate for full-length, native fIX in the complex was equivalent to that of the substrate EEL. Thus, intramolecular movement within the Gla domain to reposition new Glu's for catalysis is as rapid as diffusion-limited positioning of a small substrate, and the Gla domain is not sterically constrained by the rest of the fIX molecule during carboxylation. The rate of carboxylation of fIX in the preformed complex was 24-fold higher than for fIX modified by free carboxylase, which supports carboxylase processivity and which indicates that binding and/or release is the rate-limiting step in protein carboxylation. These data indicate a model of tethered processivity, in which the VKD proteins remain bound to the carboxylase throughout the reaction via their propeptide, while the Gla domain undergoes intramolecular movement to reposition new Glu's for catalysis to ultimately achieve comprehensive carboxylation.

All vitamin K-dependent (VKD)[1] proteins undergo an unusual posttranslational modification, the conversion of glutamyl residues to γ-carboxyglutamyl residues (or Gla's), required for their activities in hemostasis, growth control, Ca^{2+} homeostasis, and possibly signal transduction (*1*−*4*). This modification is carried out by the VKD or γ-carboxylase, a 95 kDa endoplasmic reticulum integral membrane enzyme (*5*, *6*). The carboxylase transduces energy from the oxygenation of vitamin K hydroquinone (KH_2) to abstract a hydrogen from the γ-glutamyl carbon to generate a carbanion intermediate which subsequently undergoes nucleophilic attack of CO_2 to produce Gla. Recent studies have identified the active site of the carboxylase where epoxidation and carboxylation occur and indicated a regulatory mechanism in which the Glu substrate coordinates an active site thiol to initiate oxygenation of KH_2 (*7*).

VKD proteins are targeted to the carboxylase via a homologous sequence, which in most instances is a propeptide cleaved after carboxylation (*6*). Modification occurs in a region immediately adjacent to the propeptide, the Gla domain. Small peptides derived from the Gla domain exhibit poor affinity for the carboxylase, in the millimolar range. Covalent attachment of the propeptide confers high-affinity binding, reducing the K_m's of such peptides 10^3−10^4 fold. A recent study shows that the affinities of different VKD propeptides vary substantially (*8*).

All VKD proteins undergo multiple (3−12) carboxylations in the Gla domain. Since partially carboxylated VKD proteins exhibit poor activity, understanding the mechanism by which comprehensive carboxylation is achieved is important. One possible mechanism is a quality control system that filters out proteins that are not fully carboxylated, analogous to the

† Supported by NIH Grant HL55666.

* To whom correspondence should be addressed. Phone: (216) 445-9760. Fax: (216) 444-9263. E-mail: berknek@ccf.org.

[1] Abbreviations: VKD, vitamin K dependent; fIX, factor IX; Gla, γ-carboxylated Glu; KH_2, vitamin K hydroquinone; fIX[180], factor IX truncated after amino acid 180; fIX[456], full-length fIX; pro, propeptide.

10.1021/bi0107039 CCC: $20.00
Published on Web 08/03/2001

process for secreting glycoproteins (*9*), and there is some evidence for cellular regulation (*10*−*12*). Comprehensive carboxylation could also be achieved by a completely different mechanism based on carboxylase processivity, i.e., in which all Glu to Gla conversions in a Gla domain are effected as a consequence of a single binding event. In the only previous study to test for carboxylase processivity, the products of an in vitro reaction between the carboxylase and a factor IX (fIX) peptide comprising the propeptide and Gla domain were analyzed by mass spectrometry (*13*). Some fully carboxylated peptide (12 mol of Gla/molecule) was observed among a much larger population of undercarboxylated peptide. A probability argument, i.e., that alternative mechanisms would be unlikely to generate fully carboxylated product, was used to suggest processivity. The fact that most of the observed product was undercarboxylated peptide raises the question of whether carboxylase processivity can account for comprehensive carboxylation in vivo. While the authors attributed the partial products to being an in vitro artifact, there are other potentially interesting explanations, such as the requirement for an auxiliary factor or the possibility that some VKD proteins (like fIX) are not as efficiently processive substrates as others. The inefficient in vitro carboxylation is similar to the poor carboxylation often observed when r-VKD proteins are expressed in mammalian cells (*14*−*17*), and an obvious question is whether both observations result from inefficient carboxylase processivity.

To investigate the mechanism of carboxylation, we developed a direct method for testing processivity: the in vitro carboxylation of full-length, native fIX in a fIX/carboxylase complex was challenged using an excess amount of a distinguishable fIX variant to determine if the fIX variant disrupted carboxylation. We found that carboxylation of fIX in the complex was unaffected by the challenge protein, showing unequivocally that the carboxylase is processive. These studies also showed that the carboxylase was capable of comprehensively modifying fIX, that carboxylation of individual fIX/carboxylase complexes was nonsynchronous, and that the carboxylase has a mechanism to drive the conversion of Glu's to Gla's within the Gla domain. This direct method for proving carboxylase processivity now provides a powerful system for defining the processive mechanism.

MATERIALS AND METHODS

Generation of Cell Lines Expressing Full-Length or Truncated fIX. A *Bam*HI fragment containing a cDNA encoding full-length fIX (*18*) was subcloned into the *Bam*HI site of ZEM229 (*19*), which includes a cassette expressing dihydrofolate reductase. A cDNA encoding truncated fIX (amino acids −46 to 180) was constructed by ligating the oligonucleotides TCG GTA ACC CGG GG and GAT CCC CCG GGT TA to the 675 bp *Bam*HI−*Ava*I fragment from the full-length fIX cDNA, which introduced a termination codon after amino acid 180. The truncated cDNA was inserted into the *Bam*HI site of ZEM229. Plasmids were transfected into BHK cells and amplified by selection to 1 μM (truncated fIX) or 5 μM (full-length fIX) methotrexate. Clones were screened by ELISA (*20*) to isolate a high-producing line.

Isolation of Propeptide-Containing fIX. Full-length fIX (fIX[456]) and truncated fIX (fIX[180]) were both secreted as a mixture of propeptide-containing and mature protein. Spent media from cells cultured without serum or vitamin K was chromatographed on a column of anti-fIX propeptide Ab coupled to CNBr-activated Sepharose (2 mg/mL). The Ab was rabbit Ab prepared against the human fIX propeptide (amino acids −18 to −1) (*21*), affinity purified over a column of immobilized fIX propeptide. Purified fIXs were sequenced by Edman degradation, which detected only the propeptide-containing forms (data not shown). Gla quantitation showed that the proteins were uncarboxylated (<0.3 mol of Gla/mol of fIX) (*17*).

Antibodies Used in the Challenge Assay. The anti-fIX MAbs used in the processivity assay, ESN1 (American Diagnostica) and anti-HFIX-40 (generously provided by William Church), were tested for their ability to immunoreact with fIX[456] but not fIX[180]. One test was an ELISA (*20*) using the MAbs as the capturing Abs and affinity-purified polyclonal anti-fIX Ab for detection. fIX[456] quantitation by ELISA gave a value identical to that obtained by BCA (0.6 μg/μL), while fIX[180] (1.6 μg/μL by BCA) could not be detected in the ELISA (<10 ng/mL). The second test was chromatography on columns of immobilized ESN1 or anti-HFIX-40. Spent media from cells expressing either fIX[180] or fIX[456] was passaged over each column (1 mg of Ab/mL of resin, 2 mL of resin); the columns were washed with 20 mL of TNEN (20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP40) and then with PBS (20 mL) and then eluted with 50 mM triethylamine, pH 11. Starting material and eluant were then analyzed in a western using anti-fIX propeptide Ab: while fIX[180] and fIX[456] were both observed in the spent media, only fIX[456] was detected in eluant.

Challenge Assay for Carboxylase Processivity. The preformed fIX/carboxylase complex was isolated from a r-human fIX[456], r-human carboxylase expressing BHK cell line (*22*) cultured in media lacking vitamin K. Solubilized microsomes were prepared (*22*), and aliquots (500 μL) were adsorbed to ESN1 or anti-HFIX-40 (100 μL of resin, 2 mg of Ab/mL) by overnight nutation at 4 °C. The resin was centrifuged (1000 rpm, 1 min) to remove unbound material and then washed five times by the addition of 1 mL of TNCPD (50 mM Tris, pH 7.4, 500 mM NaCl, 0.25% CHAPS, 0.25% phosphatidylcholine, and 5 mM DTT) followed by centrifugation at 1000 rpm, 1 min, and removal of TNCPD. All manipulations were at 4 °C. Aliquots (10 μL) of the slurry (1100 μL) were removed on the final wash to quantitate the amount of carboxylase by a peptide assay (*7*). The resins (100 μL) were then incubated in 400 μL of reaction cocktail [50 mM BES, pH 6.9, 0.06% CHAPS, 0.06% phosphatidylcholine, 0.06% sodium cholate, 0.6 M ammonium sulfate, 2 mM $NaH^{14}CO_3$ (Amersham, 55 cpm/pmol), 5 mM DTT] containing fIX[180] (3 μM). A parallel control lacking fIX[180] was also assayed, and both sets were performed in duplicate. Samples were incubated at 21 °C for 10 min, and carboxylation was then initiated by the addition of vitamin K hydroquinone [KH_2, to 150 μM, prepared as described (*23*)]. Reaction mixtures were continuously rocked, and at timed intervals, aliquots (40 μL) were removed and transferred to tubes containing 40 μL of SDS−PAGE loading buffer, which quenched the reaction. The samples were incubated at 21 °C for 20 min and then centrifuged at 4000 rpm for 5 min, and 60 μL was subjected

to SDS−PAGE. A ^{14}C-methylated bovine serum albumin (BSA) standard curve (89 μCi/mg, Amersham, passaged over a G-50 column to remove any noncovalently bound ^{14}C) was included on each gel. Following electrophoresis, gels were washed at least five times in 40% methanol and 5% acetic acid, then dried, and quantitated by PhosphorImager analysis. Both protein bands and triplicate blank regions (from the same lanes as the bands) were selected using the same sized box, and the average blank value was subtracted from that of each protein band. Because of the low gel background and long exposures used (1−4 months), signal-to-noise ratios of 2−50 could be obtained.

To assess the validity of the assay, the recovery of fIX from the resin was measured. The fIX[456]/carboxylase complex adsorbed to ESN1 or anti-HFIX-40 was in vitro carboxylated for 30 min, quenched with cold $NaHCO_3$ (to 50 mM), then incubated in SDS−PAGE loading buffer, and centrifuged as above. The samples were gassed with CO_2 to remove free $NaH^{14}CO_3$ and then quantitated by scintillation counting. Comparison of cpm before and after centrifugation showed reproducible, quantitative recovery of radiolabeled protein.

Gla Quantitation of Carboxylated fIX[456]. In vitro carboxylated fIX[456] from the fIX[456]/carboxylase complex was purified and subjected to Gla analysis. Carboxylation of the complex was as above, except that the complex was immobilized on anti-carboxylase Ab (100 μL of resin, 2 mg/mL). The Ab was rabbit Ab against the C-terminus of human carboxylase (amino acids 744−758), affinity purified using immobilized peptide. The fIX[456]/carboxylase complex bound to anti-carboxylase Ab was in vitro carboxylated with or without fIX[180] (3 μM) as in the challenge assay described above and then analyzed by SDS−PAGE/PhosphorImager. A separate sample was in vitro carboxylated for 30 min, and the entire reaction mix (400 μL of cocktail and 100 μL of resin) was diluted 10-fold into 1% Triton X-100, which disrupted the fIX[456]/carboxylase complex. After centrifugation (2000 rpm, 5 min, 4 °C) to remove the resin, the sample was chromatographed on an anti-fIX Ab column (ESN1, 200 μL, 2 mg/mL), followed by washes with TNEN (2 mL) and then PBS (2 mL) and elution with 50 mM triethylamine, pH 11 (500 μL). The fIX[456] was precipitated with trichloroacetic acid (to 30%), and the precipitate was processed for base hydrolysis and HPLC to quantitate Gla residues, as previously described (*22*).

RESULTS

Establishing a Model System To Assess Carboxylase Processivity. A direct test for carboxylase processivity was developed by using a fIX variant (fIX[180]) to challenge the carboxylation of full-length fIX (fIX[456]) in a preformed complex with carboxylase. In a fully processive mechanism, the kinetics of fIX[456] carboxylation would be unaffected by the challenge protein. In a distributive mechanism, the presence of excess challenge protein would compete with fIX[456] released from the complex, thereby decreasing the rate and extent of fIX[456] carboxylation.

The fIX[180] challenge protein was a truncated variant deleted in the serine protease domain so that it would be distinguishable from fIX[456] on SDS−PAGE. The two proteins were identical in amino acids −46 to 180, which comprise the leader sequence, propeptide, Gla domain, growth factor domain, and activation peptide. The propeptide-containing form of fIX[180] was used, and was obtained by exploiting the fact that propeptide processing is saturated when VKD proteins are expressed at high levels in BHK cells (*17*). This approach allowed us to use a challenge protein that contained all of the Gla domain and avoided having to introduce mutations into the substrate. Both fIX[180] and a fIX[456] control were purified using an anti-fIX propeptide Ab column, and N-terminal sequencing confirmed the presence of the propeptide. Both proteins were isolated from cell lines cultured without vitamin K and so were uncarboxylated, as verified by Gla quantitation. When analyzed by SDS−PAGE, both purified proteins exhibited a doublet, presumably due to heterogeneous glycosylation (Figure 1). For simplicity, the propeptide-containing forms of fIX[180] and fIX[456] will be referred to as fIX[180] or fIX[456] throughout, since only the propeptide forms were used in these studies.

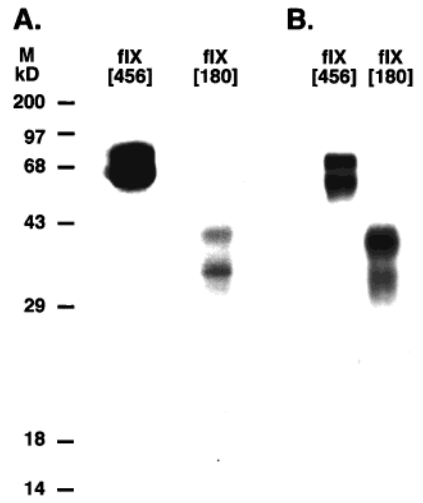

FIGURE 1: Silver stain and western analysis of fIX substrates. Purified propeptide-containing fIX[456] and fIX[180] were analyzed by SDS−PAGE and silver staining (part A), using 10 μg due to weak reactivity of fIX[180] in silver (and also in Coomassie) staining. Aliquots (50 ng) were also analyzed by a western (part B) using anti-fIX propeptide Ab.

Table 1: Kinetic Analysis of fIX Substrates[a]

substrate	V_{max} (cpm/min)	K_m (μM)	V_{max}/K_m (cpm min^{-1} μM^{-1})
fIX[456]	101	0.45	224
fIX[180]	47	0.37	127

[a] Purified fIX[180] and fIX[456] (Figure 1) were incubated at varying concentrations (0.15−3 μM) in 250 μL of reaction cocktail with r-carboxylase (50 nM). The carboxylase was a pure preparation fractionated over Q-Sepharose to remove free propeptide (*7*). Following the addition of KH_2 (to 150 μM), aliquots (50 μL) were removed (at 10, 20, and 30 min) and quantitated by SDS−PAGE and PhosphorImager using ^{14}C-BSA standard curves run on each gel as a reference.

To assess the suitability of fIX[180] as a substrate for challenging processivity, carboxylation of fIX[180] and fIX[456] was compared (Table 1). Both proteins exhibited similar kinetics: the K_m's were the same and the V_{max} for fIX[180] was only slightly lower than for fIX[456].

The fIX[456]/carboxylase complex being challenged was an in vivo preformed complex isolated from BHK cells coexpressing human r-carboxylase and human r-fIX[456].

Table 2: Isolation of the r-fIX[456]/r-Carboxylase Complex[a]

		total activity	
	fraction	(cpm/h) × 10^{-4}	%
(A) r-fIX[456]/r-carboxylase	microsomes	1200	100
	unbound material	150	13
	αfIX Ab resin	1080	90
(B) r-carboxylase	microsomes	600	100
	unbound material	610	102
	αfIX Ab resin	0.1	0.02

[a] Solubilized microsomes (1 mL) from BHK cells expressing r-fIX[456] and r-carboxylase (part A) or just r-carboxylase (part B) (*22*) were adsorbed to anti-HFIX-40 resin (200 μL) overnight at 4 °C and then centrifuged (1000 rpm, 1 min, 4 °C) to remove unbound material. The resins were washed five times with 1 mL of TNCPD, and on the final wash aliquots (10 μL of the 1200 μL slurry) were removed and assayed for carboxylase peptide activity (*7*), along with aliquots (10 μL) of the starting slurry and unbound material. An ELISA on the solubilized microsomes and unbound sample showed that all (>99%) of the fIX[456] was adsorbed to the resin (in part A). Similar results were obtained using ESN1 resin (not shown).

These cells overexpressed carboxylase 70-fold, as determined both by an activity assay and by a quantitative western, and so 99% of the complexes contained human (versus hamster) carboxylase. The cells were cultured without vitamin K so the complex was uncarboxylated. The fIX[456] in the complex was shown by western analysis with anti-fIX propeptide Ab to contain the propeptide (data not shown), as expected. An important component of the isolation was the removal of free carboxylase. We initially screened cell lines with different ratios of r-fIX to r-carboxylase (*2*−*20*) and found that all isolations yielded similar amounts of free carboxylase. The optimal cell line used in these studies contained r-fIX in ∼2-fold excess of r-carboxylase, and 13% free carboxylase was observed (Table 2). Because free carboxylase would complicate analyses, the fIX[456]/carboxylase complex was adsorbed to anti-fIX Ab resin and in vitro carboxylation reactions were performed using the immobilized complex. Immobilization of the complex did not yield detectable free carboxylase (see below), indicating tight fIX[456]/carboxylase binding and an extremely slow off rate for carboxylase dissociation. Antibodies against the serine protease of fIX[456] were used so that the challenge fIX[180], which lacks this domain and is not recognized by these Abs (Materials and Methods), would be fully accessible to carboxylase in the assay. Two different MAbs were used, which gave indistinguishable results.

We showed that immobilization of the fIX[456]/carboxylase complex on the resin was specific for fIX. Thus, while 90% of the carboxylase from a cell line expressing both r-fIX and r-carboxylase was adsorbed to anti-fIX Ab resin, only 0.02% carboxylase adsorption was observed when a cell line expressing only r-carboxylase was used (Table 2). We also validated the assay by showing that radiolabeled fIX could be quantitatively recovered from the resin (Materials and Methods). A final test showed that the concentration of the complex on the resin did not affect the kinetics: a linear response was obtained when the ratio of complex to resin was varied over a 20-fold range (data not shown). Therefore, immobilization of the complex on resin did not introduce unexpected artifacts into the assay.

Immobilization of fIX[456] via the serine protease provided a several hundred amino acid "spacer" between the site of Ab binding and the Gla domain, and so immobilization would not be expected to interfere with carboxylation. Nonetheless, to assess the effect of immobilization on carboxylation, immobilized complex was compared to free complex (Figure 2). The extent of fIX[456] carboxylation was unaffected by immobilization, and the rate for the immobilized complex was only slightly lower than for the free complex. It should be noted that because fIX[180] is not immobilized like fIX[456] in the challenge assay, if carboxylase is released from partially carboxylated fIX[456] in a distributive mechanism, the fIX[180] would have a kinetic advantage over fIX[456].

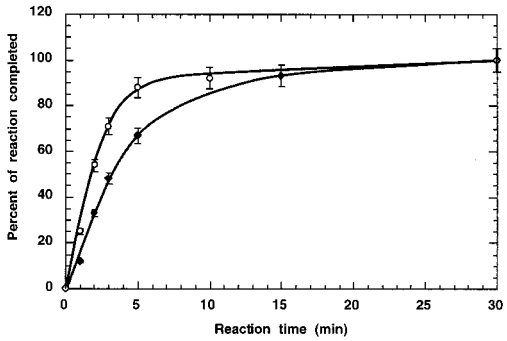

FIGURE 2: In vitro carboxylation of the free versus immobilized fIX[456]/carboxylase complexes. The fIX[456]/carboxylase complex from solubilized microsomes [100 μL, 14 pmol of carboxylase [as quantitated by peptide activity (*7*)], open circles] or immobilized to anti-HFIX-40 (100 μL of resin, 54 pmol of carboxylase, closed diamonds) was incubated at 21 °C for 5 min in 400 μL of reaction cocktail, and the reaction was then initiated with KH_2 (to 150 μM). Aliquots withdrawn at the indicated times were processed through SDS−PAGE and PhosphorImager, along with ^{14}C-BSA, to quantitate ^{14}C incorporation into fIX[456]. The 100% value for the solubilized complex or immobilized complex corresponded to 9000 or 32 000 cpm of ^{14}C-fIX[456], respectively.

Carboxylation of the fIX[456]/Carboxylase Complex in the Absence of Challenge Protein. SDS−PAGE analysis of the in vitro carboxylation reaction provided important information because fIX[456] underwent a shift in mobility upon carboxylation of the 12 Glu's (Figure 3). While a homogeneous (i.e., uncarboxylated) population of the fIX[456]/carboxylase complex was used to start the reaction, within a minute after the addition of KH_2, the fIX[456] forms were heterogeneous in extent of carboxylation. By the end of the reaction (30 min, Figure 3), only a discrete fIX[456] band was observed, which was shown to be fully carboxylated (described below).

Quantitation of the time course (Figure 4A) showed that carboxylation of fIX[456] was linear over most of the reaction. Linearity was observed until ∼5 min, a time point which corresponded to 70% carboxylation of fIX[456] (Figure 4A), and at later times the rate slowed. In the experiments presented here, the next time point sampled was 15 min. However, we initially tested several different reactions with varying time points to determine the best conditions for monitoring the entire reaction, and in all cases the rate was linear only for ∼70% of the reaction.

^{14}C incorporation into fIX[456] at the end of the reaction suggested that it was fully carboxylated. It also indicated that the free fIX[456] immobilized along with the fIX[456]/carboxylase complex on the anti-fIX Ab resin was not efficiently carboxylated, which was consistent with studies

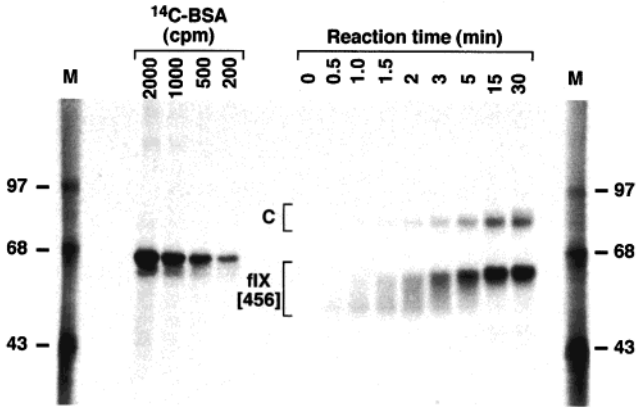

FIGURE 3: In vitro carboxylation of the fIX[456]/carboxylase complex. The fIX[456]/carboxylase complex (75 nM) isolated from a cell line coexpressing r-fIX[456] and r-carboxylase (Table 2) was immobilized on anti-HFIX-40 resin and incubated for 10 min in reaction cocktail (400 μL). Carboxylation was initiated by the addition of KH_2, and aliquots removed at the indicated times were electrophoresed on an 8% gel, along with ^{14}C-BSA and molecular weight markers (M). The gel was dried and exposed for 2 months to quantitate ^{14}C incorporation into fIX[456] and the carboxylase (labeled C).

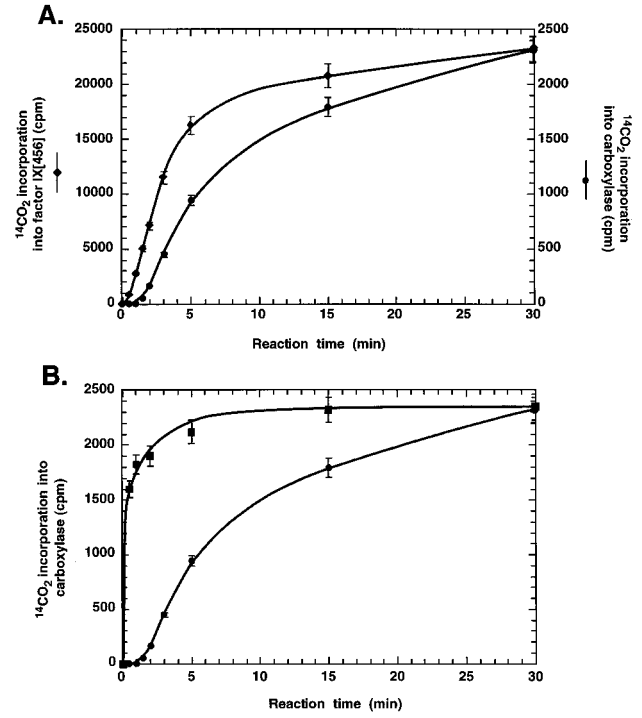

FIGURE 4: Quantitation of in vitro fIX[456]/carboxylase complex carboxylation. (A) Duplicate gels (Figure 3) were quantitated by PhosphorImager for fIX[456] (diamond symbols) and carboxylase (circles) carboxylation. (B) Carboxylase carboxylation of free carboxylase (square symbols) versus fIX[456]-bound carboxylase (circles) was compared. Free carboxylase was immobilized on resin by adsorbing carboxylase from cells expressing only r-carboxylase (Table 2) on anti-C-terminal carboxylase Ab (100 μL of resin). Immobilized fIX[456]-bound carboxylase was prepared as in Figure 3. Resins (100 μL) were incubated in 400 μL of reaction cocktail with KH_2 (150 μM), and aliquots withdrawn at the indicated times were quantitated by SDS−PAGE and PhosphorImager.

showing a large difference in rates for free versus carboxylase-bound fIX[456] (discussed below). To independently quantitate the extent of fIX[456] carboxylation, the protein was purified and subjected to Gla analysis. For this experiment, the fIX[456]/carboxylase complex was immobilized on anti-carboxylase Ab instead of anti-fIX Ab (Materials and Methods), so that the only fIX[456] present was in a fIX-[456]/carboxylase complex. The time course of carboxylation was identical to that shown in Figure 4A, and quantitation of purified fIX[456] from the end of the reaction (30 min) by Gla analysis showed comprehensive conversion of Glu's to Gla's (11.6 ± 0.3 mol of Gla/mol of fIX).

Table 3: Rates of Peptide, Protein, and Complex Carboxylation[a]

	substrate	carboxylase source	Glu turnover (min^{-1})
a	fIX[456]	pure	0.2
b	fIX[180]	pure	0.3
c	EEL	pure	4.4
d	fIX[456]	complex	4.8
e	EEL	complex	5.1
f	fIX[456]	immobilized complex	2.2
g	EEL	immobilized complex	5.0

[a] (a−c) fIX[456] and fIX[180] (both at 2.1 μM, or 5 times over K_m, Table 1) and EEL [1.5 mM, or 8 times over K_m (*7*)] were incubated with 35 nM r-carboxylase (purified as described in the legend to Table 1) in 200 μL of reaction cocktail. Propeptide (10 μM) was included in the EEL assay (c). Following the addition of KH_2 (to 150 μM), aliquots (50 μL) were withdrawn (over 10−30 min) and quenched by dilution into 2 mL of 0.1% CHAPS. Samples were boiled and quantitated by scintillation counting. (d−g) Solubilized microsomes containing 90% of the carboxylase complexed to fIX[456] (Table 2) were either incubated in reaction cocktail (200 μL, 35 nM carboxylase/fIX[456]) (d) or were immobilized on anti-HFIX-40 resin (50 μL) and then in vitro carboxylated in reaction cocktail (200 μL, 90 nM fIX[456]/carboxylase) (f). Duplicate aliquots were incubated with propeptide (to disrupt the complex) and 3 mM EEL (e, g), and EEL carboxylation was quantitated (over 5−15 min) as TCA nonprecipitable counts. fIX[456] carboxylation in the complex (d, f) was quantitated both by SDS−PAGE/PhosphorImager, so that the small amount of carboxylase carboxylation did not contribute to total cpm, and by scintillation counting (which gave values ∼10% higher), so that EEL and fIX[456] cpm could be compared using the same assay. The numbers shown (d, f) are from the gel assay. Aliquots (50 μL, taken over 1−3 min) were quenched with SDS−PAGE loading buffer or with CHAPS, as above.

Carboxylase in the fIX[456]/carboxylase complex was also carboxylated, occurring after fIX[456] carboxylation (Figure 4A). We had previously reported the fact that the carboxylase is carboxylated both in vitro and in vivo (*22*). The much longer exposure times used in the present studies (2 months) increased the sensitivity of detection and permitted a better analysis of the temporal appearance of carboxylase versus fIX[456] carboxylation. Carboxylase carboxylation was not detected until 90 s after the addition of KH_2, at a time when 22% of the fIX[456] was carboxylated (Figure 4A). In contrast, carboxylation of free carboxylase did not exhibit such a delay (Figure 4B).

The rate of carboxylation of fIX[456] in the complex was similar to the rate for a small peptide (EEL). The fIX[456]/carboxylase complex was incubated with or without EEL, and carboxylation of both fIX[456] and EEL was monitored (Table 3d−g). Both the immobilized and nonimmobilized fIX[456]/carboxylase complexes were tested. The rate of fIX[456] carboxylation in the nonimmobilized complex was ∼2-fold higher than for the immobilized complex, as expected (Figure 2), and the rate of the nonimmobilized complex was equivalent to the rate of EEL carboxylation (Table 3, d and e). The rate of EEL carboxylation by the complex was similar to the value obtained using pure carboxylase (Table 3, c and d).

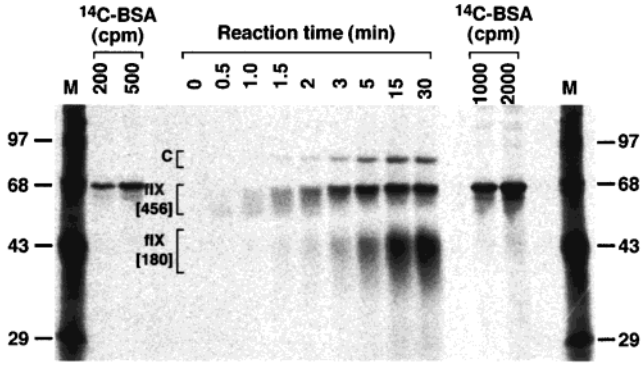

FIGURE 5: In vitro carboxylation of the fIX[456]/carboxylase complex in the presence of the fIX[180] challenge protein. The fIX[456]/carboxylase complex was in vitro carboxylated as described in the legend to Figure 3, except that fIX[180] (3 μM) was included in the reaction cocktail and the proteins were analyzed on a 12% gel.

The rate of fIX[456] carboxylation in the preformed complex was 24-fold higher than the rate for pure carboxylase reacted with free fIX[456] (Table 3, a and d). This difference most likely reflected the different number of fIX-[456] turnovers that occurred in each reaction. With free carboxylase and free fIX[456], the fIX[456] was in excess and underwent multiple turnovers, while with the preformed complex the carboxylase and fIX[456] were stoichiometric and so fIX[456] could only undergo one turnover. In a processive mechanism, binding and release would be reflected in the rate of carboxylation of free carboxylase and fIX[456] but not of complex. In a distributive mechanism, binding and release would be reflected in the rate for both reactions. The 24-fold difference in rates (Table 3, a and d) therefore suggests a processive reaction with binding and/or release being the rate-limiting step in carboxylation.

Carboxylation of the fIX[456]/Carboxylase Complex in the Presence of Challenge Protein. The fIX[456]/carboxylase complex was in vitro carboxylated in the presence of fIX-[180], and the products were analyzed by SDS−PAGE and PhosphorImager (Figure 5). The concentration of fIX[180] was 3 μM, which was 8-fold above the K_m for this substrate (Table 1) and 40-fold in excess of the complex. As with the complex carboxylated in the absence of fIX[180], a heterogeneous population of fIX[456] forms was observed shortly after the addition of KH_2, and the temporal profile of fIX-[456] forms was similar for the complex incubated with or without challenge protein (Figures 3 and 5). The presence of fIX[180] did not affect either the rate or the extent of fIX[456] carboxylation (Figure 6). The plateau represented full carboxylation, as shown by purifying the fIX[456] and performing Gla quantitation (Materials and Methods). Thus, comprehensive fIX[456] carboxylation was achieved even in the presence of excess fIX[180] challenge protein.

Efficient fIX[180] carboxylation was observed at later times in the reaction (Figure 7A), consistent with the data showing that fIX[180] was suitable as a challenge protein (Table 1). The onset of fIX[180] carboxylation was concurrent with that of carboxylase carboxylation, at 90 s (Figure 7B). Interestingly, fIX[180] carboxylation began at a time when fIX[456] carboxylation was only 23% completed (Figure 7). Thus, carboxylation of the two fIX forms occurred without fIX[180] disrupting fIX[456] carboxylation (Figure 6).

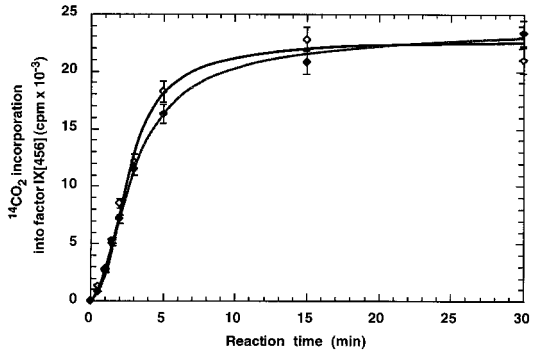

FIGURE 6: The fIX[180] challenge substrate does not disrupt carboxylation of fIX[456] in the fIX[456]/carboxylase complex. The fIX[456]/carboxylase complex in vitro carboxylated in the presence (open diamonds) or absence (closed diamonds) of fIX-[180] was subjected to SDS−PAGE (Figures 3 and 5), and duplicate gels were quantitated by PhosphorImager. The entire experiment was performed twice.

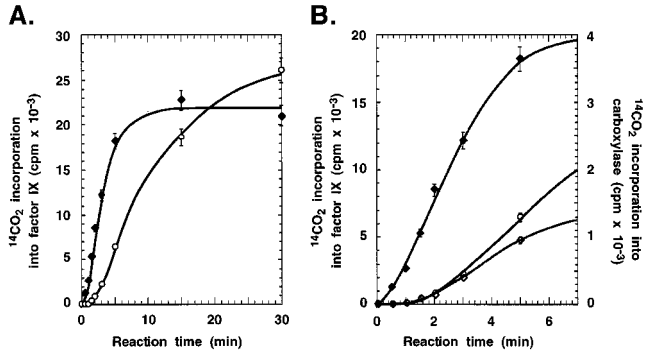

FIGURE 7: Quantitation of fIX[456], fIX[180], and carboxylase carboxylation in the challenge reaction. Duplicate gels of the fIX-[456]/carboxylase complex carboxylated in the presence of fIX-[180] (Figure 5) were quantitated by PhosphorImager for fIX[456] (closed diamonds), fIX[180] (open circles), and carboxylase (open diamonds). Part A is the entire time course of fIX[456] and fIX-[180] carboxylation, and part B is the initial portion of the reaction showing fIX[456], fIX[180], and carboxylase carboxylation.

Detection of fIX[180] carboxylation correlated with the appearance of free carboxylase, which could be monitored because the fIX[456]/carboxylase complex was immobilized on anti-fIX MAb resin (Table 4). At a time soon after the addition of KH_2 (one min) when neither carboxylase nor fIX-[180] carboxylation was detected, only 2% free carboxylase was observed. With time, an increasing amount of carboxylase was released from the anti-fIX MAb resin, and the time course for appearance of free carboxylase paralleled that of fIX[180] and carboxylase carboxylation (Figure 7B). These results, and the observed heterogeneity in fIX[456] forms (Figures 3 and 5), suggest a nonsynchronous reaction, i.e., in which repositioning within the Gla domain for sequential carboxylation of Glu's is not identical among individual complexes. Nonsynchronicity would lead to complexes completing fIX[456] carboxylation at different times, and the free carboxylase generated would therefore account for the observed fIX[180] and carboxylase carboxylation.

Carboxylation of a Small Gla Domain-Derived Peptide by the fIX[456]/Carboxylase Complex. To determine if a small peptide can access the carboxylase active site during fIX[456]/carboxylase complex carboxylation, the peptide EEL was reacted with the fIX[456]/carboxylase complex, and both EEL and fIX[456] carboxylation were monitored.

Table 4: Release of Carboxylase from fIX[456]/Anti-fIX MAb Resin during in Vitro Carboxylation[a]

time (min)	unbound carboxylase (%)	time (min)	unbound carboxylase (%)
0	0	7	20
1	2	9	20
3	8	15	25
5	13		

[a] The fIX[456]/carboxylase complex immobilized on anti-HFIX-40 resin (100 μL, 60 nM complex) was incubated for 10 min in 400 μL of reaction cocktail with fIX[180] (3 μM). KH_2 (to 150 μM) was added, and two aliquots were removed at the times indicated. One set (20 μL) was analyzed by SDS−PAGE and PhosphorImager, which showed the same kinetics as in Figure 5. The other set (40 μL) was centrifuged (2000 rpm, 20 s), 10 μL of the supernatant was transferred to a new tube, and the remaining supernatant was discarded. Supernatant and resin were assayed for carboxylase peptide activity (*7*), and the unbound carboxylase was determined by activity in the supernatant versus the sum of supernatant and resin. In a control experiment lacking KH_2, the percent unbound carboxylase at 15 min was the same as at 0 min, showing that carboxylase release over time was specific to carboxylation. The experiment was performed at 21 °C, twice, in duplicate.

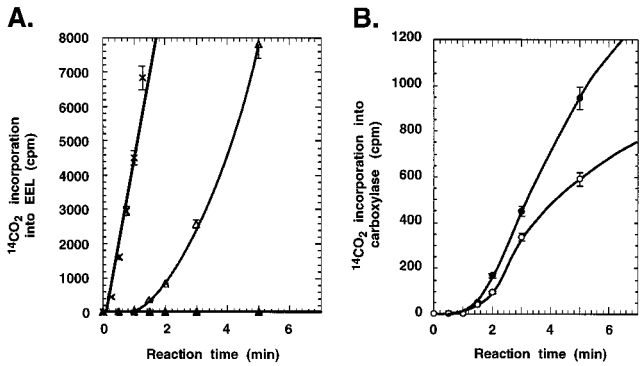

FIGURE 8: Carboxylation of EEL by the fIX[456]/carboxylase complex. (A) The fIX[456]/carboxylase complex was in vitro carboxylated as in Figure 3, except that the reaction was performed in the absence (closed triangles) or presence (open triangles) of EEL. Carboxylation of EEL by free carboxylase was also measured (× symbols) using solubilized microsomes from baculovirus- (r-carboxylase-) infected insect cells (*34*) adsorbed to anti-C-terminal carboxylase Ab resin. Resins (100 μL) were incubated in 400 μL of reaction cocktail and 150 μM KH_2 with or without 3 mM EEL. All three reactions were performed in duplicate, and aliquots removed at the indicated times were quantitated for EEL by measuring TCA nonprecipitable counts. (B) The initial part of the time course is shown for carboxylase in the fIX[456]/carboxylase complex carboxylated in the absence (closed circles) or presence (open circles) of EEL as monitored in duplicate time courses by SDS−PAGE and PhosphorImager.

The peptide, which lacks a propeptide and should therefore not displace fIX[456] from carboxylase, was present at 3 mM, which was 15-fold over the K_m (*7*) and 40 000-fold in excess of the complex. The rate of fIX[456] carboxylation was identical to that observed in the absence of peptide (Figure 3 and data not shown). However, EEL carboxylation was not detected until 90 s after the addition of KH_2 (Figure 8A), which was not due to limitations in detection and which contrasted the much earlier onset obtained with free carboxylase. The delayed onset of EEL and carboxylase carboxylation was identical (90 s, Figure 8B) and paralleled the delay observed for fIX[180] carboxylation (Figure 7B). The delayed onset and lack of effect of EEL on fIX[456] carboxylation together indicate a tight association of the fIX-[456] Gla domain with the carboxylase which blocks EEL access during fIX[456] carboxylation.

DISCUSSION

All VKD proteins require multiple carboxylations for their biological activity. To determine how multiple carboxylation is accomplished, we developed a model system to directly and conclusively test for carboxylase processivity. The results show that the efficiency of carboxylase processivity is high and sufficient to account for comprehensive carboxylation in vivo and provide several new insights into the mechanism by which processive carboxylation is accomplished.

Tethered Processivity of the Carboxylase. The fIX[456]/carboxylase complex showed identical kinetics in the presence or absence of excess challenge fIX[180] (Figure 6), and both reactions resulted in comprehensive carboxylation of fIX[456]. The observations that neither the rate nor extent of fIX[456] carboxylation was affected is consistent only with a processive mechanism in which fIX[456] release does not occur during the multiple Glu carboxylations. A distributive mechanism would result in the observed data only if the fIX[180] was a poor substrate; however, fIX[180] was efficiently carboxylated (Table 1, Figure 7). A partially distributive mechanism, i.e., in which release occurs after several carboxylations, can also be ruled out. Such a mechanism would prevent full fIX[456] carboxylation, which was not observed (Figure 6). The only other alternative is one in which partially carboxylated forms released from the carboxylase outcompete uncarboxylated protein for binding. However, this possibility is also ruled out since carboxylated fIX[180] was generated early in the reaction but did not disrupt fIX[456] carboxylation (Figures 6 and 7).

Carboxylation of VKD proteins, then, occurs by a mechanism of tethered processivity in which the proteins are bound to the carboxylase throughout the reaction via the propeptide, with the Gla domain undergoing intramolecular movement to reposition Glu's for catalysis (Figure 9). The Gla domain is tightly associated with the carboxylase during the multiple modifications, since the presence of fIX in the carboxylase active site blocked EEL carboxylation (Figure 8A).

Carboxylation of individual fIX[456]/carboxylase complexes was nonsynchronous. A fairly homogeneous population of fIX forms was observed at the first time point sampled, but at later times both a heterogeneous population of partially and fully carboxylated fIX[456] forms and free carboxylase were observed (Figures 3 and 5 and Table 4). The reaction was initiated using complexes lacking vitamin K. However, it is unlikely that the lack of cofactor during in vivo complex formation was responsible for nonsynchronicity, since the population was so homogeneous at the first time point of the reaction, after vitamin K binding and carboxylation corresponding to ~1 mol of Gla/fIX. The nonsynchronicity is more likely due to nonidentical intramolecular movement within the Gla domain. The average rate of carboxylation of Glu's within the Gla domains occurred at the same rate as carboxylation of the peptide EEL (Table 3), showing that intramolecular movement to reposition Glu's for catalysis is very rapid, equivalent to peptide access by diffusion. This result is also significant because the fIX[456] substrate was the full-length molecule. Thus, the efficient

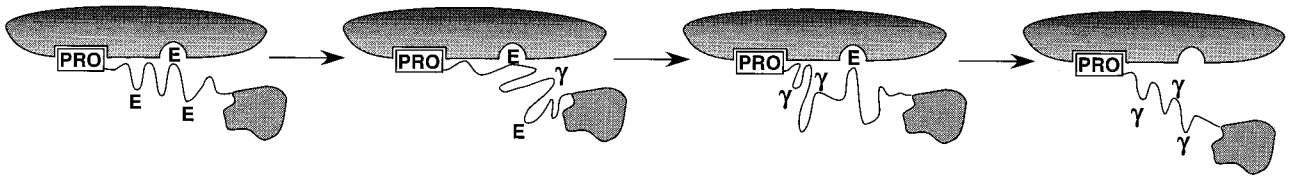

FIGURE 9: Tethered processivity of the carboxylase. VKD proteins remain tethered to the carboxylase via the propeptide (PRO), while the Gla domain undergoes intramolecular movement to reposition Glu's for catalysis. Efficient movement is not constrained by the remainder of the VKD protein. Distinction of Glu's from Gla's drives the reaction, and comprehensive carboxylation is ultimately achieved.

rate shows that the Gla domain, which represents only ~10% of the fIX molecule, is not sterically constrained by the rest of the protein during carboxylation.

Determination of the rate of Glu carboxylation in a VKD protein/carboxylase complex allows the determination of the catalytic rate independent of VKD protein binding and release, because the reaction is processive. The rate for complex carboxylation, which is the first such reported value, was 24-fold higher than for free carboxylase incubated with free fIX (Table 3), showing that the rate-limiting step in VKD protein carboxylation is protein binding and/or release. The rate of complex carboxylation is also of interest with respect to the previous study on carboxylase processivity (*13*). In that work, the carboxylation products isolated after a 27 h reaction were shown to be mostly undercarboxylated peptide. However, our data show that fIX[456] in the complex turned over in ~10 min (Figure 4), and so >99% of the product would be predicted to be fully carboxylated by 27 h. The difference indicates poor processivity in those studies, which may have been due to the use of a peptide substrate and/or the fact that the peptide contained mutations in both the propeptide and Gla domains, in contrast to our use of full-length native protein.

While the challenge fIX[180] did not perturb carboxylation of the fIX[456]/carboxylase complex (Figure 6), its carboxylation was detected early in the course of the reaction (Figure 7). These results can be explained by nonsynchronous fIX[456]/carboxylase carboxylation. The onset of fIX[180] carboxylation (90 s) was identical to that observed for carboxylase and EEL carboxylation (Figures 4, 7, and 8). At this time, 23% of the fIX[456] was carboxylated and a small amount of free carboxylase was detectable (Table 4). As the reaction proceeded, the amount of free carboxylase increased, along with the appearance of the slow-migrating fIX[456] form which comigrated with fully carboxylated fIX[456] (Figure 5). The simplest interpretation of the data, then, is that nonsynchronous carboxylation generates a subpopulation of fully carboxylated fIX[456] and free carboxylase, which is now available for fIX[180], EEL, and self-carboxylation.

The carboxylase mechanism is unusual with respect to other known processive enzymes (*24*−*26*). The substrates for most of those enzymes are quite repetitive versus the nonrepetitive Gla domain. Most of the enzymes move along the substrate, binding at the actual site of catalysis. In contrast, the carboxylase remains bound to the same site on the substrate (the VKD propeptide), and a separate region being catalyzed, the Gla domain, undergoes conformational changes to effect multiple modifications. Many processive enzymes move a finite distance along the substrate and are then released. With the carboxylase, however, comprehensive fIX carboxylation was achieved, showing that the carboxylase released fIX only at the end of all modifications. Other processive enzymes have been shown to contain multiple, related binding sites which facilitate interaction with their repetitive substrates (*27*). Given the differences between these enzymes and the carboxylase, however, it is unlikely that the carboxylase uses a similar mechanism.

Implications for Comprehensive Carboxylation. One of the most striking observations in these studies was the linearity in rate over most of the reaction (Figure 4). Processive carboxylation limits the modification of Glu's within each complex to a single Gla domain, so the ratio of Glu's to Gla's will change as the reaction proceeds (from 12:0 to 0:12). The probability of binding a Glu for catalysis will therefore decrease over time with a concomitant decrease in rate if Glu's and Gla's are bound by the carboxylase with equal affinity. A linear rate for most of the reaction therefore suggests that the Gla domain continuously undergoes rapid intramolecular movement with the carboxylase sampling amino acids within the domain, and when a Glu is exposed, it is bound tightly to initiate catalysis. Conversion of Glu to a Gla would eliminate the preferential binding, resulting in release from the binding site. Thus, we propose that the carboxylase distinguishes Glu's from Gla's, e.g., by charge or size, to provide a driving force for the reaction.

A mechanism for distinguishing Glu's from Gla's could explain how the carboxylase achieves comprehensive carboxylation. Only Glu's in the Gla domain are carboxylated, and plausible explanations for such restriction include distance requirements between the propeptide and Gla domain or structural barriers adjacent to the Gla domain (e.g., the highly folded growth factor domain) that inhibit downstream carboxylation. What is unknown, however, is how comprehensive carboxylation is obtained. Both fully and partially carboxylated fIX have the propeptide, yet processive carboxylation resulted in the preferential release of only fully carboxylated fIX. The ability of the carboxylase to distinguish partially versus fully carboxylated fIX could be based on its ability to distinguish Glu's from Gla's. Thus, comprehensive carboxylation would lead to consequent loss of Glu residues in the Gla domain, which may weaken propeptide binding and result in release of the VKD product.

The importance of Glu binding to carboxylation has support in previous studies. Asp-containing substrates are very poorly carboxylated, indicating specificity of the carboxylase for Glu residues (*28*). In addition, a small Glu-containing substrate stimulated KH_2 epoxidation, indicating potential regulation by Glu's (*29*). Finally, mutation of either of the two active site Cys which effect epoxidation and carboxylation resulted in an increase in K_m for the substrate EEL (*7*), suggesting that these two Cys's participate in coordinating Glu's. These data thus support the proposal that distinction of Gla's from Glu's drives the reaction and acts

as a sensor for comprehensive carboxylation and consequent VKD protein release.

At present, there are conflicting results as to whether the Gla domain affects processive carboxylation or whether the propeptide alone is sufficient for efficiently processive and comprehensive carboxylation. Analysis of in vitro carboxylated bone Gla protein indicates some order to carboxylation, showing that Glu selection within the Gla domain is not random (*30*). Mutations of Asp for Glu at individual positions within the Gla domain impair overall carboxylation, which could be due to an effect of the Gla domain upon processivity (*31*). However, a prothrombin mutant with the propeptide fused to a non-Gla domain sequence (and deleted in the natural Gla domain) was secreted as multiply carboxylated protein (*32*), and a synthetic propeptide−non-Gla domain sequence with 5 Glu's gave products with 2−4 mol of Gla/mol of peptide upon in vitro carboxylation (*33*). The propeptide therefore appears to be sufficient for directing multiple carboxylations. It is important to note that multiple carboxylation is significantly different than comprehensive carboxylation, which is required for VKD protein activity and which we showed could be achieved using a natural fIX substrate. A role for the Gla domain in processive and comprehensive carboxylation cannot therefore be ruled out.

Determining how full carboxylation is obtained in vivo will require a detailed understanding of the processive mechanism. Important questions will include whether individual VKD proteins exhibit differential processivity, how release of fully carboxylated product is accomplished, and whether carboxylase carboxylation affects processivity. The carboxylase mechanism is unlike that of other processive enzymes which have been described, and the model system we have developed will be a powerful tool in addressing questions about carboxylase processivity and defining the mechanism.

ACKNOWLEDGMENT

The authors thank Dr. William Church for generously providing large amounts of the HFIX-40 anti-fIX MAb and ZymoGenetics for performing the N-terminal sequence analysis on the factor IX substrates. We also thank Drs. Steve Olson and Kurt Runge for their helpful comments in critiquing the manuscript.

REFERENCES

1. Price, P. A. (1988) *Annu. Rev. Nutr. 8*, 565−583.
2. Manfioletti, G., Brancolini, C., Avanzi, G., and Schneider, C. (1993) *Mol. Cell. Biol. 13*, 4976−4985.
3. Kulman, J. D., Harris, J. E., Haldeman, B. A., and Davie, E. W. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 9058−9062.
4. Kulman, J. D., Harris, J. E., Xie, L., and Davie, E. W. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 1370−1375.
5. Berkner, K. L. (2000) *J. Nutr. 130*, 1877−1880.
6. Furie, B., Bouchard, B. A., and Furie, B. C. (1999) *Blood 93*, 1798−1808.
7. Pudota, B. N., Miyagi, M., Hallgren, K. W., West, K. A., Crabb, J. W., Misono, K. S., and Berkner, K. L. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 13033−13038.
8. Stanley, T. B., Jin, D. Y., Lin, P. J., and Stafford, D. W. (1999) *J. Biol. Chem. 274*, 16940−16944.
9. Chen, W., Helenius, J., Braakman, I., and Helenius, A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 6229−6233.
10. Tokunaga, F., Wakabayashi, S., and Koide, T. (1995) *Biochemistry 34*, 1163−1170.
11. Wu, W., Bancroft, J. D., and Suttie, J. W. (1996) *Thromb. Haemostasis 76*, 46−52.
12. Wu, W., Bancroft, J. D., and Suttie, J. W. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 13654−13660.
13. Morris, D. P., Stevens, R. D., Wright, D. J., and Stafford, D. W. (1995) *J. Biol. Chem. 270*, 30491−30498.
14. Suttie, J. W. (1986) *Thromb. Res. 44*, 129−134.
15. Yan, S. C., Razzano, P., Chao, Y. B., Walls, J. D., Berg, D. T., McClure, D. B., and Grinnell, B. W. (1990) *Bio/Technology 8*, 655−661.
16. Bond, M., Jankowski, M., Patel, H., Karnik, S., Strang, A., Xu, B., Rouse, J., Koza, S., Letwin, B., Steckert, J., Amphlett, G., and Scoble, H. (1998) *Semin. Hematol. 35* (No. 2, Suppl. 2), 11−17.
17. Berkner, K. L. (1993) *Methods Enzymol. 222*, 450−477.
18. Busby, S., Kumar, A., Joseph, M., Halfpap, L., Insley, M., Berkner, K., Kurachi, K., and Woodbury, R. (1985) *Nature 316*, 271−273.
19. Foster, D. C., Holly, R. D., Sprecher, C. A., Walker, K. M., and Kumar, A. A. (1991) *Biochemistry 30*, 367−372.
20. Lingenfelter, S. E., and Berkner, K. L. (1996) *Biochemistry 35*, 8234−8243.
21. Kurachi, K., and Davie, E. W. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6461−6464.
22. Berkner, K. L., and Pudota, B. N. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 466−471.
23. Sadowski, J. A., Esmon, C. T., and Suttie, J. W. (1976) *J. Biol. Chem. 251*, 2770−2775.
24. Jager, J., and Pata, J. D. (1999) *Curr. Opin. Struct. Biol. 9*, 21−28.
25. Dong, H. (1998) *Methods Mol. Biol. 77*, 33−42.
26. Von Hippel, P. H., Fairfield, F. R., and Dolejsi, M. K. (1994) *Ann. N.Y. Acad. Sci. 726*, 118−131.
27. delCardayre, S. B., and Raines, R. T. (1994) *Biochemistry 33*, 6031−6037.
28. Suttie, J. W. (1985) *Annu. Rev. Biochem. 54*, 459−477.
29. Sugiura, I., Furie, B., Walsh, C. T., and Furie, B. C. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 9069−9074.
30. Benton, M. E., Price, P. A., and Suttie, J. W. (1995) *Biochemistry 34*, 9541−9551.
31. Zhang, L., Jhingan, A., and Castellino, F. J. (1992) *Blood 80*, 942−952.
32. Furie, B. C., Ratcliffe, J. V., Tward, J., Jorgensen, M. J., Blaszkowsky, L. S., DiMichele, D., and Furie, B. (1997) *J. Biol. Chem. 272*, 28258−28262.
33. Stanley, T. B., Wu, S. M., Houben, R. J., Mutucumarana, V. P., and Stafford, D. W. (1998) *Biochemistry 37*, 13262−13268.
34. Berkner, K. L., and McNally, B. A. (1997) *Methods Enzymol. 282*, 313−333.

BI0107039