Binding of the Factor IX γ-Carboxyglutamic Acid Domain to the Vitamin K-dependent γ-Glutamyl Carboxylase Active Site Induces an Allosteric Effect That May Ensure Processive Carboxylation and Regulate the Release of Carboxylated Product*

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Propeptides of the vitamin K-dependent proteins bind to an exosite on γ-glutamyl carboxylase; while they are bound, multiple glutamic acids in the γ-carboxyglutamic acid (Gla) domain are carboxylated. The role of the propeptides has been studied extensively; however, the role of the Gla domain in substrate binding is less well understood. We used kinetic and fluorescence techniques to investigate the interactions of the carboxylase with a substrate containing the propeptide and Gla domain of factor IX (FIXproGla41). In addition, we characterized the effect of the Gla domain and carboxylation on propeptide and substrate binding. For the propeptide of factor IX (proFIX18), FIXproGla41, and carboxylated FIXproGla41, the K_d values were 50, 2.5, and 19.7 nM and the k_{off} values were 273×10^{-5}, 9×10^{-5}, and 37×10^{-5} s^{-1}, respectively. The k_{off} of proFIX18 is reduced 3-fold by FLEEL and 9-fold by the Gla domain (residues 1–46) of FIX. The pre-steady state rate constants for carboxylation of FIXproGla41 was 0.02 s^{-1} in enzyme excess and 0.016 s^{-1} in substrate excess. The steady state rate in substrate excess is 4.5×10^{-4} s^{-1}. These results demonstrate the following. 1) The pre-steady state carboxylation rate constant of FIXproGla41 is significantly slower than that of FLEEL. 2) The Gla domain plays an allosteric role in substrate-enzyme interactions. 3) Carboxylation reduces the allosteric effect. 4) The similarity between the steady state carboxylation rate constant and product dissociation rate constant suggests that product release is rate-limiting. 5) The increased dissociation rate after carboxylation contributes to the release of product.

The vitamin K-dependent γ-glutamyl carboxylase is an integral membrane protein located in the endoplasmic reticulum. It catalyzes the post-translational modification of specific glutamic acid residues to γ-carboxylglutamic acid in a number of vitamin K-dependent proteins. In these vitamin K-dependent proteins, multiple glutamic acid residues in the amino-terminal Gla[1] domain are modified (1–3). The existence of these multiple γ-carboxylglutamic acid residues allows the Gla domain to form the calcium-dependent conformation required for the activity of these vitamin K-dependent proteins (4, 5). Under-carboxylated vitamin K-dependent proteins cannot form this calcium-dependent structure and, as a result, possess poor affinities for phospholipid surfaces, endothelial cells, or activated platelets (6–8).

Previous studies indicate that all or nearly all of the Glu residues to be carboxylated are modified during a single substrate binding event (9, 10). The loss of as few as three carboxylations can markedly decrease the activities of vitamin K-dependent proteins (8). Therefore, because the most frequently used anticoagulant, warfarin, causes under-carboxylation by reducing vitamin K concentration, understanding the mechanism by which processivity yields functional enzymes is important.

A tethered model has been proposed to account for how carboxylase accomplishes full carboxylation of pro-factor IX (9, 11). In this hypothesis, the primary interaction between carboxylase and its substrates is mediated by a propeptide sequence of 18 amino acids. Presumably, the propeptide anchors the Gla domain of the substrate near the carboxylase active site for a time sufficient for modification of all or most of the Glu residues of the Gla domain.

Several lines of evidence are consistent with the idea that full carboxylation is the result of a stochastic, processive mechanism, *i.e.* the degree of carboxylation is controlled by the balance between the carboxylation rate of the substrate and the dissociation rate of the product. First, bone Gla protein, whose propeptide has an extremely low affinity ($K_i > 500$ μM) for the carboxylase and therefore a rapid dissociation rate (12, 13), is routinely under-carboxylated in the general population. However, diets supplemented with vitamin K, which should increase the rate of carboxylation, resulted in full carboxylation of bone Gla protein in the individuals studied (14, 15). Second, individuals with mutations in the FIX propeptide (A–10T, A–10V, and N–9K) were identified because warfarin therapy affected the activity of factor IX much more than expected (16, 17). These mutations also reduce the affinity of the propeptide for the carboxylase 10–600-fold. The extent of the warfarin effect correlates with both the affinity of the mutant propeptide for the carboxylase and the concentration of vitamin K. Finally, about 70% of factor X is uncarboxylated when expressed in cell

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[1] The abbreviations used are: Gla, γ-carboxyglutamic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; EEL, peptide Glu-Glu-Leu; FLEEL, pentapeptide Phe-Leu-Glu-Glu-Leu; f-peptide, peptide modified at its amino terminus with 5(6)-carboxyfluo-

rescein; FIXGla1–46 the 46-mer peptide containing first 46 residues of FIX (sequence 1–46); FIXproGla41, 59-amino acid peptide containing human factor IX propeptide and first 41 residues of FIX Gla domain (sequence -18–41); Glu, glutamic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; proFIX18, 18-mer peptide, TVFLDHEN-ANKILNRPKR.

culture. The propeptide of factor X binds tighter to the carboxylase than the propeptide of any other coagulation protein. Thus, when factor X was overexpressed in cell culture (substrate excess), we predicted that product release would be rate-limiting, and a large fraction of the factor X would be secreted having by-passed the carboxylase. We predicted that changing the propeptide to one with lower affinity (but not so low as to lead to premature dissociation) would increase the turnover rate and result in increased carboxylation. This prediction was realized as decreasing the affinity of the propeptide from ~2.6 to ~277 nM resulted in the percentage of fully carboxylated factor X increasing from about 32% to about 85% (18). All these examples can be explained by a balance between the rate of carboxylation and the rate of product dissociation, *i.e.* either reducing the rate of carboxylation or increasing the rate of dissociation would lead to increased amounts of under-carboxylated clotting proteins.

In contrast to the clear role of the propeptide in carboxylation, the contribution of the Gla domain to binding has not been well defined. Price *et al.* (19) proposed that a conserved sequence within the Gla domains of vitamin K-dependent proteins E(*X*C)*XX*(ERE*X*C) is important for substrate-carboxylase interaction. In a typical Gla domain, the two cysteines in this motif form a disulfide bond. In support of this hypothesis, mutating either of the two conserved cysteines in the Gla domain of protein C results in under-carboxylated protein C (20). Furie *et al.* (21), however, report that mutation of either of the equivalent cysteines in the Gla domain of prothrombin does not affect carboxylation.

Both *in vitro* and *in vivo* experiments suggest that the propeptide is sufficient for carboxylation of a Glu-containing non-Gla domain substrate. Bovill *et al.* (7) made a substrate consisting of the propeptide of prothrombin attached to a random portion of its catalytic domain. This construct was fully carboxylated in cell culture. We joined a random sequence containing five glutamic acid residues to the propeptide of factor IX, and we examined its carboxylation *in vitro*. Our results indicate that the major product of the reaction was carboxylated at least four times in a processive manner (11). The substrate was probably fully carboxylated as it has since been shown that Gla moieties are preferentially ejected during mass spectrometry (22).

Although the contribution of a specific Gla domain sequence is still in question, evidence clearly suggests that substrates affect the binding of the propeptide of FIX to carboxylase, increasing its affinity about 3-fold. Presnell *et al.* (23) propose a working model to explain the effect of Glu residues on the carboxylase-substrate interaction. In this model, the binding energy necessary for full carboxylation of vitamin K-dependent proteins is conferred by the propeptide of the substrate; the affinity of the propeptide is in turn regulated by the occupancy of Glu at the carboxylase active site.

We have demonstrated previously (24) that the propeptides of the vitamin K-dependent proteins have very different affinities for the carboxylase. However, the affinity for a propeptide-containing substrate has not been reported previously. To test the contribution of the Gla domain in the interaction of carboxylase and its substrate, we have measured the affinities of the propeptide of factor IX covalently attached to the first 41 residues of the Gla domain of factor IX (FIXproGla41). Our results show that the affinity for carboxylase increases by a factor of 20 when the propeptide is covalently attached to a Gla domain. In addition, we measured the k_{off} of propeptide, FIXproGla41, and carboxylated FIXproGla41. We also measured the rate of carboxylation of a carboxylase-bound FIXproGla41 under conditions of excess enzyme. Our studies indicate that a linkage

exists between the active site and the propeptide-binding site and that this linkage effect may be important for the processivity of carboxylase. Furthermore, the k_{off} of a fully carboxylated substrate FIXproGla41 is about three times faster than its non-carboxylated counterpart. This difference in dissociation rate may be significant for product release.

EXPERIMENTAL PROCEDURES
Materials

All chemicals were reagent grade. FLEEL and *t*-butoxycarbonyl-Glu-*t*-butyl ester were purchased from Bachem (Philadelphia, PA). Peptide FLDDL was from Chiron Mimotopes (Clayton Victoria, Australia). CHAPS was from Pierce. Leupeptin, pepstatin, aprotinin, and phenylmethylsulfonyl fluoride were from Roche Applied Science. Vitamin K_1 was from Sigma and was reduced to hydroquinone (KH_2), as described previously (25). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine was from Avanti Polar Lipids (Alabaster, AL). Bovine serum albumin (fraction V, heat shock) was from Roche Applied Science. Fluorescein-labeled and unlabeled peptides, based on the human FIX propeptide, proFIX18, or based on human pro-factor IX sequence–18-41, FIXproGla41 (and its fully carboxylated counterpart), were chemically synthesized, purified, and verified by ion spray mass spectrometry by Chiron Mimotopes (Clayton Victoria, Australia) or by Genemed Synthesis (South San Francisco, CA). For synthesis of the fluorescein-labeled propeptides, 5(6)-carboxyfluorescein was conjugated to the amino terminus of the propeptide. All peptides were ~95% pure as determined by mass spectrometry and high pressure liquid chromatography. Labeling of the peptide with fluorescein was found to have no significant effect on carboxylase-propeptide interaction (23) or on the K_m and V_{max} for FIXproGla41 carboxylation (data not shown). The pSK⁻ vector was from Stratagene (La Jolla, CA). The pVL1392 vector was from Pharmingen (San Diego, CA). The BacVector 3000 baculoviral DNA was from Novagen (Madison, WI). Sf9 insect cells were obtained from the Lineberger Cancer Center at the University of North Carolina, Chapel Hill. High Five insect cells were provided by Dr. Thomas Kost of Glaxo Wellcome. HPC4 antibody affinity resin was obtained from Dr. Charles Esmon (Oklahoma Medical Research Foundation). SP-Sepharose was from Amersham Biosciences. All other materials were from Sigma and were reagent grade. Carboxylase was >98% pure as described previously (24) and was titrated to determine the concentration of active enzyme (23).

Methods

Fluorescence Anisotropy—All measurements were performed in a 400-μl fluorimetric quartz cuvette (Starna Cells, Atascadero, CA) at 17 °C, with a final volume of 300 μl. Before analysis, enzymes and peptides were pre-incubated for 1 h in buffer A, containing 50 mM MOPS (pH 7.5), 500 mM NaCl, 5% glycerol, 6.3 mM dithiothreitol, 66 μM EDTA, 0.16% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 0.16% CHAPS, and 0.2% bovine serum albumin. The excitation and emission wavelengths were 490 and 525 nm, respectively. Anisotropy was measured with an OLIS modified T-format SLM spectrofluorimeter (On-Line Instruments, Bogart, GA) essentially as described (23, 26).

Determining the K_d of ProFIX18 and of FIXproGla41 for Carboxylase—For each sample, the desired concentration of carboxylase was pre-incubated with either 10 nM fluorescein-labeled proFIX18 (f-proFIX18), 10 nM fluorescein-labeled FIXproGla41 (f-FIXproGla41), or 10 nM fluorescein-labeled carboxylated FIXproGla41 (f-carboxylated FIXproGla41) in buffer A and at 17 °C for 1 h. No significant loss of carboxylase activity at 17 °C was detected within 2 h in the absence of substrate or within 24 h in the presence of saturating substrate. The fraction of bound fluorescein-labeled peptide and the K_d values were determined from anisotropy, as described previously (23, 26).

Kinetic Studies—For enzyme excess studies of FIXproGla41 carboxylation, we pre-incubated 250 nM active carboxylase with 50 nM FIXproGla41 in buffer A at 17 °C for 1 h. Reactions were initiated by the addition of 120 μM vitamin K hydroquinone and 10 μCi of NaH¹⁴CO₃ (specific activity 55 mCi/mmol). Control experiments included 800 nM proFIX18 added after the 1-h incubation to determine whether reassociation of any released partially carboxylated product occurred during the reaction. At each time interval, we removed 100 μl of the sample from the 1.5-ml reaction mixture and determined the incorporation of $^{14}CO_2$ as described previously (25). Backgrounds were determined from reactions that omitted FIXproGla41. Both [^{14}C]alanine (93 mCi/mmol) and a ^{14}C standard purchased from Amersham Biosciences were used to determine the molar concentration of $^{14}CO_2$ incorporated

into product. We also used a molar extinction coefficient of 1520 to calculate the concentration of the substrate (27).

For substrate excess studies, 1500 nM FIXproGla41 and 25 nM active carboxylase were pre-incubated in buffer A at 17 °C for 1 h, and the assay was performed as described above for the enzyme excess experiments. The turnover rate constant of FIXproGla41 molecule during the steady state was determined by dividing the $^{14}CO_2$ incorporation rate during the post-burst, slower linear part of the curve in Fig. 2b by 305.6, the y intercept of the steady state curve in Fig. 2b. This burst size represents the concentration of $^{14}CO_2$ incorporated into 25 nM FIXproGla41 in one round of reaction (10). The kinetic studies of FLEEL, EEL, and t-butoxycarbonyl-Glu-t-butyl ester carboxylation were performed in buffer A using 25 nM active carboxylase at 17 °C for 30 min as described previously (23, 26).

Dissociation Rate Measurement—We measured the time course of fluorescein-labeled peptide release from carboxylase as described previously (23, 26). We pre-incubated 20 nM fluorescein-labeled peptide with 100 nM carboxylase in buffer A at 17 °C for 1 h to allow the mixture to come to equilibrium and then added a 200-fold excess of unlabeled peptide (4 μM) at time 0. To determine the effects of Glu-containing substrates on the dissociation rate of f-proFIX18, 4.8 mM FLEEL or 250 μM FIXGla1–46 were pre-incubated with 20 nM fluorescein-labeled peptide and 100 nM carboxylase in buffer A. We took anisotropy measurements at 20-s intervals and calculated the fraction bound of fluorescein-labeled peptide at each data point as described previously. We conducted all off-rate experiments at 17 °C and determined the rate constants as described previously (26).

Measurement of the Induced Fluorescence Intensity Changes of the Carboxylase-bound, Fluorescein-labeled ProFIX18 by FLEEL—For each sample, 10 nM fluorescein-labeled proFIX18 and 350 nM carboxylase were pre-incubated with a desired concentration of FLEEL, or FLDDL in buffer A at 10 °C for 1 h. In addition to the FDDL control, a second control included heat-inactivated carboxylase instead of active enzyme. The fluorescence intensity of carboxylase bound fluorescein-labeled proFIX18 was determined as described previously (23).

RESULTS

K_d Values of ProFIX18, FIXProGla41, and Fully Carboxylated FIXProGla41 for Carboxylase—The contribution of the Gla domain to the affinity of the substrate was determined by measuring the changes in anisotropy caused by adding increasing concentrations of carboxylase to fixed concentrations of f-proFIX18, f-FIXproGla41, or f-carboxylated FIXproGla41. The K_d values at 17 °C are 50.0 ± 1.7 nM for f-proFIX18, 2.5 ± 0.1 nM for f-FIXproGla41, and 19.7 ± 2.0 nM for f-carboxylated FIXproGla41 (Fig. 1).

Kinetics of FIXproGla41 Carboxylation with Enzyme in Excess of Substrate—The time course for carboxylation of FIXproGla41 in the presence of excess carboxylase appears in Fig. 2a. The conditions of the experiments ensure that more than 99% of the substrate is bound to the carboxylase at the beginning of the reaction. There was no appreciable dissociation and re-association during the reaction because control experiments that included 800 nM proFIX18, a competitive inhibitor of FIXproGla41 carboxylation with a K_d 50 nM, added at time 0 did not significantly affect carboxylation of carboxylase-bound FIXproGla41 (Fig. 2a). The observed carboxylation rate constant for CO_2 incorporation, determined from the linear portion of the curve in Fig. 2a, is 0.020 ± 0.003 s^{-1}. In addition, at the completion of the reaction, the amount of radioactivity incorporated corresponds to 11.5 of the 12 Glu residues being carboxylated.

Kinetics of FIXproGla41 Carboxylation with Substrate Excess Over Enzyme—Fig. 2b shows that the time course of carboxylation of 1500 nM FIXproGla41 by 25 nM carboxylase consists of a burst phase followed by a long steady state rate. The burst phase represents the accumulation of enzyme-product complex during the carboxylation of the first 12 glutamic acid residues. The rate determined from the burst phase of Fig. 2b, 0.016 s^{-1}, agrees, as it should, with the value (0.02 s^{-1}) determined from the enzyme excess experiments Fig. 2a. The agreement of these two values testifies to their accuracy. In addition,
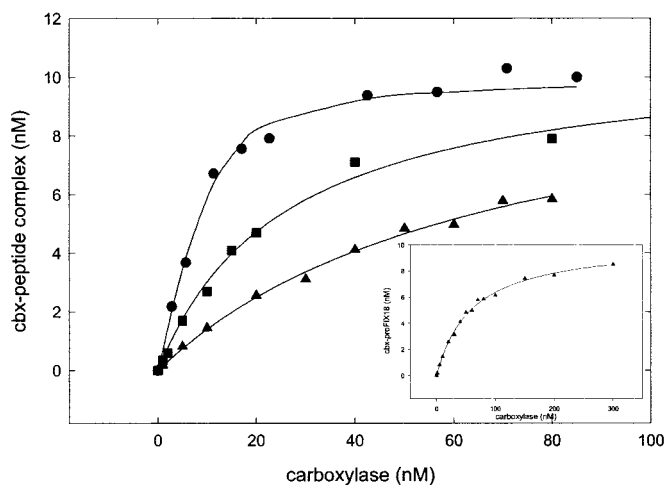
FIG. 1. **Determination of the affinities of fluorescently labeled proFIX18, FIXproGla41, or carboxylated FIXproGla41.** The points represent samples containing varying concentrations of purified carboxylase and 10 nM proFIX18 (*triangle*), 10 nM FIXproGla41 (*circle*), or 10 nM carboxylated FIXproGla41 (*squares*). The concentration of peptide bound was calculated from fluorescence anisotropy changes. The K_d values were obtained by fitting the titration data to the appropriate quadratic equation. The K_d values of carboxylase for proFIX18, FIXproGla41, and carboxylated FIXproGla41 were 50 ± 1.7, 2.5 ± 0.1, and 19.7 ± 2.0 nM, respectively. The *inset* represents the full titration curve of proFIX18 against carboxylase.

the y intercept of the slower steady state phase is 305 nM which, when divided by the number of Gla residues modified during one round of carboxylation, 12, estimates that the concentration of active enzyme is 25.4 nM. We estimated that our enzyme concentration was 25 nM by titration with a tight binding propeptide (23). Again, the agreement between these two values emphasizes the accuracy of our method for determining the concentration of active enzyme.

The slower linear rate shown in Fig. 2b depicts the steady state rate of FIXproGla41 carboxylation. The steady state rate constant, 4.5×10^{-4} s^{-1}, was determined by dividing the slope by the enzyme concentration and the number of Glu residues, 12, expected to be modified.

K_{off} Values for f-proFIX18, f-FIXproGla41, and Carboxylated f-FIXproGla41—Because the balance between the rate of carboxylation and the rate of substrate release influences full carboxylation, we measured the dissociation rates of these peptides from carboxylase by using the same conditions that we routinely use for determining *in vitro* rates of carboxylation. Figs. 3 and 4 show that a single exponential decay curve satisfactorily fits the time for dissociation of a carboxylase-substrate complex, allowing us to determine the k_{off}. The rate constants for f-proFIX18, f-FIXproGla41, and carboxylated f-FIXproGla41 are shown in Table I. It is significant that the rate of dissociation (3.7×10^{-4} s^{-1}) is so similar to the steady state rate constant (4.5×10^{-4} s^{-1}) measured above.

The Effects of Glutamic Acid-containing Peptides on the Dissociation Rate of ProFIX18—It is known that binding of the propeptide to the carboxylase reduces the K_m of a small substrate (FLEEL) (26, 28, 29). Moreover, under turnover conditions (in the presence of FLEEL, vitamin K, CO_2, and O_2), both the K_d and the k_{off} values of proFIX18 decrease relative to those in the absence of substrates (23). This indicates that occupancy of sites other than the propeptide site stabilizes the enzyme-propeptide complex. This effect could be because of Glu binding to the active site, binding of vitamin K, CO_2, or O_2, or a combination of all of these. To assess the effects of glutamate-containing substrates alone on the affinity of proFIX18 for carboxylase, we measured the k_{off} of proFIX18 in the presence
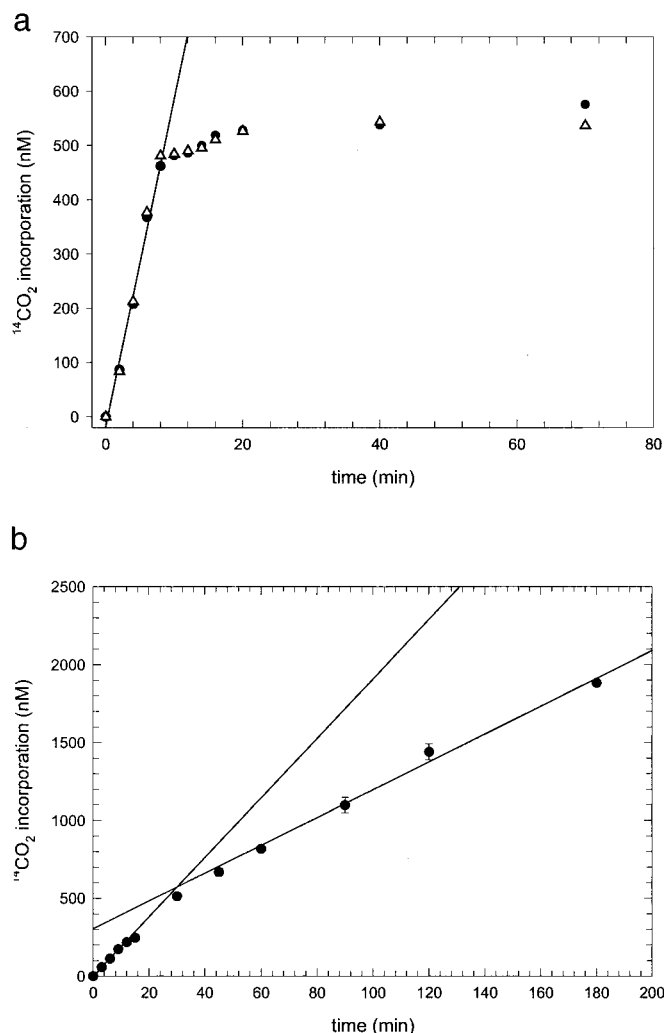
a

b

FIG. 2. **The time course of carboxylation of the pre-formed carboxylase-FIXproGla41 complex at 17 °C.** *a*, reactions in the absence of proFIX18 (*filled circles*) and in the presence of 800 nM proFIX18 (*triangles*) were performed as described under "Experimental Procedures." Each reaction contained 250 nM active carboxylase and 50 nM FIXproGla41. The *line* represents the linear regression of the first four points. *b*, carboxylation time course with FIXproGla41 in excess of carboxylase. Reactions were performed using 25 nM purified carboxylase and 1500 nM FIXproGla41 at 17 °C as described under "Experimental Procedures." The slow linear part represents the steady state of FIXproGla41 carboxylation, and the *straight line* represents the linear regression of the last five points. The fast linear part is the burst phase that is determined from the first 10 min of reaction. *Points* represent the average of three independent experiments.

of FLEEL or the entire Gla domain (FIXGla1–46). As shown in Table I, the off-rate constant of proFIX18 in the presence of FLEEL is decreased 3-fold, whereas FIXGla1–46 decreases the rate of dissociation 9-fold. Addition of reduced vitamin K or CO_2 had no effect on the dissociation rate.

The Effect of FLEEL on the Fluorescence Intensity of Carboxylase-bound Fluorescein-labeled Propeptide—Fluorescence intensity is sensitive to its local environment. Hence, any conformational change, accompanied by a change in environment at the propeptide-binding site, may affect the intensity of a fluorescein-labeled proFIX18 bound to carboxylase. The concentration of FLEEL required for half-maximal intensity change is 0.62 mM (Fig. 5); this is similar to the K_m of carboxylase for FLEEL (0.8 mM in this study) in the presence of propeptide. In contrast, FLDDL induced no intensity change, indicating the importance of Glu residues for the allosteric effect.
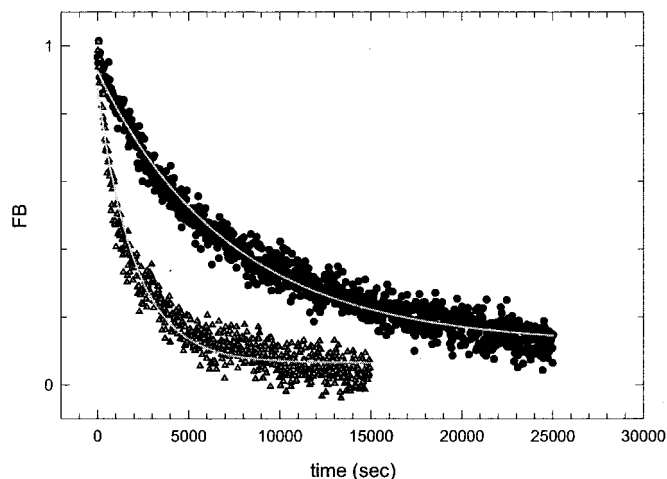

FIG. 3. **Dissociation time course from carboxylase of f-FIXpro-Gla41 (*circles*) and carboxylated f-FIXproGla41 (*triangles*).** Experiments were done at 17 °C using 20 nM f-peptide and 100 nM carboxylase as described under "Experimental Procedures." 4 μM unlabeled peptide was used at time 0 to minimize the re-association of f-peptide to carboxylase. Rate constants were determined by fitting data to the equation for single exponential decay.

DISCUSSION

The purpose of this study was to further understand the mechanism of the processive carboxylation of factor IX. We have proposed that the degree of carboxylation is controlled by the balance between the dissociation rate of the product and the rate of carboxylation of the substrate (30). This hypothesis was based upon our measurements of the k_{off} values of propeptides (23) from the carboxylase and on our rationale that the rate of carboxylation of a propeptide-containing substrate, FIXproGla41, would be at least as fast, and probably faster, than that of FLEEL because we expected the concentration of Glu in a bound substrate would be much higher than the non-tethered FLEEL. Thus, we were surprised to find that the apparent average rate constant for carboxylation of a FIXproGla41 substrate during one binding event was about one-fourteenth that of the FLEEL carboxylation rate (Table II) The rate constants for FIXproGla41 were determined both in enzyme excess where the concentration of the substrate is required to determine the rate constant and in substrate excess where the concentration of enzyme is required.

There are three important pieces of information that can be derived from the substrate excess data. First, we can measure the carboxylation rate constant from the linear burst phase and determine the average carboxylation rate of the factor IX Gla domain while it is tethered to the carboxylase by the propeptide of FIX; this rate, 0.016 s^{-1}, agrees very well with the rate determined in enzyme excess (0.02 s^{-1}). Second, from the slower linear portion of the curve, we can determine the steady state turnover rate; this rate agrees very well with the k_{off} value of the fully carboxylated product calculated from anisotropy. Third, we can estimate the enzyme concentration from the *y* intercept of the steady state rate. The *y* intercept varied from about 287 to 305 nM depending upon which points were chosen for drawing the line. When divided by the number of expected Gla residues, 12, the enzyme concentration was calculated to be between 23 and 25 nM. A Dynafit model of the data predicted a value of 27 nM for the enzyme. According to our titration with a consensus propeptide, the concentration of enzyme in the substrate excess experiments was estimated to be 25 nM, so the agreement is very good.

We also measured the rate constant for the carboxylation of FIXproGla41 with enzyme in excess over substrate; in this case
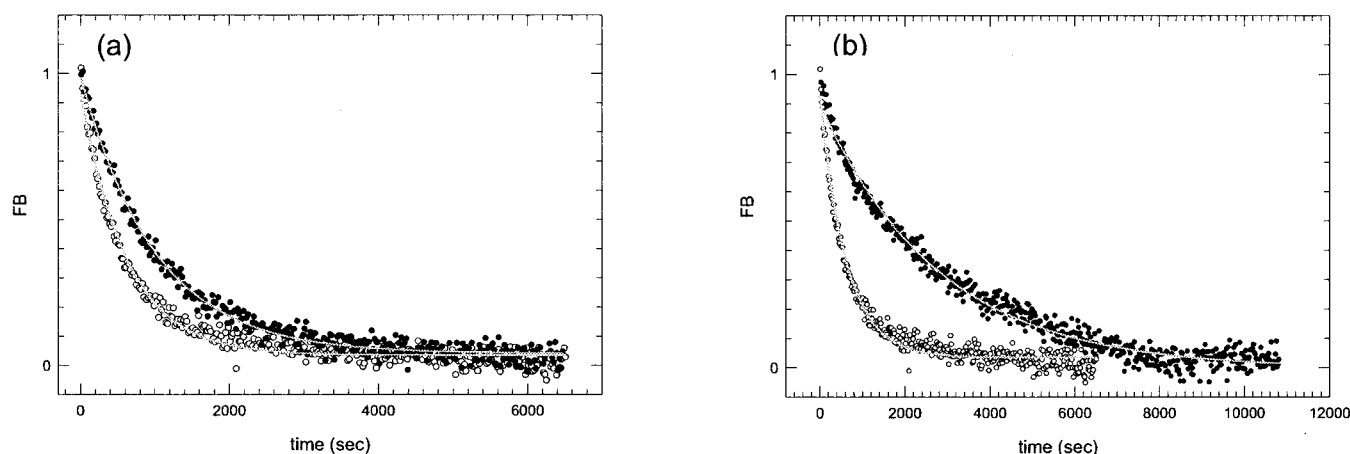
The Processive Mechanism of γ-Glutamyl Carboxylase

FIG. 4. **The effects of Glu-containing peptides on the dissociation rate of proFIX18.** The faster decay curves (*open circles*) in *a* and *b* represent the dissociation time course of proFIX18 in the absence of a Glu-containing peptide. The slower decay curves (*closed circles*) represent the dissociation of proFIX18 from carboxylase in the presence of 4.8 mM FLEEL (*a*) or 250 μM FIXGla1–46 (*b*). Experiments were performed as described in the legend of Fig. 3.

TABLE I

The k_{off} values of propeptide-containing substrates from carboxylase

Each k_{off} represents the average of results from three independent measurements.

Fluorescein-labeled peptide	Glu-containing peptide	k_{off} ($\times 10^{-5}$ s^{-1})
f-FIXproGla41		9 ± 1
f-Carboxylated FIXproGla41		37 ± 2
f-pro FIX18		273 ± 9
f-pro FIX18	4.8 mM FLEEL	90 ± 5
f-pro FIX18	250 μM FIXGla 1–46	31 ± 4

TABLE II

The kinetic properties of EEL, FLEEL and FIXproGla41 carboxylation

Substrate	K_m	k_{cat}
	μM	s^{-1}
Boc-Glu-OtBu[a]	3450 ± 425	0.08 ± 0.01
EEL[a]	328 ± 23	0.21 ± 0.03
FLEEL[a]	804 ± 39	0.28 ± 0.07
FIXproGla41[b]		0.020 ± 0.004

[a] Determined in the presence of 5 μM proFIX18. OtBu is *t*-butyl ester.
[b] $^{14}CO_2$ incorporation rate constant during a single binding event, determined from the experiments with enzyme in excess (Fig. 2).
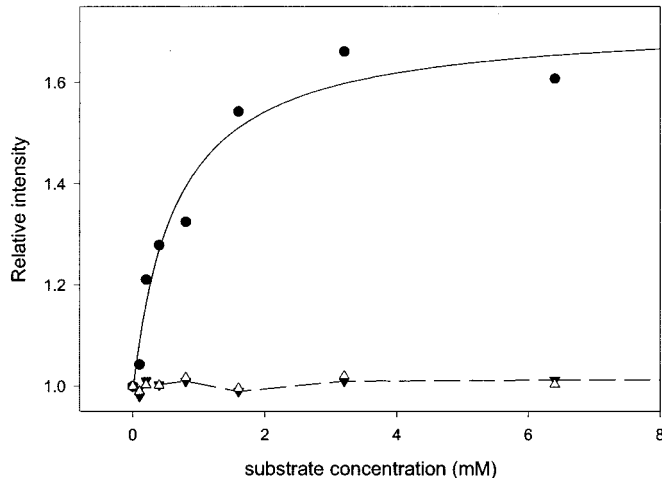

FIG. 5. **The effect of FLEEL on the fluorescence intensity of the carboxylase-bound fluorescein-labeled proFIX18.** The intensity changes induced by small peptides were determined by measuring the fluorescence intensity of 10 nM fluorescein-labeled proFIX18 that was incubated with 350 nM active carboxylase at varying concentrations of FLEEL (*filled circles*) or FLDDL (*filled triangles*). In a control experiment, 350 nM heat-inactivated carboxylase was incubated with varying concentrations of FLEEL and 10 nM fluorescein-labeled proFIX18 (*open triangles*). The excitation wavelength is 490 mm and the emission wavelength is 525 mm. The *curve* represents the hyperbolic regression of *filled circles*.

the rate is dependent only upon the substrate concentration because all of the substrate is bound to the carboxylase. In the enzyme excess experiments, the rate constant measured from the linear portion of the curve was 0.02 s^{-1}. The excellent agreement with the calculation from the substrate excess experiments discussed above (0.016 s^{-1}) suggests that for the conditions and substrates used in our experiments the calcu-

lated rate is reasonably accurate. Our experiments in enzyme excess (Fig. 2*a*) make several additional important points regarding the observed carboxylation rate of a propeptide-containing substrate. Within experimental error, the rate of carboxylation is linear up to about 85% completion. This suggests that for the first 10 carboxylations, the effective concentration of Glu does not change because the observed rate remains the same, whereas the actual concentration of Glu decreases. In agreement with Stenina *et al.* (9), this indicates the enzyme distinguishes between Glu and Gla and binds Glu preferentially, *i.e.* Gla does not compete with Glu for the active site. The linearity also suggests that the individual carboxylation rates for the first 10 of the 12 Glu residues in the Gla domain of the FIX are approximately the same and are controlled by the same slow rate-limiting step. The observation that the last two Gla residues are added slowly may be related to the observation that factor IX produced in cell culture has a significant amount of FIX lacking the Gla modifications at Glu[36] and Glu[40] (31). The affinity of the propeptide of FIX is in the nanomolar range, and a complex with this high affinity is expected to be relatively rigid. If true, this expectation means that the multiple carboxylations must occur either by diffusion of the appropriate Glu residues of the substrate into the active site or by movement of the active site relative to the substrate. Therefore, releasing Gla from the active site or rearranging the conformation of the Gla domain or the movement of the carboxylase active site after each carboxylation may control the observed carboxylation rate of FIXproGla41 rendering the rate of carboxylation of the propeptide-bound Gla domain slower than the rate of FLEEL carboxylation.

In the only other study using concentrations that approximate single turnover, Stenina *et al.* (9) reported that the rates of carboxylation for profactor IX and that of the small substrate EEL are similar. The k_{cat} value they report for EEL was about

4-fold slower than our value for FLEEL. When we used their conditions and studied EEL kinetics, we obtained results similar to theirs. However, no matter whether we use their rate constants or ours to predict the extent of carboxylation, the rate is still too slow to result in full carboxylation, if the substrate dissociation rate is dependent solely on the affinity of the propeptide.

Our rate constant measurements for a tethered substrate suggest that, for complete carboxylation to occur, factors other than the propeptide must contribute to substrate binding. Therefore, we measured the K_d and k_{off} values for FIXproGla41 and fully carboxylated FIXproGla41. We found that the K_d value of FIXproGla41 for carboxylase was 20-fold lower than that of proFIX18, and that the k_{off} value for proFIX18 was 30-fold faster than that of FIXproGla41. This indicates that the Gla domain of factor IX contributes significantly to the interaction between profactor IX and the carboxylase. The importance of these measurements to our proposed mechanism is that the k_{off} of FIXproGla41 is about 220-fold slower than the rate constant of carboxylation. This slow dissociation rate should ensure nearly full carboxylation because 95% of the substrate should still be associated with the carboxylase 10 min after binding, and carboxylation is nearly complete in 10 min (Fig. 2a).

A further important point derived from our measurements of the k_{off} for carboxylated FIXproGla41 is that its k_{off} (3.7×10^{-4} s^{-1}) is nearly identical with the steady state rate of carboxylation (4.5×10^{-4} s^{-1}) measured in the substrate excess experiment; this means that the overall rate of carboxylation is determined by the release of the product. A further point is that while the k_{off} of carboxylated FIXproGla41 is 4-fold faster than that of FIXproGla41, the difference in K_d is 8-fold. This suggests that the association rate constant of the product is slower than that of the substrate.

The effect of the Gla domain on propeptide binding is primarily the result of its allosteric effect on the propeptide-binding site; the addition of 250 μM FIXGla1–46 causes a 9-fold decrease in k_{off}. The true allosteric effect is probably greater because the solubility of the FIXGla1–46 limits its achievable concentration to two times higher than its K_m. Even so, the 3-fold difference in k_{off} between proFIX18 in the presence of the Gla domain and FIXproGla41 indicates that a major part of the effect of the Gla domain on propeptide binding is allosteric.

We demonstrated previously that, under turnover conditions, the affinity or the propeptide for the carboxylase was increased (23). In this work we demonstrate that the effect is due to FLEEL and not to another component of the reaction mix such as vitamin K. Control experiments with FLDDL have no measurable effect on propeptide k_{off}. In addition FLEEL, but not FLDDL, caused a fluorescence intensity change of a fluorescein-labeled propeptide bound to the carboxylase. This suggests that FLEEL induces a conformational change at the propeptide-binding site, thus stabilizing the carboxylase-propeptide complex.

The effect of FLEEL on propeptide binding strongly suggests that occupancy of the active site by glutamic acid is critical for the allosteric effect observed when the substrate binds to the enzyme. The importance of Glu at the active site is further emphasized by the 4-fold increase in the k_{off} of fully carboxylated FIXproGla41 compared with FIXproGla41. It is worth emphasizing that the binding of FLEEL to the active site results in a 3-fold decrease in the off rate of the propeptide from the carboxylase, whereas the k_{off} of fully carboxylated FIXpro-Gla41 is 4-fold faster than its uncarboxylated counterpart. In both cases, the difference appears to be the presence or absence of Glu. The lower affinity, although the difference is modest, of

the carboxylated form relative to the uncarboxylated form of FIXproGla41 suggests that the change in dissociation rate that accompanies complete carboxylation contributes to product release.

Hallgren *et al.* (32) proposed an active release mechanism based upon their observation that, in a chase experiment, the amount of carboxylated factor IX-carboxylase complex remains unchanged unless a competing propeptide is present. They concluded that another propeptide binds to a second propeptide-binding site, facilitating the release of the carboxylated proFIX. However, in their experiments, the concentrations of substrate and carboxylase were approximately equal and, given the affinity of the propeptide of FIX for the carboxylase, any dissociated product would have re-associated unless a competitive substrate was present. Furthermore, our estimate of the stoichiometry of propeptide to carboxylase is 1:1 (23). Our observation that the steady state turnover rate of FIXproGla41 is nearly identical to the dissociation rate of the carboxylated product in the absence of other substrates or cofactors suggests that the rate-limiting step of carboxylation relies on the affinity of the enzyme-product complex; thus, there is no reason to increase the complexity of the models by invoking a second propeptide-binding site.

In this study, we report for the first time the K_d value of carboxylase for a peptide containing both the propeptide and Gla domain of factor IX, and we show that the Gla domain significantly alters the affinity of the substrate for the enzyme. We also determined the k_{off} and the carboxylation rate constant of a propeptide-containing substrate *in vitro*. Our results indicate that the primary contribution of the Gla domain to binding is an allosteric effect on propeptide binding and that this effect is potentially important for processive carboxylation of factor IX. Our results additionally show that product release is the rate-limiting step in profactor IX carboxylation. We do not know to what extent this allosteric effect contributes to the interaction of other vitamin K-dependent proteins with carboxylase. Do the Gla domains of those vitamin K-dependent proteins whose propeptides have lower apparent affinities for carboxylase than proFIX have different affinities for the carboxylase? Will propeptides with very high affinity be modulated by their Gla domains? Have individual Gla domains evolved to work best with their own propeptides? Further studies to discover how the Gla domain interacts with carboxylase and how it repositions the new Glu residues to the carboxylase active site will contribute to understanding the processive mechanism of carboxylase.

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