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The Putative Vitamin K-dependent γ-Glutamyl Carboxylase Internal Propeptide Appears to Be the Propeptide Binding Site*

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The vitamin K-dependent γ-glutamyl carboxylase binds an 18-amino acid sequence usually attached as a propeptide to its substrates. Price and Williamson (*Protein Sci.* (1993) 2, 1997–1998) noticed that residues 495–513 of the carboxylase shares similarity with the propeptide. They suggested that this internal propeptide could bind intramolecularly to the propeptide binding site of carboxylase, thereby preventing carboxylation of substrates lacking a propeptide recognition sequence. To test Price's hypothesis, we created nine mutant enzyme species that have single or double mutations within this putative internal propeptide. The apparent K_d values of these mutant enzymes for human factor IX propeptide varied from 0.5- to 287-fold when compared with that of wild type enzyme. These results are consistent with the internal propeptide hypothesis but could also be explained by these residues participating in propeptide binding site *per se*. To distinguish between the two alternative hypotheses, we measured the dissociation rates of propeptides from each of the mutant enzymes. Changes in an internal propeptide should not affect the dissociation rates, but changes to a propeptide binding site may affect the dissociation rate. We found that dissociation rates varied in a manner consistent with the apparent K_d values measured above. Furthermore, kinetic studies using propeptide-containing substrates demonstrated a correlation between the affinity for propeptide and V_{max}. Taken together, our results indicated that these mutations affected the propeptide binding site rather than a competitive inhibitory internal propeptide sequence. These results agree with our previous observations, indicating that residues in this region are involved in propeptide binding.

The vitamin K-dependent γ-glutamyl carboxylase is a polytopic integral membrane protein that resides in the endoplasmic reticulum (1). It catalyzes the post-translational modification of a number of vitamin K-dependent proteins (*e.g.* the coagulation proteins prothrombin, factor VII, factor IX, factor X, protein S, protein C, and protein Z) (2, 3). Other known vitamin K-dependent proteins are the bone-related proteins osteocalcin and matrix Gla protein, the growth arrest protein Gas 6, and four proteins of unknown function: proline-rich Gla proteins I and II and TMG proteins 3 and 4 (4–9). Vitamin K-dependent carboxylase utilizes the substrates: reduced vitamin K, carbon dioxide, oxygen, and a propeptide-containing substrate. Multiple glutamic acid residues of the polypeptide substrate, within about 40 residues of the propeptide, are usually modified to γ-carboxyglutamate during a single binding event (10).

The primary interaction between the vitamin K-dependent carboxylase and its substrates is mediated by the 18-amino acid propeptide sequence (11, 12), which in all known vitamin K-dependent proteins, except for matrix Gla protein, is removed prior to secretion. The role of the substrate's propeptide is to anchor it to the carboxylase for a time sufficient for multiple carboxylations (13). In addition to its role in anchoring the substrate to the carboxylase, binding of the propeptide to carboxylase also significantly stimulates the incorporation of CO_2 into non-propeptide-containing substrates (12, 14).

We have recently demonstrated that, at 20 °C, the affinities (measured as K_i) of the various propeptides for carboxylase vary from about 5 nM for factor X to greater than 500 μM for bone Gla protein (15). We further demonstrated that a consensus propeptide bound considerably tighter to carboxylase than any known naturally occurring propeptide (16).

Knobloch and Suttie (12) found that the propeptide of factor X decreased the K_m and increased the V_{max} of the carboxylase for the small Glu-containing substrate FLEEL.[1] They proposed that this stimulation resulted in propeptide-containing substrates being selectively modified by the carboxylase. Based upon this suggestion, Price and Williamson (17) searched the carboxylase for a sequence that could compete with the propeptide for its substrates. They searched the 24-amino acid sequence of exon 3 of the human matrix Gla protein and found a sequence within the carboxylase homologous with the propeptide sequence. Based upon the earlier observation that the propeptide stimulated incorporation of CO_2 into FLEEL, they postulated that residues 495–513 of the carboxylase functioned as an internal propeptide, and could bind intramolecularly to the propeptide binding site. They further suggested that this intramolecular interaction would prevent carboxylation of proteins lacking the propeptide docking sequence. To test this hypothesis, they proposed that one should reduce the affinity of the internal propeptide for the propeptide binding site by mutating the highly conserved phenylalanine that corresponded to −16 of the propeptide. With our knowledge that the consensus sequence propeptide binds tightly to carboxylase (16), we set

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[1] The abbreviations used are: FLEEL, the pentapeptide Phe-Leu-Glu-Glu-Leu; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; FproCon, the 19-mer peptide fluorescein-GAVFLSREQANQVLQRRRR modified at its N terminus with 5(6)-carboxyfluorescein; FproFIX19, the 19-mer peptide fluorescein-TVFLDHENANKILNRPKRY modified at its N terminus with 5(6)-carboxyfluorescein; proFIX19, the 19-mer peptide, TVFLDHENANKILNRPKRYFIX(Q/S), 59-amino acid peptide containing human factor IX propeptide, and first 41 residues of FIX Gla domain (sequence −18 to 41).

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Consensus          AVFLSREQANQVLQRRRR-
Internal_Propep    -PFQRTSWVQPLLNDLSPW
F496A              -PAQRTSWVQPLLNDLSPW
P495V/Q497L        -VFLRTSWVQPLLNDLSPW
V502A              -PFQRTSWAQPLLNDLSPW
V502A/P504Q        -PFQRTSWAQQLLNDLSPW
Q503N              -PFQRTSWVNPLLNDLSPW
Q503R              -PFQRTSWVRPLLNDLSPW
P504Q              -PFQRTSWVQQLLNDLSPW
S510A              -PFQRTSWVQPLLNDLAPW
W512A              -PFQRTSWVQPLLNDLSPA
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FIG. 1. **The alignment of the proposed internal propeptide region with the consensus human propeptide and the corresponding region of the mutations used.** *Yellow residues* on a *black background* represent mutations that make the internal propeptide less like the consensus sequence. Residues mutated to be more like the consensus sequence are shown in black on a yellow background. Residues Ser^{510} and Trp^{512} were mutated to test our alternative hypothesis and are shown with a *brown background*.

out to test Price's hypothesis. We hypothesized that if we made the putative internal propeptide sequence more (or less) like the consensus sequence, the intramolecular binding should be tighter (or weaker) and that it would require higher (or lower) concentrations of propeptide to stimulate CO_2 incorporation into the small substrate FLEEL. To test the hypothesis that there was an internal propeptide, we made nine single or double mutations in the putative internal propeptide of human carboxylase and tested their effect on the carboxylase molecule. Our results strongly suggest that the proposed internal propeptide contributes to the propeptide binding site rather than to a self-regulatory domain.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade. FLEEL was purchased from Bachem (Philadelphia, PA). CHAPS was from Pierce. Leupeptin, pepstatin, aprotinin, and phenylmethylsulfonyl fluoride were from Roche Molecular Biochemicals. Vitamin K1 was from Sigma and was reduced to hydroquinone (KH_2) as previously described (18). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine was from Avanti Polar Lipids (Alabaster, AL). Bovine serum albumin (fraction V, heat shock) was from Roche Molecular Biochemicals. Fluorescein-labeled and unlabeled peptides, based on the human FIX propeptide, proFIX19, and consensus sequences (16) were chemically synthesized, purified, and verified by ion spray mass spectrometry by Chiron Mimotopes (Clayton, Victoria, Australia). In the synthesis of the fluorescein-labeled propeptides, 5(6)-carboxyfluorescein was conjugated to the amino terminus of the propeptide. Purity of all peptides was determined by mass spectrometry and high pressure liquid chromatography and was found to be ~95%. 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, NC). 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.

Expression and Purification of Recombinant Wild Type and Mutant Carboxylase—The cDNA of human γ-glutamyl carboxylase was cloned into the pSK^- vector and was modified to the desired sequence by site-directed mutagenesis as previously described (19). All constructs having carboxylase sequence were flanked by sequences coding for the FLAG (DYKDDDDK) tag at their amino termini and by the HPC4 tag (EDQVDPRLIDGK) (20) at their carboxyl termini. The presence of these tags does not appear to alter the properties of the carboxylase molecule; *i.e.* its activity and kinetic properties are not measurably different from the wild type enzyme. The DNA sequence of each construct was determined in its entirety by the DNA sequence facility at the University of North Carolina at Chapel Hill. The engineered DNAs were subcloned into the pVL1392 vector, and the proteins were expressed in baculovirus-infected High Five cells as previously described (15). Solubilization and isolation of microsomes from High Five cells were done as previously described (15). All enzyme preparations were purified to homogeneity (purity > 98%) as previously described (15).

Determining Protein Concentrations—The enzyme preparations were preincubated with 0.067 M iodoacetamide at 37 °C for 15 min to

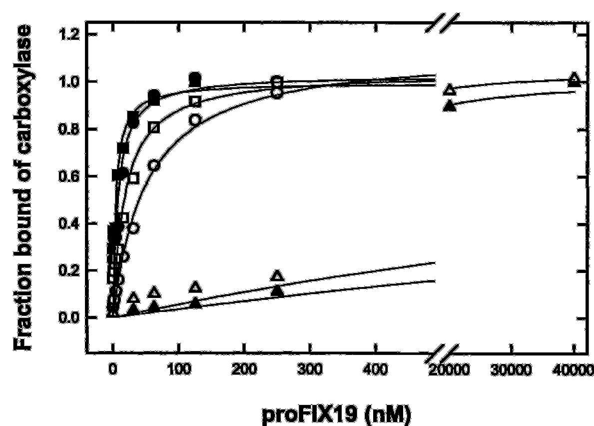

FIG. 2. **Determining the apparent K_d of proFIX19 for wild type and mutant carboxylase.** Carboxylation was measured by $^{14}CO_2$ incorporation as described under "Experimental Procedures," and reactions were performed, at varied proFIX19 concentrations, with 20 nM active wild type enzyme (●), V502A (○), Q503R (■), Q503N (□), S510A (▲), or W512A (△). The fraction of bound enzyme was determined as described under "Experimental Procedures." Only results for mutants that have altered affinities for proFIX19 are shown.

eliminate the effects of dithiothreitol on the protein assay reagents. The total protein concentrations (including both active and inactive enzyme) were determined by using Bio-Rad detergent-compatible protein assay kits and IgG protein standard following the supplier's protocol. The active concentration of our enzyme preparations was determined by titration with FproCon as described below.

Fluorescence Anisotropy—All of the measurements were performed in a 400-μl fluorimetric quartz curette (Starna Cells, Atascadero, CA) at 4.5 °C in a final sample volume of 300 μl. Enzymes and propeptides were preincubated for 1 h in standard buffer containing 100 mM MOPS (pH 7.5), 166 mM NaCl, 3.5% glycerol, 6.3 mM dithiothreitol, 66 μM EDTA, 0.1% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 0.28% CHAPS, and 0.4% bovine serum albumin. The fluorescence intensity of plane polarized light at four mutually perpendicular polarizing filter settings (I_{VV}, I_{VH}, I_{HV}, and I_{HH}) of samples was measured with an SLM-Aminco 8100 spectrofluorimeter equipped with Glan-Thompson polarizing filters in the excitation and emission beams; each point represents the average of six readings. The excitation and emission wavelengths were 490 and 525 nm, respectively. Anisotropy was calculated from the four measured intensities as previously described (21, 22).

Titration for Active Enzyme Concentrations—The concentration of active enzyme in a purified carboxylase preparation was determined by measuring the number of propeptide binding sites in the preparation. Previous work indicates a correlation between the presence of a propeptide binding site and catalytic activity and indicates that only active enzyme can bind propeptide. Therefore, a measurement of the concentration of propeptide binding sites in a carboxylase preparation is a measurement of the concentration of the active fraction of enzyme (22). For each sample, the fraction of FproCon bound to carboxylase was determined from the anisotropy value, and the concentration of propeptide binding sites (active enzyme) was determined from the fraction bound *versus* [carboxylase] plot as previously described (22). Briefly, when appropriate conditions are chosen (concentration of FproCon $\gg K_d$), anisotropy of FproCon increases linearly with increasing carboxylase concentration to about 75% saturation. The equivalence point of active carboxylase concentration was determined from the intersection of a linear regression of the data points (up to 75% fraction bound) with a horizontal line determined from the saturation value (100% fraction bound). All enzyme concentrations used in the calculation of kinetic constants were the active enzyme concentrations determined by its ability to bind propeptide (22).

Off Rate Measurement—The time course of FproFIX19 release from wild type and mutant carboxylases was measured essentially as described previously (22). For the wild type or P495V/Q497A, F496A, and Q503R carboxylases, 10 nM active enzyme was preincubated with 25 nM FproFIX19 in standard buffer at 4.5 °C for 1 h to allow the mixture to come to equilibrium. A 350-fold excess of unlabeled proFIX19 was added at time 0. The sample was added to a microcuvette and was placed into the sample holder of the SLM-Aminco 8100 spectrofluorimeter. Intensity measurements were taken at 20-s intervals as previously described (22), and the anisotropy at each data point was calcu-

TABLE I
The apparent K_d for proFIX19 and the maximal stimulated FLEEL carboxylation velocities for wild type and mutant carboxylase

Enzyme	Apparent K_d for proFIX19	Difference	Maximal stimulated activity	Difference
	nM	*-fold*	*pmol $^{14}CO_2$/min/pmol enzyme*	*-fold*
Wild type	7.3 ± 0.5	1.0	29.1 ± 1.1	1.0
P495V/Q497A	9.4 ± 1.3	1.3	26.2 ± 2.6	0.9
F496A	8.8 ± 0.7	1.2	27.1 ± 1.6	0.9
V502A	53.7 ± 3.1	7.4	28.9 ± 1.3	1.0
V502A/P504Q	51.2 ± 4.9	7.0	28.2 ± 1.2	1.0
Q503N	24.4 ± 1.7	3.3	28.0 ± 1.2	1.0
Q503R	4.2 ± 0.1	0.6	26.6 ± 0.4	0.9
P504Q	11.7 ± 1.5	1.6	28.0 ± 1.6	1.0
S510A	2200 ± 50	287	25.9 ± 0.9	0.9
W512A	1770 ± 58	242	28.9 ± 1.2	1.0

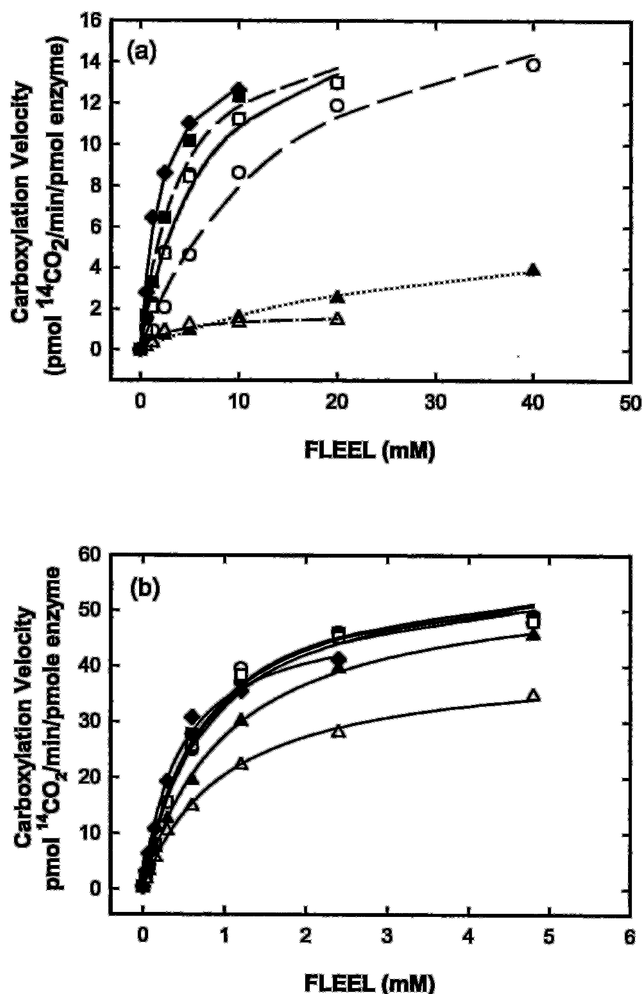

FIG. 3. **Kinetic studies of FLEEL carboxylation.** Carboxylation of FLEEL, measured by $^{14}CO_2$ incorporation as described under "Experimental Procedures," was performed in the absence of (*a*) or in the presence of saturating proFIX19 (*b*). Reactions contained 20 nM of active wild type enzyme (●), V502A (○), Q503R (■), Q503N (□), S510A (▲), or W512A (△). Only results for mutants that have altered affinities for proFIX19 are shown.

lated (21). For the mutants V502A, V502A/P504Q, S510A, and W512A, 100 nM active carboxylase was premixed with 100 nM FproFIX19 to achieve a starting anisotropy value significantly above base line. For the mutants Q503N and P504Q, 50 nM carboxylase was premixed with 100 nM FproFIX19. All off rate experiments were carried out at 4.5 °C, and all data were well fitted to a model of propeptide release described by a single exponential decay,

$$a_t = (a_i - a_f)\exp(-k*t) + a_f \quad \text{(Eq. 1)}$$

where a_t is the anisotropy at time t, a_i and a_f are the anisotropies at initial and final time, respectively, k is the first-order rate constant, and t is time. Time course data were fitted to Equation 1 by minimizing the parameters a_i, a_f, and k. The changes in fluorescent intensity of Fpro-FIX19 upon binding to the wild type and all mutant enzymes were found to be insignificant and did not affect the fraction bound, so rate constants could be accurately determined directly from the anisotropy data (22).

Determining the Apparent K_d of proFIX19 for Wild Type and Mutant Carboxylase—Reactions were performed with 20 nM active carboxylase in a buffer containing 25 mM MOPS (pH 7.5), 500 mM NaCl, 0.16% CHAPS, 0.16% phosphatidylcholine, 222 μM vitamin K hydroquinone, 6 mM dithiothreitol, 10 μCi of $NaH^{14}CO_3$ (specific activity, 54 mCi/mmol), and 1.5 mM FLEEL. The desired concentration of proFIX19 was included in each reaction. Reactions were at 20 °C for 30 min as previously described (19). The fraction of bound enzymes (f_b) was determined using the following equation: $f_b = (V_{obs} - V_f)/(V_{sat} - V_f)$. The V_{obs}, V_f, and V_{sat} refer to FLEEL carboxylation velocity in the presence of a certain concentration of proFIX19, in the absence of proFIX19, and in the presence of saturating proFIX19 respectively. The apparent K_d of wild type and mutant carboxylase was determined from f_b as previously described (22, 23).

Kinetic Studies—For kinetic studies of FLEEL carboxylation, 20 nM active carboxylase was used for each reaction. Reactions were performed in the presence or absence of saturating proFIX19 at 20 °C for 30 min and were carried out as described above, except that the concentration of the substrate FLEEL was varied from 0 to 40 mM for V502A and S510A and from 0 to 20 mM for other mutants in the absence of proFIX19. In the presence of saturating proFIX19, the concentration of the substrate FLEEL was varied from 0 to 4.8 mM. For kinetic studies of propeptide-containing substrates, FIX Q/S, consisting of residues −18 to +41 of human FIX with mutations at −4 (Arg → Gln), −1 (Arg → Ser), and 19 (Met → Ile), was used. Previous studies have shown that there is no significant difference in kinetic properties between a wild type substrate and the FIX Q/S (24). Twenty nM active carboxylase was used for each reaction. Reactions were performed at 20 °C for 60 min in a buffer containing 25 mM MOPS (pH 7.5), 500 mM NaCl, 0.16% CHAPS, 0.16% phosphatidycholine, 222 μM vitamin K hydroquinone, 6 mM dithiothreitol, and 10 μCi of $NaH^{14}CO_3$. The concentration of the propeptide-containing substrate FIX Q/S, was varied from 0 to 2500 nM.

On Rate Measurement—To measure the rate of FproCon to carboxylase, 150 nM of wild type, V502A, S510A, or W512A enzyme was added to standard buffer containing 10 nM FproCon at time 0. 20 s after adding enzymes, the anisotropy values were measured at 5-s intervals. The anisotropy at each time point was converted to fraction bound of FproCon as previously described (21, 22). Our previous results indicate that once FproCon bound to wild type, V502A, S510A, or W512A carboxylase, it would not come off within 12 h (data not shown). Thus, the contributions to the changes of anisotropy in our experiments of the release of FproCon from carboxylase can be ignored. All measurements were performed at 4.5 °C, and all data were fitted to a pseudo-first order reaction equation,

$$c = b*(1 - \exp(-k*a*t)) \quad \text{(Eq. 2)}$$

where c represents the concentration of carboxylase-FproCon complex, b is the total concentration of FproCon, a is the total concentration of enzyme, t is time in seconds, and k is the rate constant.

RESULTS

Mutations—To investigate the function of the putative internal propeptide region of carboxylase, we made several mutations in the region identified by Price and Williamson. These

TABLE II
Comparison of kinetic parameters for FLEEL

Enzyme	FLEEL without proFIX19		FLEEL with saturating proFIX19	
	K_m	V_{max}	K_m	V_{max}
	mM	*pmol $^{14}CO_2$/min/pmol enzyme*	*mM*	*pmol $^{14}CO_2$/min/pmol enzyme*
Wild type	3.3 ± 0.4	17.0 ± 2.6	0.78 ± 0.12	50.4 ± 1.7
P495V/Q497A	3.8 ± 0.3	16.3 ± 2.0	0.82 ± 0.07	48.0 ± 2.6
F496A	3.6 ± 0.2	17.5 ± 3.0	0.84 ± 0.09	48.2 ± 2.0
V502A	8.0 ± 0.1	16.5 ± 0.8	0.79 ± 0.07	49.6 ± 1.2
V502A/P504Q	7.6 ± 0.7	16.5 ± 2.1	0.80 ± 0.16	48.7 ± 2.9
Q503N	4.7 ± 0.4	15.6 ± 1.5	0.79 ± 0.04	46.0 ± 2.6
Q503R	1.9 ± 0.2	17.2 ± 2.5	0.78 ± 0.11	51.8 ± 3.7
P504Q	3.4 ± 0.3	16.5 ± 3.5	0.79 ± 0.13	48.6 ± 3.3
S510A	>30	8.1 ± 0.8	0.93 ± 0.08	47.4 ± 1.1
W512A	3.2 ± 0.2	2.0 ± 0.2	0.76 ± 0.06	38.2 ± 2.8

mutations were designed to make the putative internal propeptide sequence either more like or less like a consensus propeptide sequence. The consensus sequence, which was determined by selecting the most prevalent amino acid at each position in all known propeptides, binds to the carboxylase with an affinity much higher than any known, naturally occurring, propeptide (16). Fig. 1 demonstrates the alignment of the proposed internal propeptide region with the consensus human propeptide and the corresponding region of the mutations used in this paper. If region 495–513 contains an internal propeptide, then mutations rendering it more like the consensus sequence (*e.g.* V502A, Q503N, P504Q, and the double mutations P495V/Q497L and V502A/P504Q) would be expected to cause stronger intramolecular interactions with the propeptide binding site. These mutations would cause a decrease in the apparent affinity for the propeptide. Conversely, mutations making the region diverge from the consensus sequence (*e.g.* F496A and Q503R) should reduce the binding between the propeptide binding site and the putative internal propeptide, resulting in an apparent increased affinity for propeptide. S510A and W512A were mutated to test the alternative hypothesis.

Determining the Apparent K_d of proFIX19 for Wild Type and Mutant Carboxylase—The free propeptide of the vitamin K-dependent proteins has been shown to stimulate the rate of carboxylation of FLEEL (12); therefore, the apparent K_d of propeptide can be determined by measuring the velocities of FLEEL carboxylation at varied propeptide concentrations. Fig. 2 shows a plot of enzyme fraction bound *versus* proFIX19 concentrations. The apparent K_d of proFIX19 for wild type enzyme was 7.3 ± 0.5 nM, whereas the apparent K_d for mutant enzymes varied from 4.2 ± 0.1 to 2200 ± 50 nM (Table I). However, P495V/Q497A and F496A were not significantly different from wild type enzyme (Table I). It is notable that the maximum stimulated rates of FLEEL carboxylation were comparable for all mutants and wild type enzyme.

FLEEL Carboxylation—The binding sites for glutamate and propeptide appear to be functionally linked (12, 14, 22). We therefore investigated the effects of these mutations on the kinetics of CO_2 incorporation into FLEEL. Kinetic constants for FLEEL carboxylation were determined in the presence or absence of saturating proFIX19. In the absence of proFIX19, Q503R (which has the highest affinity for proFIX19) was found to have the lowest K_m (1.90 mM). In contrast, the K_m values for FLEEL of V502A, Q503N, and S510A vary from 4.7 to more than 30 mM and are higher than the 3.33 mM of wild type enzyme. The affinity of these mutants (V502A, Q503N, Q503R, and S510A) for FLEEL appears to increase or decrease in parallel with their affinities for proFIX19 (Fig. 3*a* and Table II). As shown in Fig. 3, although the affinities in the absence of propeptide (inferred from the K_m values toward FLEEL) are different from that of wild type enzyme, their V_{max} values are similar to that of the wild type enzyme in the presence of saturating proFIX19. The kinetic constants for FLEEL carboxylation in the presence of saturating proFIX19 were also determined. Fig. 3*b* demonstrates that, in the presence of saturating proFIX19, the K_m and V_{max} values of all mutants were similar to wild type enzyme. It is notable that, except for S510A and W512A, the V_{max} values of most mutations and the wild type enzyme were consistently increased 3-fold when the mutant enzymes were saturated with proFIX19 (Table II).

The Off Rate of FproFIX19 from Carboxylase—Thus far, our data are consistent with Price's hypothesis (17). However, we might obtain the same effects if we were mutating the propeptide binding site instead of an internal propeptide. We realized that if mutations were in the proposed internal propeptide site, then, neglecting linkage, they should not affect the off rate of a bound propeptide. If, however, the region mutated was part of the propeptide binding site, the off rate might be affected. As shown in Fig. 4, the time course of anisotropy loss due to FproFIX19 release can be satisfactorily fit to a single exponential decay curve, which allows us to determine the off rate constants. The off rate constant for FproFIX19 release from wild type carboxylase was found to be $6.77 \pm 0.19 \times 10^{-4}$ s^{-1}, whereas the rate constants for mutants varied from $3.46 \pm 0.12 \times 10^{-4}$ s^{-1} for Q503R to $102 \pm 10 \times 10^{-4}$ s^{-1} for V502A/P504Q (Table III). Our results suggest that these mutations affect the propeptide binding site rather than an internal propeptide.

The On Rate of FproCon to Carboxylase—The apparent K_d and off rate data suggest that these mutations may also affect the association rate of propeptide and carboxylase (Table IV). Therefore, we measured the association rate of FproCon to carboxylase. As shown in Fig. 5 and Table IV, the association rate constants for FproCon binding to wild type or mutant enzymes varied from 1.3×10^4 to 1.2×10^5 s^{-1} M^{-1}.

Kinetics with a Propeptide-containing Substrate—We chose four mutations exhibiting the greatest difference in propeptide binding kinetic properties for additional studies. The kinetic parameters of FIX Q/S were consistent with a change in affinity of the carboxylase for propeptide. The K_m and V_{max} values for wild type are 68 ± 6 nM and 23.4 ± 0.6 pmol of $^{14}CO_2$ per pmol of active enzyme/h, respectively. The K_m values for V502A, Q503R, S510A, and W512A varied from 41 ± 2 to 560 ± 20 nM, and the V_{max} varied from 18.0 ± 0.6 to 55.8 ± 1.2 pmol of $^{14}CO_2$/pmol of active enzyme/min (Table V and Fig. 6).

DISCUSSION

Peptide substrates bind to the vitamin K-dependent γ-glutamyl carboxylase through their propeptide. This propeptide binds to a specific region of the carboxylase and positions the substrate's glutamic acids, destined for modification, into the active site of the enzyme. Price and Williamson observed that residues 495–513 of human and bovine carboxylase had homology to the propeptide of matrix Gla protein (17). They proposed

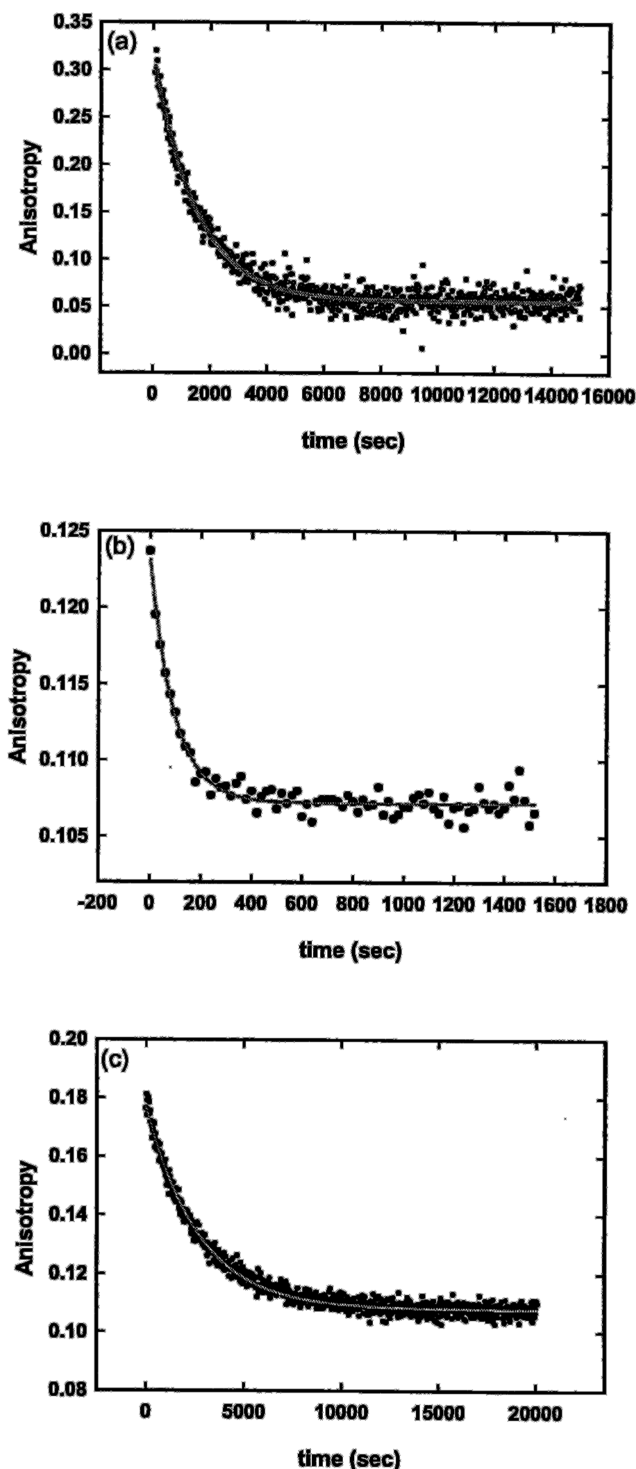

FIG. 4. **Measurements of dissociation rates of proFIX19 from wild type (*a*), V502A (*b*), and Q503R (*c*).** Fluorescein-labeled proFIX19 was preincubated with wild type or mutant carboxylase to reach a measurable anisotropy value. A 350-fold excess of unlabeled proFIX19 was then added to compete for the propeptide binding site. Off rates were determined by measuring the anisotropy decrease over time. Rate constants were determined by fitting data to a single exponential decay equation. Experiments were done at 4.5 °C.

TABLE III
Comparison of the dissociation rate constants of wild type and mutant carboxylase

Enzyme	Off rate constant	Difference
	10^{-4} s^{-1}	*-fold*
Wild type	6.77 ± 0.19	1.0
P495V/Q497A	7.76 ± 0.24	1.1
F496A	7.57 ± 0.22	1.1
V502A	96.5 ± 14.0	14.2
V502A/P504Q	102 ± 10	15.1
Q503N	21.9 ± 1.7	3.2
Q503R	3.46 ± 0.12	0.5
P504Q	16.7 ± 0.1	2.5
S510A	94.9 ± 6.6	14.0
W512A	39.5 ± 3.7	5.8

TABLE IV
Comparison of the association rate constants of wild type and mutant carboxylase

Each rate constant represents the average of two measurements.

Enzyme	On rate constant	Difference
	10^{4} s^{-1} M^{-1}	*-fold*
Wild type	12	1
V502A	8.9	0.74
S510A	1.4	0.12
W512A	1.3	0.11

that this propeptide-like sequence or "internal propeptide" could react intramolecularly with the propeptide binding site of carboxylase and thus block the active site of the enzyme. An authentic substrate with a propeptide would displace the internal propeptide and allow the reaction to proceed. This would prevent the promiscuous modification of glutamates of substrates lacking a propeptide. Based on this model, we imagined that equilibrium between closed (the less active) and open (the more active) forms of carboxylase exist and that a shift toward the open form would occur in the presence of a propeptide or a propeptide-containing substrate. According to this hypothesis, shifting the equilibrium toward the closed form, thus making the internal propeptide bind tighter to the propeptide binding site, should produce a carboxylase with lower apparent affinity for the propeptide. Conversely, reducing the affinity of the internal propeptide for the propeptide binding site should result in a carboxylase molecule that has a higher apparent affinity for the propeptide.

To test this model, we made mutant carboxylases with changes in the putative internal propeptide that we predicted would either increase or decrease the apparent affinity of carboxylase for the propeptide. We were aided in the design of our experiments by results from our previous studies in which we demonstrated that a peptide with the consensus sequence containing the most prevalent amino acid at each position in the known propeptides binds to the carboxylase with subnanomolar affinity. Therefore, we expressed five different mutant carboxylase molecules, F496A, V502A, Q503R, Q503N, and P504Q. Each mutation was intended to make the enzyme's putative internal propeptide more or less like the consensus propeptide sequence. Phe^{496} would correspond to residue −16, and the change from the consensus phenylalanine to alanine should reduce the affinity of the proposed internal propeptide for the propeptide binding site. On the other hand, Val^{502} corresponds to position −10, and the change to alanine, the consensus residue, should increase the affinity of the proposed internal propeptide for the propeptide binding site. While not highly conserved in propeptides, the most prevalent amino acid at −9 is asparagine. In the "internal propeptide," the corresponding position is glutamine 503. Based on our studies with the factor IX propeptide and on substitution studies done on the human prothrombin propeptide, changing Gln^{503} to Arg (16) should reduce the affinity of the putative internal propeptide for the propeptide binding site. Changing Gln^{503} to Asn, the consensus residue might cause a modest increase in the apparent affinity. Pro^{504} corresponds to position −8, which is not a highly conserved position; however, the propeptide may

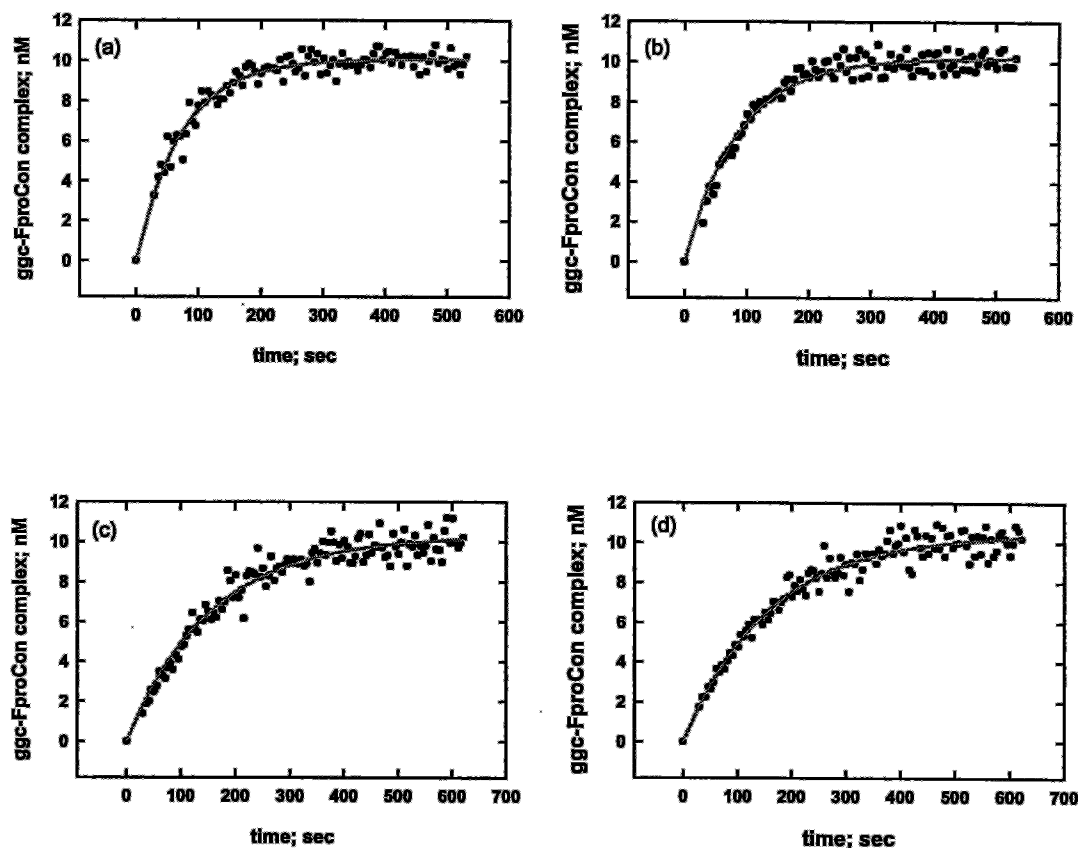

FIG. 5. **Measurements of association rates of FproCon to wild type (*a*), V502A (*b*), S510A (*c*), and W512A (*d*).** Reactions were performed at 4.5 °C as described under "Experimental Procedures."

TABLE V
Kinetic parameters for FIX Q/S carboxylation

Enzyme	K_m	Difference	V_{max}	Difference
	nM	*-fold*	*pmol* $^{14}CO_2$*/h/pmol enzyme*	*-fold*
Wild type	68 ± 6	1	23.4 ± 0.6	1
V502A	232 ± 16	3.4	53.4 ± 1.8	2.3
Q503R	41 ± 2	0.6	18.0 ± 0.6	0.8
S510A	560 ± 20	8.2	55.8 ± 1.2	2.4
W512A	328 ± 21	4.8	46.2 ± 1.8	2.0

have an α-helical structure (25), and, if this is the case, the proline might disrupt the helix. Therefore, changing proline 504 to the consensus sequence residue, glutamine, should increase the affinity of the internal propeptide for its binding site.

To measure the relative affinity of the carboxylase molecules for proFIX19, we determined the apparent K_d for proFIX19. As expected, V502A and Q503N have lower affinities for proFIX19 than does wild type carboxylase. This result was consistent with the hypothesis that residues 495–513 are functioning as an internal propeptide. Also consistent with the hypothesis, Q503R caused a slight decrease in the apparent K_d for proFIX19. P504Q caused only a small increase in the K_d for proFIX19. In contrast, F496A, which corresponds to the most highly conserved residue in the propeptide, should have decreased the apparent affinity for propeptide but had no effect. Our results then were not completely consistent with the idea that residues 495–513 are acting as an internal propeptide. Furthermore, our results could be interpreted to support an alternative hypothesis (*i.e.* residues 495–513 are part of a propeptide binding site). In addition, this possibility is consistent with our previous report that the propeptide binding site resides within a 14-kDa cyanogen bromide fragment beyond residue 438.

At this point in our study, we shifted focus to distinguishing between the two hypotheses. We reasoned that, if we mutated residues within an internal propeptide, once a propeptide bound to the carboxylase, its dissociation rate should be unaffected by mutations at the site. This, of course, would be contingent on the mutation not causing a gross conformational change in the enzyme. If, on the other hand, we mutated residues within the propeptide binding site, the off rates should change in parallel with the apparent K_d for proFIX19. The propeptide dissociation rates from the five mutant carboxylases described above were consistent with the alternative hypothesis that these residues participate in propeptide binding (Table III and Fig. 4).

To further characterize this site, we made additional mutant carboxylases to test the effect on propeptide binding (Table I and Fig. 2): P495V/Q497A, V502A/P504Q, S510A, and W512A. The double mutation P495V/Q497A had no significant effect on any measured parameter. The double mutant V502A/P504Q caused a 6-fold increase in the apparent K_d for proFIX19. This effect could be attributed, predominantly, to the single point mutation, V502A, described above. Mutation of tryptophan 512 and serine 510 both caused a greater than 200-fold increase in the apparent K_d for proFIX19. In all cases, the apparent affin-

ities and off rate results were consistent and supported the propeptide binding site model. Whereas the apparent affinities for proFIX19 and off rate data are qualitatively consistent in all cases, the differences in the apparent K_d determined by stimulation of FLEEL carboxylation are higher relative to wild type than the differences in off rates for S510A and W512A (Table VI). This implies that these mutations affect the association rate of the propeptide for carboxylase. The affinities of S510A and W512A were so low that we could not measure the on rates using the proFIX19, so we used the consensus propeptide for these measurements. Results shown in Table IV and Fig. 5 indicate that the on rates for S510A and W512A are indeed about 10-fold slower than that of the wild type enzyme.

We have shown that another affect of altered propeptide affinity is on the overall rate of carboxylation of propeptide-containing substrates. The rate is at least partially dependent upon the affinity of the propeptide for the carboxylase (13, 26). In other words, product release appears to be the rate-determining step. Therefore, substrates whose propeptides have relatively low affinities for the carboxylase have increased rates of turnover. Conversely, substrates with high affinity for the carboxylase exhibit slower turnover rates. Thus, we expected mutations that reduced the affinity of the carboxylase for the propeptides to increase the V_{max} (*i.e.* incorporation of CO_2 into a propeptide-containing substrate would be expected to increase in the mutations with higher off rates, and a carboxylase with higher affinity toward the propeptide should exhibit a lower V_{max}). In contrast, in the intramolecular interaction model, the proposed internal propeptide sequence would be a competitive inhibitor. In this case, we would expect changes in K_m for propeptide-containing substrate rather than in V_{max} when the mutations were made at this region. Again, our results agree with our predictions and are consistent with these residues being involved in propeptide binding (Fig. 6 and Table V).

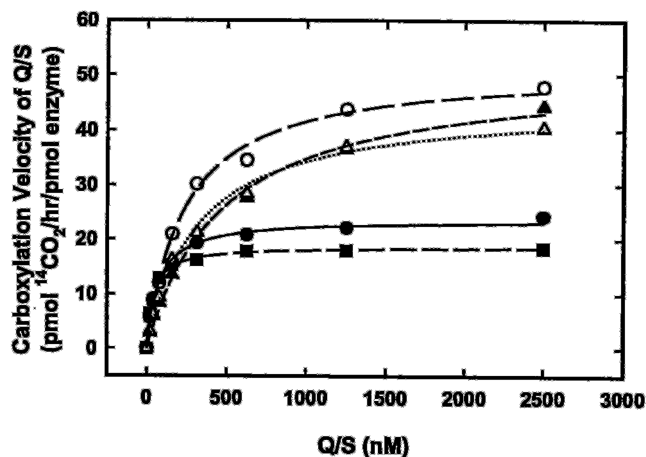

FIG. 6. **Carboxylation of FIX Q/S.** Carboxylation of FIX Q/S by wild type enzyme (●), V502A (○), Q503R (■), S510A (▲), or W512A (△) was performed at 20 °C for 1 h as described under "Experimental Procedures." The concentrations of FIX Q/S varied from 0 to 2500 nM.

Although our kinetic studies with the small pentapeptide FLEEL do not shed light directly on the issue of whether the residues we mutated are involved in propeptide binding, they are of interest for two reasons. First they show that in most cases the mutations specifically affect propeptide binding, and second the mutations that do affect catalysis are of interest in terms of linkage between the substrate binding site and the propeptide binding site. Knobloch and Suttie reported that the propeptide of factor X stimulates CO_2 incorporation into FLEEL and suggested that this was due to such a linkage phenomenon (12, 14, 27). We predicted, based on this hypothesis, that mutation of certain propeptide binding residues will affect the interaction of carboxylase with non-propeptide-containing substrates. Conversely, certain mutations at the substrate-binding site may affect propeptide binding. In fact, while our results overwhelmingly indicate that the residues mutated in this study are important for propeptide binding, in the absence of proFIX19, the K_m for FLEEL for V502A is higher than that of the wild type enzyme. Additionally, both the K_m and V_{max} for S510A are affected, whereas the V_{max} for W512A is significantly lower than that of wild type (Fig. 3 and Table II). Q503R has a slightly lower K_m than that of wild type. Thus, we have found mutations that not only affect propeptide binding but also increase or decrease the catalytic efficiency of the carboxylase toward FLEEL. Moreover, the observed catalytic differences of the mutant carboxylases disappear in the presence of propeptide (Fig. 3 and Table II). Therefore, it is likely that binding of the propeptide to its binding site results in a conformational change, which alters the affinity of the small-substrate binding site. This conclusion is consistent with our previous result describing a residue involved in small substrate binding (L394R), mutation of which affects propeptide binding (19).

Since we used stimulation of FLEEL carboxylation as one measure of propeptide affinity, this linkage may affect the apparent K_d values we report, especially for S510A, which has a K_m at least 10 times that of wild type. Whereas our off rate, on rate, and FIX Q/S kinetic results all support the idea that the residues in our study are important in propeptide binding, we assessed linkage effects by measuring the apparent K_d values for S510A, V502A, and wild type carboxylase at higher FLEEL concentrations. The largest effect was on the apparent K_d for S510A that was about 3-fold lower when we performed the experiments with 60 mM FLEEL as compared with 1.25 mM FLEEL (results not shown). Under these conditions, the K_d of S510A was still 100-fold higher than that of wild type carboxylase; therefore, linkage effects do not affect our conclusions.

In summary, our results support the conclusion that residues 495–513 in human carboxylase do not represent an internal propeptide involved in control of substrate binding but rather are part of the enzyme that binds the substrates' propeptide directly. In addition, some of these residues are important for functional linkage of the propeptide binding site to the substrate Gla domain binding site.

TABLE VI
Comparison of the apparent K_d for proFIX19, kinetic parameters for FLEEL, off rates, and kinetic parameters for FIX Q/S

Enzyme	Apparent K_d for proFIX19	V_{max}/k_m for FLEEL carboxylation[a]	Off rate × 10^4	V_{max} for FIX Q/S carboxylation
	nM	*pmol $^{14}CO_2$/min/pmol enzyme/mM*	*s^{-1}*	*pmol $^{14}CO_2$/h/pmol enzyme*
Wild type	7.3 ± 0.5	64.6 ± 2.2	6.8 ± 0.2	23.4 ± 0.6
V502A	53.7 ± 3.1	62.8 ± 1.6	96.5 ± 14.0	53.4 ± 1.8
Q503R	4.2 ± 0.1	66.4 ± 4.7	3.5 ± 0.1	18.0 ± 0.6
S510A	2200 ± 50	51.5 ± 1.2	94.9 ± 6.6	55.8 ± 1.2
W512A	1770 ± 58	50.3 ± 3.7	39.5 ± 3.7	46.2 ± 1.8

[a] In the presence of saturating proFIX19.

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