The vitamin K-dependent carboxylase, a constituent of the endoplasmic reticulum membrane, catalyzes the conversion of reduced vitamin K to vitamin K epoxide and the concomitant conversion of glutamic acid to \(\gamma\)-carboxyglutamic acid. To study structure-function relationships in the enzyme, seventeen clusters of charged residues of the bovine \(\gamma\)-glutamyl carboxylase were substituted with alanines using site-specific mutagenesis. Wild-type and mutant carboxylase species were expressed in Chinese hamster ovary cells with an immunodetectable octapeptide inserted at their amino-terminal ends. Out of 17 mutant carboxylase species that contain a total of 41 charged residue to alanine substitutions, K217A/K218A (CBX217/218), R234A/H235A (CBX234/235), R359A/H360A/K361A (CBX359/360/361), R406A/H408A (CBX406/408), and R513A/K515A (CBX513/515) had impaired carboxylation activity compared with the wild-type enzyme. The vitamin K epoxide activities of these mutants were reduced in parallel with the carboxylase activities. CBX217/218 appears to be inactive. High propeptide concentrations were required for stimulation of carboxylation of FLEEL by CBX234/235, CBX406/408, and CBX513/515, suggesting defects in the propeptide binding site. CBX359/360/361 showed normal affinity for the propeptide, FLEEL, proPT28, and vitamin K hydroquinone but exhibited a low catalytic rate for carboxylation. These results suggest that residue 217, residue 218, or both are either critical for catalysis or for maintaining the structure of a catalytically active enzyme. Regions around residues 234, 406, and 513 define in part the propeptide binding site, while the regions around residue 359 are involved in catalysis.

Vitamin K-dependent \(\gamma\)-glutamyl carboxylase is a membrane-associated endoplasmic reticulum resident enzyme that catalyzes the posttranslational conversion of specific glutamic acids of the vitamin K-dependent proteins to Gla. The vitamin K-dependent blood clotting and regulatory proteins have clusters of 9–12 Gla residues near their N termini. A Ca\(^{2+}\) -stabilized three-dimensional structure of the Gla domain is essential for binding of the protein to phospholipid vesicles or cell membranes (1).

Carboxylase activity has been found in such diverse tissues as liver, testis, skin, lung, and kidney. The Gla-containing proteins are found not only in blood plasma but also in calcified tissue, spermatozoa, urine, and lung surfactant (2). Protein S and a homologous protein, Gas6 (3), have been identified as ligands for Tyro3 and Axl, respectively, members of a family of receptor tyrosine kinases (4, 5) although the role of protein S as a receptor tyrosine kinase ligand has been questioned (6). Cellular responses to activation of these receptors by protein S (4) and Gas6 (5) requires vitamin K, suggesting a requirement of \(\gamma\)-carboxylation of the ligands. Thus, the vitamin K-dependent carboxylase appears to play important roles in many physiologic processes in addition to blood clotting.

The carboxylase, a bifunctional enzyme, catalyzes the conversion of glutamic acid and vitamin K hydroquinone (vitamin KH\(_2\)) \(^3\) to Gla and vitamin K epoxide in the presence of CO\(_2\) and O\(_2\). Studies using the crude enzyme showed that the epoxidation and the carboxylation activities are stoichiometrically coupled when CO\(_2\) is saturating (7). Characterization of purified bovine liver carboxylase has confirmed that these reactions are carried out by the same protein (8). According to the basicity enhancement model proposed recently (9), the driving force for the carboxylation of glutamic acid derives from enzymatic oxygenation of vitamin KH\(_2\).

The propeptide, linked at the N-terminal end of the Gla domain of the precursor forms of the vitamin K-dependent blood proteins, directs carboxylation by mediating the interaction of the substrate protein and the carboxylase. The carboxylation recognition site (\(\gamma\)-CRS) located near the amino terminus of the propeptide functions as a docking element for the carboxylase (10–12), while the free propeptide functions as a stimulatory element for carboxylation of small Gla-containing peptides (13, 14). The bovine carboxylase has been purified (15, 16) utilizing the affinity of carboxylase for the \(\gamma\)-CRS of the propeptide of vitamin K-dependent proteins, and the full-length human cDNA (17) and bovine cDNA (18) have been cloned. The hydropathy analysis of the primary amino acid sequence predicted that the enzyme has a short N-terminal hydrophilic region (residues 1–50), a hydrophobic region (residues 51–314), and a long C-terminal hydrophilic region (residues 315–758). The hydrophobic region contains three to five putative transmembrane domains. The C-terminal hydrophilic region of the propeptide functions in the interaction of the vitamin K-dependent propep-
Substrate Binding Sites on the Vitamin K-dependent Carboxylase

Cells from selected colonies were grown to confluence in 12-well plates, and the level of expression of recombinant carboxylase was assayed by Western blotting. Cells were harvested, washed with 1 ml of PBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, and 6.5 mM Na₂HPO₄), resuspended in 50 μl of PBS with protease inhibitor complex (P1; 2 mM diithiothreitol, 2 mM EDTA, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 2 μg/ml aprotinin), and subjected to Western blotting. Three independent clones that expressed recombinant carboxylase were selected for further analysis.

Preparation of Cell Lysate—Confluent cells in two 140 x 20-mm plates were suspended with PBS, 5 mM EDTA, washed once with 10 ml of PBS, and resuspended in 400 μl of PBS, 20% glycerol, P1. Cell lysates were also cell-suspended, and a 50 μl aliquot of PBS, 1% CHAPS, 0.2% phosphatidylcholine, 20% glycerol, P1 and sonicated on ice for 5 s twice with an Ultrasonic processor W-220 fitted with a microprobe (Heatsystems-Ultrasonics, Inc.). After centrifugation (16,000 x g, 10 min, 4°C), the solubilized cell supernatant was stored at -80°C.

Western Blotting—Monoclonal anti-FLAG M2 antibody was used to detect wild-type FLAG-CBX and mutant FLAG-CBX species in cell lysate. A monoclonal rabbit polyclonal antipeptide antibody directed against residues 86–99 of bovine carboxylase, anti-CBX-(86–99) (22), was employed for detecting wild-type CBX in cell lysate and bovine liver carboxylase in solubilized bovine liver microsome. Western blots were prepared by electrophoretic proteins separated in 10% SDS-polyacrylamide gel. In addition, a 8 M urea, 1% diiodomide membrane. After blocking with 5% nonfat dry milk in PBS with 0.05% Tween 20 (PBS/T), membranes were incubated with anti-FLAG M2 monoclonal antibody (1 μg/ml) or anti-CBX-(86–99) antibody (1 μg/ml) in PBS/T at 4°C overnight. Membranes were then incubated with peroxidase-conjugated goat anti-mouse immunoglobulin (0.2 μg/ml) or peroxidase-conjugated goat anti-rabbit immunoglobulin (0.05 μg/ml) in PBS/T at room temperature for 1 h. Bound antibodies were detected with the ECL detection kit (Amersham). The light emission produced was detected by autoradiography on Hyperfilm ECL (Amersham).

Quantitation of Wild-type FLAG-CBX, Mutant FLAG-CBX Species, Wild-type CBX, and Liver Microsomal Carboxylase—Quantitation was performed by densitometry of the Western blots prepared as described above. Concentration of wild-type FLAG-CBX in a CHO cell lysate was determined from Western blots using amino-terminal FLAG-bacterial alkaline phosphatase of known concentration as a primary standard. This preparation of FLAG-CBX was used as the secondary standard for quantitation of wild-type or mutant FLAG-carboxylase in other cell lysates. Six different aliquots of standard FLAG-CBX lysate containing from 4 to 12 μg of FLAG-CBX were used to generate a standard curve. The developing antibody was either the anti-FLAG M2 antibody or anti-CBX-(86–99) antibody. Autoradiographs were analyzed with an UltraScan XL laser densitometer (Pharmacia Biotech Inc.).

Assay of Carboxylase Activity—The amount of [¹⁴C]O₂ incorporated into exogenous peptide substrates (3.6 mM FLEEL or 5 mM proPT28) was determined by the recombinant enzyme assay. The extent of partially purified bovine carboxylase was measured in reaction mixtures of 125 μl containing 25 mM MOPS (pH 7.0), 0.5 mM NaCl, 0.16% CHAPS, 0.16% phosphatidylcholine, 8 mM diithiothreitol, 888 μM chemically reduced vitamin K₇, and 1.4 mM Na[¹⁴C]O₃ (10 μCi; Amersham). When FLEEL was used as a substrate, ammonium sulfate (0.8 M) and proFIX18 (16 μM) were included in the assay. All the assay components except carboxylase were prepared as a master mixture. The reaction was initiated by adding the master mixture to a cell lysate or liver microsomal carboxylase. Incorporated [¹⁴C]O₂ was assayed as described previously (12).

Kinetic Studies—In order to determine kinetic constants for three substrates, FLEEL, proPT28, and vitamin K₇, the initial rates of [¹⁴C]O₂ incorporation was determined at six or more different concentrations of substrate up to 2-fold Kₘ. Since FLEEL and vitamin K₇ showed apparent substrate inhibition as previously reported (8), concentrations of FLEEL and vitamin K₇ were used below the inhibitory concentrations. Fixed concentrations of FLEEL, proPT28 of 3.6 mM and 5 mM, and various kinetic conditions using these substrates were determined. Vitamin K₇ was generally used at 222 μM when determining kinetic parameters for FLEEL and proPT28. However 56 μM vitamin K₇ was used in assays for CBX153/515. Kinetic constants were determined by nonlinear regression analysis using the Michaelis-Menten equation (Deltagraph PRO 3, DeltaPoint).

Assay of Vitamin K Epoxide Activity—Carboxylase assays were performed in a 125-μl mixture as described above except that NaH[¹⁴C]O₃ was replaced with the same concentration of NaHCO₃ (final concentration, 1.4 mM). Vitamin K epoxide formation was deter-
mired as described previously (21). Briefly, upon completion of the 30-min incubation at 25 °C in sealed tubes, the reaction mixture was extracted with 250 μl of ethanol and then 750 μl of hexane. The organic and aqueous phases were separated by centrifugation at 1000 × g for 10 min. The organic phase was removed, and the solvent evaporated to dryness. The residue was redissolved in 200 μl of methanol. Half of this solution was injected onto a reverse-phase C18 HPLC column (HYPER- SIL ODS, 5 μm, 4.6 × 250 mm, Custom LC, Inc.). The column was developed with a mobile phase of 10% dichloromethane, 90% methanol, which had been saturated with nitrogen. The flow rate was 1 ml/min. Vitamin K derivatives were detected at 226 nm, and vitamin K epoxide was quantitated using a purified standard.

RESULTS

Expression of Wild-type CBX and Wild-type FLAG-CBX in CHO Cells—Wild-type CBX and wild-type FLAG-CBX were expressed in CHO cells. The proteins in cell lysates from either transfected or untransfected cells were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting using the anti-CBX-(86–99) polyclonal antibody and the anti-FLAG M2 monoclonal antibody. Anti-FLAG M2 antibody identified only wild-type FLAG-CBX, which migrated as a single band of 99 kDa, and did not cross-react with endogenous CHO cell carboxylase, wild-type CBX, or bovine liver carboxylase (Fig. 1A). Anti-CBX-(86–99) antibody identified wild-type FLAG-CBX, wild-type CBX, and bovine liver carboxylase but did not detect endogenous CHO cell carboxylase (Fig. 1B). Assuming the same specific activity for hamster and bovine carboxylase, about 0.6 ng of hamster carboxylase was loaded on the gel. This is within the detectable range for bovine carboxylase in our Western blotting system, suggesting that hamster carboxylase at these concentrations does not cross-react with anti-bovine CBX-(86–99). Thus, wild-type bovine carboxylase and wild-type bovine FLAG-CBX could be quantitated in the cell lysates by Western analysis and densitometry without interference from the endogenous CHO cell carboxylase. However, endogenous CHO cell carboxylase is detectable in assays of enzyme activity (Table 1). Thus, in kinetic analyses the endogenous CHO cell carboxylase activity was subtracted from the activity of transfected CHO cells prior to evaluation of kinetic constants.

Recombinant carboxylase in cell lysate that includes 0.5% CHAPS, 0.1% PC, 20% glycerol, and PIC was stable to multiple freeze-thaw cycles. The kinetic constants for carboxylation of FLEEL by wild-type FLAG-CBX were compared with those of the bovine liver carboxylase. Bovine liver carboxylase and wild-type FLAG-CBX exhibited K_m values for FLEEL of 1.0 ± 0.5 mM and 1.5 ± 1.0 mM, respectively. The k_cat values for the bovine carboxylase and wild-type FLAG-CBX were 0.75 s^{-1} and 0.6 s^{-1}. The stability of the enzyme in the cell lysate and the similarity of the kinetic parameters for bovine carboxylase and wild-type FLAG-CBX indicate that expression of mutant carboxylases in CHO cells provides an adequate system for evaluating structure-function relationships.

Expression and Screening of Carboxylase Mutants—Because of their presumed exposure to solvent, 10 hydrophilic regions in the bovine carboxylase were selected (Fig. 2) for evaluation by site-directed mutagenesis. Seventeen clusters of charged residues were mutated to alanines by overlap extension PCR (Table 1). The mutant carboxylase species were expressed in CHO cells, and the specific activities of the mutant enzymes were determined in cell lysates.

The concentrations of the carboxylase species were established from Western blots of SDS-gels run under reducing conditions. Expression levels varied widely among the mutants and among clonal cell lines expressing the same mutant. Levels of expression did not appear to correlate with preservation or loss of function. Some mutant carboxylase species with specific activities comparable with wild-type enzyme expressed poorly on average, while some mutants with defective specific activity expressed at high levels on average. A single protein was identified in cell lysates for each species. As shown in Fig. 3, mutants CBX217/218 and CBX234/235 yielded two bands, while CBX359/360/361 had a slightly faster mobility than wild-type FLAG-CBX. These differences were confirmed by co-analysis of mutant carboxylase cell lysate mixed with FLAG-CBX cell lysate. Mutants CBX217/218 and CBX234/235 yielded two bands, while CBX359/360/361, CBX406/408, and CBX513/515 migrated with wild-type FLAG-CBX. These differences may be due to alterations in glycosylation.

The specific carboxylase activities of 17 mutants are shown in Fig. 4 and compared with the specific activities of bovine liver carboxylase, wild-type CBX, and wild-type FLAG-CBX. The specific activities of bovine liver carboxylase, wild-type CBX, and wild-type FLAG-CBX were similar to that observed for purified bovine liver carboxylase (15). Twelve mutant carboxylase species, which together include 30 mutations of charged residues to alanines and included all mutations C-terminal of residue 566, had specific activities indistinguishable from that of wild-type carboxylase. Five mutant carboxylase species, CBX217/218, CBX234/235, CBX359/360/361, CBX406/408, and CBX513/515, exhibited significantly decreased specific activity. These five mutant carboxylase species were further characterized.

The expression levels of the wild-type FLAG-CBX and the five mutant carboxylase species with decreased specific activity ranged from 0.5 fmol to 17 fmol/10^6 cells (Table 1). Expression of wild-type FLAG-CBX in CHO cells at 11.5 fmol/10^6 cells led to a 140-fold increase in carboxylase activity over untransfected CHO cells. The mutants are expressed at levels that would permit detection of carboxylase activity above the level of endogenous CHO cell carboxylase activity if the mutant species had the same specific activity as the wild-type
Nomenclature for mutant carboxylase

| Mutants  | Mutations             |
|----------|-----------------------|
| CBX177/178 | H177A,H178B          |
| CBX189/190/191 | R189A,K190A,R191A   |
| CBX217/218  | K217A,K218A          |
| CBX234/235  | R234A,H235A          |
| CBX346/347  | K346A,R347A          |
| CBX359/360/361 | R359A,H360A,K361A |
| CBX406/408  | R406A,H408A          |
| CBX416/417  | R416A,D417A          |
| CBX438/439/440 | K438A,D439A,H440A |
| CBX513/515  | K513A,K515A          |
| CBX567/568  | R567A,K569A          |
| CBX612/624  | K612A,D614A          |
| CBX622/623/624 | K622A,E623A,K624A |
| CBX661/662  | R661A,R662A          |
| CBX671/672/673 | R671A,R672A,R673A |
| CBX678/679/680 | H678A,E679A,R680A |
| CBX687/688  | R687A,K688A          |

* CBX177/178 indicates that this mutant has alanine substitutions at positions 177 and 178.
* The naturally occurring amino acids, histidine at position 177 and histidine at position 178, were mutated to alanines. Amino acids are shown using single letter code.

The naturally occurring amino acids, histidine at position 177 and histidine at position 178, were mutated to alanines. Amino acids are shown using single letter code.

Comparison of Epoxidase and Carboxylase Activity of the Five Mutant Carboxylase Species—Specific epoxidase activity and specific carboxylase activity were determined for each of the five mutant carboxylase species. Since the endogenous CO₂ concentration in the reaction mixtures is approximately equal to the exogenously added CO₂ (27–29), the true specific carboxylase activity is approximately 2-fold that recorded in Table III. Thus, the ratio of carboxylation to epoxidation of wild-type enzyme is about 1. The ratios of the carboxylase mutants CBX234/235, CBX359/360/361, and CBX406/408 were similar to that of wild-type FLAG-CBX. Mutants CBX217/218 and CBX513/515 have slightly different ratios of carboxylation to epoxidation, although the low specific activities of CBX217/218 make that distinction more difficult to discern.

Stimulation of Carboxylase Activity by the Propeptide of Factor IX—The propeptide of the vitamin K-dependent proteins functions as a recognition element for the carboxylase (10, 11) as well as a stimulator of carboxylase activity (13, 14). To investigate the stimulatory effect of the propeptide on the mutant carboxylases, we studied their ability to carboxylate FLEEL at varying concentrations of the factor IX propeptide, proFIX18 (Fig. 5A). ProFIX18 had no effect on the activity of CBX217/218. The activation of CBX359/360/361 followed a similar dependence on proFIX18 concentration as that of wild-type FLAG-CBX, with half-maximal stimulation at about 0.06 μM. However, the maximal stimulation of CBX359/360/361 was reduced from 5-fold to 2-fold. This suggests that wild-type FLAG-CBX and CBX359/360/361 have similar affinities for proFIX18. In contrast, proFIX18 concentrations yielding half-maximal stimulation of CBX234/235 and CBX406/408 were 10-fold higher (0.7 and 0.8 μM, respectively.) CBX234/235 and CBX406/408 approached maximal stimulation comparable with the wild-type carboxylase, but much higher concentrations of propeptide were required. Although the CBX513/515 had
were more susceptible to inhibition by vitamin KH2 than 

In contrast, CBX234/235, CBX406/408, and CBX513/515 were more susceptible to inhibition by vitamin KH2 than the wild-type and mutant carboxylase species. Assays were performed at 56 µM vitamin K for CBX513/515 and at 222 µM vitamin K for the other mutants and the wild-type enzyme. 

Effect of Vitamin KH2 Concentration on Carboxylation—Carboxylation of FLEEL by bovine liver carboxylase is inhibited by high concentrations of vitamin KH2 (8). It has been shown that this inhibitory effect is not caused by solvent and lipid in the vitamin K preparation (8) and that vitamin K (up to 400 µM) and vitamin K epoxide (up to 1 mM) are not inhibitory (30). We thus investigated the effect of vitamin KH2 concentration on the carboxylase activity of the wild-type FLAG-CBX and the mutant carboxylase species (Fig. 6). Carboxylation of FLEEL by the wild-type FLAG-CBX and CBX359/360/361 was maximal at 222 µM vitamin KH2 and inhibited by higher concentrations of vitamin KH2 in a dose-dependent manner. This is similar to the inhibition observed for purified bovine carboxylase (8). In contrast, CBX234/235, CBX406/408, and CBX513/515 were more susceptible to inhibition by vitamin KH2 than wild-type enzyme. CBX234/235 and CBX406/408 exhibited maximal activity at 111 µM vitamin KH2 and CBX513/515 at 56 µM vitamin KH2.

In order to investigate the relationship between propeptide stimulation of carboxylase activity and vitamin KH2 concentration, we studied proFIX18 stimulation of FLEEL carboxylation by wild-type FLAG-CBX at concentrations of vitamin KH2 of 222 µM, 888 µM, and 1776 µM (Fig. 5B). Increasing concentrations of vitamin KH2 blunt the stimulatory effect of the propeptide. Thus, high concentrations of vitamin KH2 may indirectly inhibit carboxylation by reducing the affinity of the carboxylase for the propeptide. Alternatively, vitamin KH2 may compete for the propeptide binding site. In either case, the apparent reduced affinities of CBX234/235, CBX406/408, and CBX513/515 for the propeptide may be related to the increased sensitivity of the mutant enzymes to inhibition by vitamin KH2.

Kinetic Analysis—Values of $K_m$ and $k_{cat}$ for carboxylation of FLEEL by wild-type FLAG-CBX and the mutant enzymes were determined under standard assay conditions in the presence of 16 µM proFIX18. The wild-type FLAG-CBX, CBX234/235, CBX359/360/361, and CBX406/408 were studied in the presence of 222 µM vitamin K. CBX513/515 was studied in the presence of 56 µM vitamin K. Kinetic parameters of CBX217/218 could not be determined because of its low activity. The $K_m$ values of the wild-type FLAG-CBX and the mutant carboxylase species for FLEEL are between 1.1 ± 0.2 and 1.9 ± 0.8 mM (Table IV). CBX234/235 and CBX406/408 had $k_{cat}$ values similar to those of the wild-type enzyme, but the $k_{cat}$ for CBX359/360/361 was 3–4-fold lower and that for CBX513/515 was about 10-fold lower than wild-type enzyme.

Initial analyses were performed at proFIX18 concentrations that approach that required for maximal stimulation for the wild-type enzyme and therefore may not be sensitive to mutations that affect propeptide binding. In order to more accurately determine the effects of the mutations, these analyses were performed at concentrations of proFIX18 of 0, 0.16, 1.6, and 16 µM. Only mutants CBX234/235 and CBX406/408 were analyzed, since the activities of the other mutants were too low to be measurable at low propeptide concentrations. As the concentration of propeptide was increased, $K_m$ values for FLEEL of the wild-type FLAG-CBX, CBX234/235 and CBX406/408 decreased slightly in a dose-dependent manner (Table V). While the maximal decrease in $K_m$ was observed at 1.6 µM for the wild-type enzyme, CBX234/235 and CBX406/408 required higher concentrations of proFIX18 to realize comparable $K_m$ values. In addition, the wild-type FLAG-CBX achieved the maximal increase in $k_{cat}$ at lower concentration of proFIX18 than did CBX234/235 and CBX406/408 (Table V). Final $k_{cat}$ values for the two mutants were approximately equal to the $k_{cat}$ of the wild-type enzyme, implying that the catalytic apparatus of these mutants is intact.

Since the natural substrates of the carboxylase include the recognition site in the propeptide covalently linked to the carboxylatable glutamate residues, we also studied proPT28 as substrate. This peptide includes the propeptide of prothrombin.
Substrate Binding Sites on the Vitamin K-dependent Carboxylase

**TABLE IV**

| Glu substrate | k<sub>m</sub> (μM) | k<sub>cat</sub> (s<sup>-1</sup>) | k<sub>cat</sub>/k<sub>m</sub> (s<sup>-1</sup>) |
|---------------|----------------|----------------|-------------------------------|
| FLAG-CBX      | FLEEL (16 μM proX18) | 1.1 ± 0.2 | 0.70 ± 0.08 | 6.4 × 10<sup>2</sup> |
| CBX234/235    |               | 1.3 ± 0.4 | 0.64 ± 0.14 | 4.9 × 10<sup>2</sup> |
| CBX359/360/361|               | 1.5 ± 0.4 | 0.19 ± 0.02 | 1.3 × 10<sup>2</sup> |
| CBX406/408    |               | 1.6 ± 0.8 | 0.72 ± 0.20 | 4.5 × 10<sup>2</sup> |
| CBX513/515    |               | 1.9 ± 0.8 | 0.07 ± 0.03 | 4.0 × 10<sup>2</sup> |
| FLAG-CBX      | proPT28       | 2.3 ± 0.2 | 0.020 ± 0.005 | 8.7 × 10<sup>2</sup> |
| CBX234/235    |               | 8.1 ± 2.3 | 0.060 ± 0.017 | 7.4 × 10<sup>2</sup> |
| CBX359/360/361|               | 4.1 ± 0.9 | 0.002 ± 0.0003 | 4.9 × 10<sup>2</sup> |
| CBX406/408    |               | 5.2 ± 0.9 | 0.042 ± 0.004 | 7.7 × 10<sup>2</sup> |
| CBX513/515    |               | 14.4 ± 2.2 | 0.056 ± 0.004 | 3.9 × 10<sup>2</sup> |

* These values are uncorrected for the presence of endogenous CO2.

**TABLE V**

| profIX18  | FLAG-CBX | CBX234/235 | CBX406/408 |
|-----------|----------|------------|------------|
| K<sub>m</sub> (μM) | k<sub>cat</sub> (s<sup>-1</sup>) | k<sub>cat</sub>/K<sub>m</sub> (s<sup>-1</sup>) |
| 0 | 2.01 ± 0.16 | 2.03 ± 0.14 | 2.06 ± 0.14 |
| 0.16 | 0.91 ± 0.06 | 1.54 ± 0.47 | 1.78 ± 0.32 |
| 1.60 | 0.78 ± 0.11 | 1.25 ± 0.18 | 1.23 ± 0.21 |
| 160 | 0.78 ± 0.04 | 0.92 ± 0.01 | 1.01 ± 0.01 |
| 0 | 0.059 ± 0.003 | 0.010 ± 0.003 | 0.015 ± 0.007 |
| 0.16 | 0.324 ± 0.052 | 0.179 ± 0.032 | 0.151 ± 0.010 |
| 16 | 0.465 ± 0.032 | 0.391 ± 0.01 | 0.347 ± 0.028 |
| 160 | 0.488 ± 0.017 | 0.645 ± 0.031 | 0.560 ± 0.017 |
| 0 | 2.9 × 10<sup>2</sup> | 0.5 × 10<sup>2</sup> | 0.7 × 10<sup>2</sup> |
| 0.16 | 3.6 × 10<sup>2</sup> | 1.1 × 10<sup>2</sup> | 0.9 × 10<sup>2</sup> |
| 16 | 6.0 × 10<sup>2</sup> | 3.1 × 10<sup>2</sup> | 2.8 × 10<sup>2</sup> |
| 160 | 6.3 × 10<sup>2</sup> | 7.0 × 10<sup>2</sup> | 5.5 × 10<sup>2</sup> |

In this study, a series of bovine mutant carboxylase species in which clusters of charged residues were replaced with alanines was expressed in CHO cells to study structure-function relationships. The carboxylase has been expressed in active insect cells, despite an advantage of the absence of endogenous carboxylase activity (22), we used a mammalian expression system. To distinguish the recombinant carboxylase species from endogenous CHO cell carboxylase, we placed a FLAG epitope at the N-terminal end of the carboxylase. The expression levels of the recombinant carboxylase antigens were sufficiently high relative to the endogenous carboxylase that the endogenous carboxylase antigen and activity could be distinguished in our analysis. In addition, kinetic studies showed that the wild-type FLAG-CBX is functionally indistinguishable from the liver microsomal carboxylase.

We used site-directed mutagenesis to alter 41 charged residues in the vitamin K-dependent carboxylase to alanines. Substitution of alanine for 30 charged residues in 12 carboxylase...
creases in mutants had no effect on carboxylase activity. Five mutants, CBX217/218, CBX234/235, CBX359/360/361, CBX406/408, and CBX513/515, showed significant decreases in γ-glutamyl carboxylase-specific activity. Initial speculation based on protein sequence data and hydrophobicity analysis was that the hydrophilic region in the carboxyl terminus of the enzyme would contain the carboxylase active site. Early studies using selective solubilization of rat liver microsomes (34) showed that the carboxylase activity is facing the lumen of the endoplasmic reticulum. The CDNA sequence of the carboxylase predicts that the C-terminal half of the enzyme is exposed to the lumen because this region has 7 putative N-linked glycosylation sites. In addition, amino acid residues 468–663 within this region have 19.3% homology to soybean seed lipoxygenase (17). However, the binding sites for a small peptide substrate and for the propeptide have been recently localized within the N-terminal third of the enzyme by affinity labeling. Kuliopulos et al. (20) showed that [125I]labeled bro-moacetyl-FLEELY, an irreversible inhibitor of carboxylase as well as a substrate, binds covalently within the first 218 amino acid residues of the enzyme. Yamada et al. (19) showed that proFIX18 in which Phe is replaced by the photoactivable cross-linking group, benzoylphenylalanine, covalently cross-links to the region between residues 184 and 225 in the carboxylase. In the current study CBX217/218 had almost undetectable carboxylase activity. The mutations in this enzyme overlap the region identified as a binding site for both FLEELY and the propeptide. CBX217/218 may have lost the ability to bind propeptide and/or a Glu-containing peptide substrate, although we cannot rule out the possibility that CBX217/218 is not folded properly. The single mutants at residues 217 and 218 will warrant attention. CBX234/235, CBX359/360/361, CBX406/408, and CBX513/515 all maintain enzyme function albeit at reduced levels. By adjustment of the standard assay conditions these mutants can be induced to behave more like the wild-type enzyme. While we cannot ascribe specific functions to the mutated residues and some of these mutations may lead to local conformational changes in the enzyme, it is unlikely that they are globally misfolded.

The mutations in CBX234/235 are located close to the predicted binding region for the propeptide. On the other hand, in the primary sequence the mutations in CBX406/408 and CBX513/515 are distant from the predicted propeptide-binding site and the mutations in CBX359/360/361 are distant from the predicted peptide-substrate binding site. It is possible that these three clusters of charged residues may be in close proximity to the predicted binding sites in the three-dimensional structure. Deletion of the C-terminal 82 residues of carboxylase resulted in an enzyme with low affinity for vitamin K3 and suggested that the hydrophobic region from residues 684 to 710 presents an important site for vitamin K3 binding (21). In contrast, deletion of the hydrophilic C-terminal 47 residues did not affect enzyme activity. Similarly, our mutations in this region, CBX678/679/680 and CBX687/688, did not alter enzyme activity. We did not obtain any mutants that affected vitamin K3-binding, probably because our study was focused on hydrophilic regions of carboxylase.

The vitamin K-dependent carboxylase has two distinct functions, vitamin K epoxidation and γ-glutamyl carboxylation. These two reactions are stoichiometrically coupled except under conditions where either CO2 or Glu substrate is not saturating (7, 29). With low CO2 concentration or with low Glu substrate concentration, the ratios of mol vitamin K epoxide formed to CO2 fixed were reported to increase from 1 to 9.7 (7) and 3.5 (29). However, further studies of mechanisms of epoxidation and carboxylation are difficult due to the existence of endogenous substrates in the case of partially purified microsomal enzyme preparations or propeptide in the case of the somal enzyme preparations or propeptide. CBX217/218 may have lost the ability to bind CO2, which is required for the stimulation of FLEELY carboxylation. CBX234/235, CBX359/361, and CBX406/408 may have lost the ability to bind proFIX18 in which Phe is replaced by the photoactivable cross-linking group, benzoylphenylalanine, covalently cross-links to the region between residues 184 and 225 in the carboxylase. In the current study CBX217/218 had almost undetectable carboxylase activity. The mutations in this enzyme overlap the region identified as a binding site for both FLEELY and the propeptide. CBX217/218 may have lost the ability to bind propeptide and/or a Glu-containing peptide substrate, although we cannot rule out the possibility that CBX217/218 is not folded properly. The single mutants at residues 217 and 218 will warrant attention. CBX234/235, CBX359/360/361, CBX406/408, and CBX513/515 all maintain enzyme function albeit at reduced levels. By adjustment of the standard assay conditions these mutants can be induced to behave more like the wild-type enzyme. While we cannot ascribe specific functions to the mutated residues and some of these mutations may lead to local conformational changes in the enzyme, it is unlikely that they are globally misfolded.

| Substrate                  | Km gM | kcat s-1 | kcat/Km M·s-1 |
|---------------------------|-------|----------|---------------|
| FLAG-CBX                  | 74 ± 14.0 | 0.66 ± 0.12 | 8.9 × 10^2 |
| Vitamin KH2 (FLEELY)      | 97 ± 34.0 | 0.60 ± 0.06 | 6.2 × 10^2 |
| CBX234/235                | 43 ± 11.7 | 0.11 ± 0.05 | 2.6 × 10^2 |
| CBX359/360/361            | 53 ± 8.7  | 0.56 ± 0.06 | 1.1 × 10^4 |
| CBX406/408                | 32 ± 2.1  | 0.10 ± 0.02 | 3.1 × 10^4 |
| CBX513/515                | 4.0 ± 0.4 | 0.020 ± 0.001 | 5.0 × 10^4 |
| FLAG-CBX                  | 7.0 ± 1.0 | 0.033 ± 0.007 | 4.7 × 10^4 |
| Vitamin KH2 (proPT28)     | 3.8 ± 0.6 | 0.025 ± 0.007 | 6.6 × 10^4 |
| CBX234/235                | 12.3 ± 0.5 | 0.018 ± 0.001 | 1.5 × 10^5 |

These values are uncorrected for the presence of endogenous CO2.

Kinetic studies of CBX234/235, CBX406/408, and CBX513/515 suggest that they bind propeptide with lower affinity than wild-type enzyme. First, these mutant enzymes have slightly lowered affinity for FLEELY relative to wild-type enzyme. This defect can be overcome by increased concentration of propeptide. In addition, studies exploring proFIX18 stimulation of FLEELY carboxylation show that CBX234/235 and CBX406/408 require higher concentrations of proFIX18 for the stimulation than the wild-type enzyme. Second, they have decreased affinity for proPT28 compared with wild-type enzyme. A significant portion of the binding energy for proPT28 interaction with carboxylase is likely derived from the propeptide region, since the Km for proPT28, 2.2–3.6 gM (12, 31), is much lower than that for FLEELY, 0.7 gM (12), and carboxylation of proPT28 is inhibited by the prothrombin propeptide, proPT18, with a Ki of 3.5 gM (12). Wild-type carboxylase has a Km for a propeptide-containing substrate that is 500-fold lower than the Km for FLEELY; this decrease is associated with a 35-fold decrease in kcat. In contrast, for mutants CBX234/235, CBX406/408, and CBX513/515, kcat values for the propeptide-containing substrate relative to FLEELY decrease by 135-fold, 320-fold, and 103-fold, respectively, while the kcat values for these mutants decrease 10-fold, 17-fold, and 1.3-fold, leaving them all with kcat values higher than that for wild-type enzyme. Finally, it is interesting that epoxidase activities of the mutant enzymes are decreased in parallel with carboxylase activity.
activity, perhaps suggesting a requirement for propeptide binding for both epoxidation and carboxylation.

The behavior of CBX359/360/361 is distinct from that of CBX234/235, CBX406/408, and CBX513/515. CBX359/360/361 has a similar dependence on proFIX18 stimulation of FLEEL carboxylation as the wild-type enzyme. However, in the presence of saturating concentrations of proFIX18, CBX359/360/361 does not achieve the same catalytic rate for carboxylation as that of wild-type enzyme. This is in contrast to CBX234/235 and CBX406/408, which, at saturating concentrations of proFIX18, achieve the same catalytic rates for carboxylation as the wild-type enzyme. Since CBX359/360/361 appears to bind to the propeptide normally, there are two possible explanations for the defect in this mutant. Binding of propeptide may not lead to the alteration in the enzyme, most likely conformational, that leads to stimulation of carboxylation, or residues 359, 360, and/or 361 include important sites for catalysis directly.

In sum the pairwise or triple replacements of adjacent charged residues by alanine mutagenesis indicates lysine 217 or lysine 218 may be key for substrate and/or propeptide recognition or for catalysis. The basic triad arginine 359, histidine 360, lysine 361 in turn may be involved in secondary roles that affect $k_{cat}$. Single mutants will allow dissection of the contributions of individual residues to the properties of this unusual carboxylation/epoxidation catalyst.

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REFERENCES
1. Furie, B., and Furie, B. C. (1988) Cell 53, 505–518
2. Vermeer, C. (1990) Biochem. J. 266, 625–636
3. Manfredi, G., Brandolini, C., Avanzi, G., and Schneider, C. (1993) Mol. Cell. Biol. 13, 4976–4985
4. Stitt, T. N., Conn, G., Gore, M., Lai, C., Bruno, J., Rudzielewski, C., Mattsson, K., Fisher, J., Gies, D. R., Jones, P. F., Masiakowski, P., Ryan, T. E., Tobkes, N. J., Chen, D. H., DiStefano, P. S., Long, G. L., Basilico, C., Goldfarb, M., Lenke, G., Glass, D. J., and Yacopoulou, G. D. (1995) Cell 80, 661–670
5. Varnum, B. C., Young, C., Elliott, G., Garcia, A., Bartley, T. D., Fridell, Y.-W., Hunt, R. W., Trail, G., Clogston, C., Toso, R. J., Yanagihara, D., Bennett, L., Sylder, M., Merewether, L. A., Tseng, A., Escobar, E., Liu, E. T., and Yamane, H. K. (1995) Nature 373, 623–626
6. Ohashi, K., Nagata, K., Yoshida, J., Nakano, T., Arita, H., Tsuda, H., Suzuki, K., and Mizuno, K. (1995) J. Biol. Chem. 270, 22681–22684
7. Larson, A. E., Friedman, P. A., and Suttie, J. W. (1981) J. Biol. Chem. 256, 11032–11035
8. Morris, D. P., Soutie, B. A. M., Vermeer, C., and Stafford, D. W. (1993) J. Biol. Chem. 268, 5735–5742
9. Ham, S. W., and Dowd, P. (1990) J. Am. Chem. Soc. 112, 1660–1661
10. Jorgensen, M. J., Cantor, A. B., Furie, B. C., Brown, C. L., Shoemaker, C. B., and Furie, B. (1987) Cell 48, 185–191
11. Foster, D. C., Rudinski, M. S., Schach, B. G., Berkner, K. L., Kumar, A. A., Hagen, F. S., Sprecher, C. A., Insole, M. Y., and Davie, E. W. (1987) Biochemistry 26, 7003–7011
12. Ulrich, M. W., Furie, B., Jacobs, M. R., Vermeer, C., and Furie, B. C. (1988) J. Biol. Chem. 263, 9697–9702
13. Knobloch, J., and Suttie, J. W. (1987) J. Biol. Chem. 262, 15334–15337
14. Cheung, A., Engels, J. A., Sanders, C., and Suttie, J. W. (1989) Arch. Biochem. Biophys. 274, 574–581
15. Wu, S.-M., Morris, D. P., and Stafford, D. W. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 2236–2240
16. Kuliopulos, A., Cierzynski, C. E., Furie, B., Furie, B. C., and Walsh, C. T. (1992) Biochemistry 31, 9436–9444
17. Wu, S.-M., Cheung, W.-F., Frazier, D., and Stafforf, D. W. (1991) Science 254, 1634–1636
18. Kuliopulos, A., Roth, D. A., Wasley, L. C., Kuliopulos, A., Walsh, C. T., Furie, B. C., and Kaufman, R. J. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 4611–4615
19. Yamada, M., Kuliopulos, A., Nelson, N. P., Roth, D. A., Furie, B., Furie, B. C., and Walsh, C. T. (1995) Biochemistry 34, 481–489
20. Kuliopulos, A., Nelson, N. P., Yamada, M., Walsh, C. T., Furie, B., Furie, B. C., and Roth, D. A. (1994) J. Biol. Chem. 269, 23164–23170
21. Roth, D. A., Whirl, M. L., Velazques-Estades, L. J., Walsh, C. T., Furie, B., and Furie, B. C. (1995) J. Biol. Chem. 270, 5305–5311
22. Roth, D. A., Rehemtulla, A., Kaufman, R. J., Walsh, C. T., Furie, B., and Furie, B. C. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8372–8376
23. Sarkar, G., and Sommer, S. S. (1990) BioTechniques 8, 404–407
24. Kaufman, R. J., Davies, M. V., Wasley, L. C., and Michnicky, D. (1991) Nucleic Acids Res. 19, 4485–4490
25. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
26. Urlaub, G., and Chasin, L. A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4216–4220
27. Jones, J. P., Gardner, E. J., Cooper, T. G., and Olson, R. E. (1977) J. Biol. Chem. 252, 7738–7742
28. McGhee, J. J., and Suttie, J. W. (1983) J. Biol. Chem. 258, 12129–12131
29. Wood, G. M., and Suttie, J. W. (1988) J. Biol. Chem. 263, 3234–3239
30. Uotila, L. (1988) Arch. Biochem. Biophys. 264, 135–143
31. Hubbard, B. R., Jacobs, M., Ulrich, M. M., Walsh, C., Furie, B., and Furie, B. C. (1989) J. Biol. Chem. 264, 14145–14150
32. Bultenhuys, H. C., Soutie, B. A. M., and Vermeer, C. (1990) Biochim. Biophys. Acta 1034, 170–175
33. Soutie, B. A. M., Ulrich, M. M. W., Watson, D. J., Maddison, J. E., Ebbertink, R. H. M., and Vermeer, C. (1992) Thromb. Haemostasis 68, 521–525
34. Carlisle, T. L., and Suttie, J. W. (1980) Biochemistry 19, 1161–1167