Expression and Characterization of the Naturally Occurring Mutation L394R in Human γ-Glutamyl Carboxylase*

Received for publication, July 28, 2000
Published, JBC Papers in Press, August 8, 2000, DOI 10.1074/jbc.M006808200

Vasanth P. Mutucumarana‡, Darrel W. Stafford‡, Thomas B. Stanley‡‡, Da-Yun Jin‡, Jesus Solera†, Benjamin Brenner†, Robert Azerad**, and Sheue-Mei Wu‡‡†‡

From the ‡Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599-3280, *Department of Molecular Genetics, Hospital Universitario La Paz, Paseo de la Castellana 261, 28046 Madrid España, †Thrombosis and Hemostasis Unit, Institute of Hematology, Rambam Medical Center, P.O. Box 9602, Haifa 31096, Israel, and **Metabolisme et Biosynthese, Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, Université René Descartes, UFR Biomédicales des Saints-Pères, 45, Rue des Saints Pères, F-75270 Paris Cedex 06, France

Patients with mutation L394R in γ-glutamyl carboxylase have a severe bleeding disorder because of decreased biological activities of all vitamin K-dependent coagulation proteins. Vitamin K administration partially corrects this deficiency. To characterize L394R, we purified recombinant mutant L394R and wild-type carboxylase expressed in baculovirus-infected insect cells. By kinetic studies, we analyzed the catalytic activity of mutant L394R and its binding to factor IX's propeptide and vitamin KH₂. Mutant L394R differs from its wild-type counterpart as follows: 1) 110-fold higher Kᵦ for FIXproGla, an active site-specific, competitive inhibitor of FLEEL; 2) 30-fold lower Vₘₐₓ/Kₘ toward the substrate FLEEL in the presence of the propeptide; 3) severely reduced activity toward FLEEL carboxylation in the absence of the propeptide; 4) 7-fold decreased affinity for the propeptide; 5) 9-fold higher Kᵦ for FIXproGla, a substrate containing the propeptide and the Gla domain of human factor IX; and 6) 5-fold higher Kᵦ for vitamin KH₂. The primary defect in mutant L394R appears to be in its glutamate-binding site. To a lesser degree, the propeptide and KH₂ binding properties are altered in the L394R mutant. Compared with its wild-type counterpart, the L394R mutant shows an augmented activated FLEEL carboxylation by the propeptide.

Vitamin K-dependent carboxylase, also known as γ-glutamyl carboxylase, an integral membrane protein residing in the rough endoplasmic reticulum, catalyzes the posttranslational modification of specific glutamic acid residues to γ-carboxyglutamic acid (Gla) in vitamin K-dependent proteins (1). Gla-containing proteins are involved in blood coagulation (2), bone metabolism (3), and regulation of cell proliferation (4). The Gla domains of blood coagulation and anticoagulation proteins mediate calcium-dependent interactions between the protein and phospholipid membranes (5), a process necessary for the biological activity of these proteins. In addition to the glutamate substrate, γ-glutamyl carboxylation requires carbon dioxide, oxygen, and the essential cofactor vitamin K hydroquinone (KH₂), which is the reduced form of vitamin K (6). The formation of Gla from glutamate is coupled with the conversion of vitamin KH₂ to vitamin K 2,3-epoxide. Both of these activities occur in the vitamin K-dependent carboxylase (7, 8). The warfarin-sensitive microsomal enzyme vitamin K epoxide reductase recycles the epoxide back to vitamin KH₂ (9), thus completing the vitamin K cycle.

There have been only a few cases of combined deficiencies of vitamin K-dependent coagulation factors reported (10–18). Patients with this disorder suffer from a bleeding diathesis due to deficiencies of prothrombin and factors VII, IX, and X. In addition, the anticoagulation activities of proteins C and S are decreased. Brenner et al. (16) reported that patients' coagulation activities were severely reduced, while their antigen levels were only moderately decreased. Abnormality of the vitamin K epoxide reductase was ruled out because the patients had undetectable serum levels of vitamin K epoxide, and the hepatic vitamin K intake and skeletal development were both normal (18). Because the coagulation activities of these patients were partially corrected by administration of vitamin K and because prothrombin with impaired Gla-mediated calcium binding was identified, a defect in the vitamin K-dependent carboxylase was suspected (16). Genetic analysis of the proband (16) and her three affected siblings (18) revealed a mutation in the γ-glutamyl carboxylase gene (18). All were homozygous for a T → G point mutation in exon 9 of the carboxylase gene. This mutation results in the substitution of arginine for leucine at residue 394, a conserved residue found in the γ-glutamyl carboxylase of human (19), bovine (20), rat (21), Drosophila (22), mouse, whale, and toadfish (23). Furthermore, leucine 394 resides in a conserved region with 90% sequence identity spanning residues 374–405 (22, 23), which implies a functional or structural importance for this region. In this report, we compare the functional properties of the L394R mutant γ-glutamyl carboxylase to those of its wild-type counterpart. Our results indicate that the major defect of mutation L394R is at or near its glutamate-binding site. Furthermore, an augmented allosteric effect between the propeptide- and glutamate-binding sites is observed in the L394R mutant.

This paper is available on line at http://www.jbc.org

32572 This paper is available on line at http://www.jbc.org

This is an Open Access article under the CC BY license.
**EXPERIMENTAL PROCEDURES**

**Materials**—FLEEL was purchased from Bachem (Philadelphia, PA). L-α-Phosphatidylcholine (type V-E), CHAPS, and pepstatin were from Sigma. Vitamin K₁ was from Abbott. FIXproGla, a 59-residue recombinant peptide containing the propeptide and first 41 residues of the Gla domain of human factor IX, was prepared and purified as described (24). The peptide ProFIX19, which contains the sequence AVFLLHENANNKKLRNRPKH, was synthesized by Dr. Frank Church (University of North Carolina, Chapel Hill). The peptide Boc-mEEV was synthesized as described (25). NaH¹⁴CO₃ (specific activity, 56 mCi/mmol) was from ICN Pharmaceuticals (Costa Mesa, CA). Leupeptin, apropin, and phenylmethylsulfonyl fluoride were from Roche Molecular Biochemicals. The pSk vector was from Stratagene (La Jolla, CA). The pVL1392 vector was from Pharmingen (San Diego, CA). The BacVector 3000 insect cell DNA was from Novagen (Madison, WI). S9 (Spodoptera frugiperda) insect cells were obtained from the Lineberger Cancer Center at the University of North Carolina (Chapel Hill, NC). High Five (Trichoplusia ni) insect cells were provided by Dr. Thomas Kost of Glaxo Wellcome. HPC4 antibody affinity resin was provided by Dr. Charles Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). The anti-FLAG M2 monoclonal antibody and Met-FLAG-bacterial alkaline phosphatase were from Sigma. Peroxidase-conjugated goat anti-mouse immunoglobulin was from Jackson ImmunoResearch (West Grove, PA). ECL Western blotting detection reagents were from Amersham Pharmacia Biotech.

**Expression and Purification of Recombinant Wild-type and L394R Mutant Carboxylase**—The cDNA for human γ-glutamyl carboxylase (19), cloned into the pSk vector, was modified by site-specific mutagenesis to make the L394R mutant. Both wild-type and mutant constructs contain the FLAG epitope (DYKDDDK) attached to their amino termini and the HPC4 tag containing the sequence EDQVD-PRLIDGK (27) at their carboxyl termini. The engineered DNA constructs, coding for wild-type and mutant carboxylase, were subcloned into the pVL1392 vector, and the proteins were expressed in baculovirus-infected High Five cells as described (28).

Isolation of microsomes from High Five cells was performed as described (29) with minor modifications. Cells (8–10 × 10⁶) harvested from 1 liter of a culture were washed with 300 ml of cold buffer containing 25 mM Tris, pH 7.4, 150 mM NaCl, and 15% glycerol. The washed cell pellet was resuspended in 120 ml of cold buffer containing protease inhibitor mixture, which contains 2 mM dithiothreitol, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml apropin, and 0.1 mg/ml phenylmethylsulfonyl fluoride, and disrupted by sonication (with 80 pulses, 1.5 s each, using a Heat Systems XL2020 sonicator at a power output of 6). The homogenate was centrifuged at 4300 × g avg at 4 °C for 15 min. The supernatant was recovered and centrifuged at 150,000 × g avg for 1 h at 4 °C. Solubilization of the microsomal pellet and the subsequent purification of carboxylase using the HPC4 antibody affinity resin was performed as described (28).

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis**—Purified carboxylase was analyzed by silver-stained SDS-polyacrylamide gel electrophoresis (10% polyacrylamide gels; Bio-Rad) and by Western blot analysis. For Western blot analysis, the proteins transferred to a polyvinylidene difluoride membrane were probed with the anti-FLAG M2 monoclonal antibody (1.4 μg/ml) and then with the peroxidase-conjugated secondary antibody (0.07 μg/ml). The FLAG-tag-containing proteins were detected by chemiluminescence following incubation of the membranes with ECL reagents (Amersham Pharmacia Biotech) and autoradiography on Hyperfilm (Amersham Pharmacia Biotech). The wild-type and mutant carboxylases were quantitated by dot blot analysis on polyvinylidene difluoride membranes using known amounts of Met-FLAG-bacterial alkaline phosphatase as standards. The FLAG-tag-containing carboxylase and the standard were detected using anti-FLAG M2 antibody. Quantification was made based on the analysis of autoradiographs using an ImageQuant densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

**Carboxylase Activity Assays**—The in vitro carboxylase activity assays were performed as described (24) with minor modifications. Reactions were performed at 20 °C in 25 mM MOPS, pH 7.4, 500 mM NaCl, 0.16% phosphatidylcholine, 0.16% CHAPS, 222 μM vitamin K₁, 1.4 mM NaH¹⁴CO₃ (10 μCi), and various concentrations of FLEEL or FIXproGla. In FLEEL carboxylation assays, ProFIX19 at 5 μM was also included.

**Vitamin K Epoxidease Activity Assay**—Carboxylations of FLEEL in the presence of 5 μM ProFIX19 were performed as described above, except that unlabeled NaHCO₃ was used. FLEEL concentrations of 1 and 12 mM were used in the wild-type and mutant reactions, respectively.

**RESULTS**

**Expression of the Recombinant Wild-type and L394R Mutant Carboxylase**—The recombinant wild-type and L394R mutant carboxylase, expressed in baculovirus-infected High Five cells, contain FLAG tags at their amino termini and HPC4 tags at their carboxyl termini. These epitopes, which lie outside the enzymatic sites, are not expected to interfere with the assay. The recombinant wild-type and L394R mutant carboxylase of high purity (Fig. 1). The estimated molecular mass of the proteins is around 95 kDa, similar to that reported for the purified carboxylase (32). Western blot analysis revealed a single antibody-reactive band at 95 kDa in both the wild-type and mutant carboxylase preparations (Fig. 1) and indicates the absence of proteolysis in either preparation.
binding properties of mutant L394R, we used the active site-specific, competitive inhibitor Boc-mEEV (25). As shown in Fig. 4, by fitting the data into the competitive inhibition equation, the $K_i$ of Boc-mEEV is 0.013 ± 0.002 mM for wild-type carboxylase compared with 1.44 ± 0.16 mM for mutant L394R. These results revealed a 110-fold difference in the apparent affinities of wild type and L394R for Boc-mEEV.

**Vitamin K Epoxide Formation**—The rate of vitamin K epoxide formation was 20.3 ± 0.5 pmol of KO/min/pmol of mutant L394R vs. 36.6 ± 3.9 pmol of KO/min/pmol of wild-type carboxylase. This 1.8-fold reduction in epoxide formation observed in mutant L394R compared with the wild type carboxylase parallels the 2.5-fold difference observed in FLEEL carboxylation, suggesting that the L394R mutant’s carboxylase and epoxidase activities remain coupled (8, 36).

**Carboxylation of FIXproGla**—FIXproGla, a peptide substrate containing the propeptide covalently linked to the Gla domain of human factor IX, mimics the physiological substrate of vitamin K-dependent carboxylase (24, 35). Carboxylation of FIXproGla by the wild-type and mutant L394R carboxylase follows Michaelis-Menten kinetics. The $K_m$ of FIXproGla by wild-type carboxylase was 0.23 ± 0.03 μM, which is 3 orders of magnitude lower than that for FLEEL and is in agreement with previous reports (24, 37, 38). As shown in Fig. 5 and Table I, the $K_m$ of FIXproGla for mutant L394R was 2.08 ± 0.23 μM for mutant L394R, which is 9-fold higher than that of the wild-type carboxylase. Interestingly, the $V_{max}$ of FIXproGla carboxylation by mutant L394R was 2-fold higher than that by the wild-type carboxylase.

**Inhibition of FIXproGla Carboxylation by Free Propeptide**—The propeptide of factor IX has been shown to be a competitive inhibitor of FIXproGla carboxylation (28, 35). To obtain a better estimate of their relative affinities for the propeptide, we compared the $K_i$ values of the wild type and L394R mutant for the inhibition of FIXproGla carboxylation by ProFIX19. The $K_i$ values determined for wild-type and mutant L394R carboxylases were 63 ± 13 and 459 ± 28 mM, respectively (Fig. 6). This observed decrease in the relative affinity toward ProFIX19 by the mutant L394R agrees with our results obtained from studies of ProFIX19 inhibition of FLEEL carboxylation.

**Effect of ProFIX19 on FLEEL Carboxylation—**The free propeptide of the vitamin K-dependent coagulation proteins has been shown to enhance the rate of carboxylation of small glutamate-containing peptide substrates that lack a covalently linked propeptide sequence (33, 34). We therefore used ProFIX19, the propeptide sequence of factor IX, to compare its effect on the carboxylation of FLEEL by the wild-type and mutant carboxylases. In the absence of the free propeptide, the wild-type carboxylase exhibits significant activity toward FLEEL carboxylation. As shown in Fig. 2, the rate of FLEEL carboxylation by the wild-type carboxylase increased 5–6-fold when saturating amounts of ProFIX19 were added. In contrast, mutant L394R showed almost undetectable activity toward FLEEL in the absence of the free propeptide. However, this deficiency can be ameliorated by adding ProFIX19. Compared with its wild-type counterpart, a higher concentration of ProFIX19 was required to achieve maximal stimulation of FLEEL carboxylation by mutant L394R. All kinetic constants were derived from the Michaelis-Menten equation using data below the inhibitory concentrations of ProFIX19 (Table I). As shown in Fig. 3, substrate inhibition was apparent at high concentrations of FLEEL. This phenomenon was previously seen in the purified bovine carboxylase (8). Inhibition occurred when FLEEL concentrations were greater than 9 mM for the wild type and 18 mM for the mutant L394R. All kinetic constants were derived from the Michaelis-Menten equation using data below the inhibitory concentrations of FLEEL. Comparison of $V_{max}$ for FLEEL carboxylation showed that the reaction rate of mutant L394R was 2.5-fold slower than that of the wild-type (Fig. 3, Table I). The $K_m$ of FLEEL for mutant L394R is 6.49 ± 0.72 mM, which is 12-fold higher than that of the wild-type (0.54 ± 0.10 mM). It was impossible to determine the $K_m$ of FLEEL for mutant L394R in the absence of the propeptide, even when FLEEL concentrations were increased up to 60 mM, because its activity is almost undetectable under such conditions.

**Active Site Inhibition Studies**—To examine the glutamate domain of human factor IX, mimics the physiological substrate of vitamin K-dependent carboxylase (24, 35). Carboxylation of FIXproGla, a peptide substrate containing the propeptide covalently linked to the Gla domain of human factor IX, mimics the physiological substrate of vitamin K-dependent carboxylase (24, 35). Carboxylation of FIXproGla by the wild-type and mutant L394R carboxylase follows Michaelis-Menten kinetics. The $K_m$ of FIXproGla by wild-type carboxylase was 0.23 ± 0.03 μM, which is 3 orders of magnitude lower than that for FLEEL and is in agreement with previous reports (24, 37, 38). As shown in Fig. 5 and Table I, the $K_m$ of FIXproGla for mutant L394R was 2.08 ± 0.23 μM for mutant L394R, which is 9-fold higher than that of the wild-type carboxylase. Interestingly, the $V_{max}$ of FIXproGla carboxylation by mutant L394R was 2-fold higher than that by the wild-type carboxylase.

**Effect of ProFIX19 on FLEEL Carboxylation—**The free propeptide of the vitamin K-dependent coagulation proteins has been shown to enhance the rate of carboxylation of small glutamate-containing peptide substrates that lack a covalently linked propeptide sequence (33, 34). We therefore used ProFIX19, the propeptide sequence of factor IX, to compare its effect on the carboxylation of FLEEL by the wild-type and mutant carboxylases. In the absence of the free propeptide, the wild-type carboxylase exhibits significant activity toward FLEEL carboxylation. As shown in Fig. 2, the rate of FLEEL carboxylation by the wild-type carboxylase increased 5–6-fold when saturating amounts of ProFIX19 were added. In contrast, mutant L394R showed almost undetectable activity toward FLEEL in the absence of the free propeptide. However, this deficiency can be ameliorated by adding ProFIX19. Compared with its wild-type counterpart, a higher concentration of ProFIX19 was required to achieve maximal stimulation of FLEEL carboxylation by mutant L394R. All kinetic constants were derived from the Michaelis-Menten equation using data below the inhibitory concentrations of ProFIX19 (Table I). As shown in Fig. 3, substrate inhibition was apparent at high concentrations of FLEEL. This phenomenon was previously seen in the purified bovine carboxylase (8). Inhibition occurred when FLEEL concentrations were greater than 9 mM for the wild type and 18 mM for the mutant L394R. All kinetic constants were derived from the Michaelis-Menten equation using data below the inhibitory concentrations of FLEEL. Comparison of $V_{max}$ for FLEEL carboxylation showed that the reaction rate of mutant L394R was 2.5-fold slower than that of the wild-type (Fig. 3, Table I). The $K_m$ of FLEEL for mutant L394R is 6.49 ± 0.72 mM, which is 12-fold higher than that of the wild-type (0.54 ± 0.10 mM). It was impossible to determine the $K_m$ of FLEEL for mutant L394R in the absence of the propeptide, even when FLEEL concentrations were increased up to 60 mM, because its activity is almost undetectable under such conditions.

**Active Site Inhibition Studies**—To examine the glutamate
300 times (compared with 5–6 times for wild-type carboxylase). Nevertheless, primarily because of its 12-fold increased $K_m$ the catalytic efficiency ($V_{\text{max}}/K_m$) of L394R toward the small sub-

| Substrate | Enzyme     | $K_m$ | $V_{\text{max}}$ | $V_{\text{max}}/K_m$ |
|-----------|------------|-------|-------------------|----------------------|
| FLEEL     | Wild type  | 541 ± 97 | 446 ± 73 | 0.87 ± 0.19 |
|           | L394R      | 6490 ± 724 | 178 ± 15  | 0.028 ± 0.005 |
| FIXproGla | Wild type  | 0.23 ± 0.03 | 47 ± 2 | 205 ± 18 |
|           | L394R      | 2.08 ± 0.23 | 98 ± 20  | 48 ± 9 |
| Vitamin KH$_2$ | Wild type | 7.0 ± 1.2 | 42 ± 3 | 6.1 ± 0.8 |
|           | L394R      | 32.8 ± 5.4 | 78 ± 8  | 2.3 ± 0.5 |

$^a$ Determined at 5 μM ProFIX19.

$^b$ Determined at 1.2 and 10.0 μM FIXproGla in wild-type and mutant enzyme reactions, respectively.

FLEEL (mM)

**Fig. 3.** Carboxylation of FLEEL. Carboxylation of FLEEL, measured by $^{14}$CO$_2$ incorporation, was performed in the presence of 5 μM ProFIX19, a concentration that maximally stimulates both wild type and mutant L394R. Concentrations of FLEEL between 0 and 12 mM were used for the wild-type carboxylase (filled squares) and between 0 and 24 mM for mutant L394R (filled circles). The $K_m$ is $0.54 ± 0.10$ mM for the wild-type carboxylase and $6.49 ± 0.72$ mM for mutant L394R.

[Graph: FLEEL (mM)]

Boc-mEEV (mM)

**Fig. 4.** Inhibition of FLEEL carboxylation by the active site specific inhibitor Boc-mEEV. The relative rates ($V_{i}/V_{o}$) of FLEEL carboxylation for the wild-type carboxylase (filled squares) and mutant L394R (filled circles) are plotted versus the concentration of Boc-mEEV. $V_{i}$ is $^{14}$CO$_2$ incorporation in the presence of Boc-mEEV, and $V_{o}$ is $^{14}$CO$_2$ incorporation at a given concentration of Boc-mEEV. The reactions were performed at 5 μM ProFIX19 with 0.8 mM FLEEL (1.5-fold $K_m$) for the wild-type carboxylase or with 10.0 μM FLEEL (1.5-fold $K_m$) for mutant L394R. The $K_i$ determined is $0.013 ± 0.002$ and $1.44 ± 0.16$ mM for the wild type and mutant L394R, respectively.

[Graph: Boc-mEEV (mM)]

FIXproGla (μM)

**Fig. 5.** Carboxylation of FIXproGla. Carboxylation of FIXproGla is measured by $^{14}$CO$_2$ incorporation. FIXproGla concentrations between 0 and 4.8 μM were used for wild-type carboxylase (filled squares) and between 0 and 12.0 μM for mutant L394R (filled circles). The $K_m$ is $0.23 ± 0.03$ μM for the wild-type carboxylase and $2.08 ± 0.23$ μM for mutant L394R.

[Graph: FIXproGla (μM)]

ProFIX19 (μM)

**Fig. 6.** Inhibition of FIXproGla carboxylation by ProFIX19. The relative rate ($V_{i}/V_{o}$) of FIXproGla carboxylation at ProFIX19 concentrations between 0 and 10 μM is shown for the wild-type carboxylase (filled squares) and mutant L394R (filled circles). $V_{o}$ is $^{14}$CO$_2$ incorporation in the absence of ProFIX19, and $V_{i}$ is $^{14}$CO$_2$ incorporation at a given concentration of ProFIX19. The reactions were performed at 0.5 μM FIXproGla (2-fold $K_m$) for the wild-type carboxylase and 4.0 μM FIXproGla (2-fold $K_m$) for mutant L394R. The $K_i$ determined is $63 ± 13$ and $459 ± 28$ mM for the wild type and mutant L394R, respectively.

[Graph: ProFIX19 (μM)]

300 times (compared with 5–6 times for wild-type carboxylase). Nevertheless, primarily because of its 12-fold increased $K_m$, the catalytic efficiency ($V_{\text{max}}/K_m$) of L394R toward the small sub-
Mutation L394R in Human γ-Glutamyl Carboxylase

394 to arginine results in a striking defect in the catalytic efficiency of the carboxylase toward the small substrate FLEEL.

In contrast to FLEEL, the more physiological substrate, FIXproGla, which has 12 Glu residues, has a slightly higher (~2-fold) $V_{\text{max}}$ and a 9-fold higher $K_m$ for the mutant enzyme than for the wild-type γ-glutamyl carboxylase. Other carboxylase mutations with higher $V_{\text{max}}$ and $K_m$ have been reported (39).

The increased $V_{\text{max}}$ for the mutant is consistent with our previous suggestions that product release is the rate-limiting step for propeptide-containing substrates (the propeptide of the vitamin K-dependent proteins is the primary binding site for the carboxylase-substrate interaction (35, 40)) and that all carboxylations occur during a single binding event (41). Since the assay measures only CO$_2$ incorporation and not complete carboxylation of a 12-Glu substrate, any decrease in peptide affinity can increase the $V_{\text{max}}$ due to an increased off-rate, and may also result in undercarboxylated products. For L394R, FLEEL’s $V_{\text{max}}$ is slower because CO$_2$ incorporation, rather than off-rate, is the rate-limiting step.

L394R also has smaller but significant effects on the propeptide and vitamin K interactions. The propeptide concentration required for half-maximal stimulation of FLEEL carboxylation was 15-fold higher, while the $K$ for the inhibition of CO$_2$ incorporation into FIXproGla by FIX’s propeptide was 7-fold higher for L394R than for the wild-type enzyme. L394R’s $K_m$ for vitamin K was also 5-fold higher than that of the wild-type enzyme. These results seem consistent with previous studies suggesting that the propeptide, vitamin K, and glutamate binding site are functionally linked (33, 34, 42). Thus, while the primary defect in L394R is glutamate substrate binding, linkage of this site to the other substrate sites results in a complex, multifaceted effect on enzyme activity.

There are several features of this mutation that make it interesting. First, the mutated residue is one of 20 contiguous amino acids that are identical in human (19), bovine (20), rat (21), Drosophila (22), mouse, whale, toadfish, and chicken (23). Second, the mutation is a drastic one, a hydrophobic residue to a charged residue. This almost certainly means that the mutation is a surface residue. If it were not, we would expect a very unstable protein with little activity. However, mutant L394R is stable and, under appropriate conditions, has substantial activity. Surface hydrophobic amino acids are often found to be important for protein-protein or protein-substrate interactions (43); therefore, it is likely that leucine 394 is a surface residue that contributes to substrate binding. Thus, although not previously implicated in glutamate binding (44), we postulate that this region of the carboxylase is a functionally important part of the glutamate-binding site.

The most interesting question, then, is how to relate our observations on the purified carboxylases to the symptoms of the patients carrying this mutation L394R. The original reports (16, 18) demonstrated that vitamin K administration partially ameliorated the patients’ bleeding problems. Therefore, we originally hypothesized that the defect in L394R is in the vitamin K interaction. As described here, however, reduced interactions with vitamin K are apparently not the principal defect. As mentioned above, it has been shown that the propeptide of the vitamin K-dependent proteins provides the primary binding site for the enzyme-substrate interaction (35, 40). We have also shown that patients who synthesize carboxylation-deficient factor IX, when the vitamin K concentration is reduced, have a factor IX propeptide with reduced affinity for the γ-glutamyl carboxylase (45). Furthermore, carboxylation appears to occur processively and, during a single binding event, modifies all of the glutamic acids that will be carboxylated (41). Thus, anything that reduces the affinity of the substrate for the carboxylase will result in a shorter residence time of the substrate on the carboxylase, resulting in its more rapid release and leading, potentially, to partially carboxylated, inactive products. Similarly, anything that decreases the rate of CO$_2$ incorporation may result in the normal residence time being insufficient for complete carboxylation before the product is released. Thus, any mutation that reduces the affinity of the substrate for γ-glutamyl carboxylase or reduces the rate of CO$_2$ incorporation has the potential to result in undercarboxylated products.

To understand how the effects listed above might explain the therapeutic efficacy of vitamin K, we examined the effect of vitamin K on the rate of carboxylation at a low FIXproGla concentration, near the $K_m$ of the FIXproGla substrate for the wild-type enzyme. It is often assumed that for maximum control of rates, substrates are present in vivo at concentrations near their $K_m$. Results (Fig. 8) show that, in contrast to high FIXproGla concentrations, L394R has a lower $V_{\text{max}}$ than wild-
type carboxylase at lower concentrations of substrate, but the activity is increased from a low level to approximately 33% of wild type by increasing vitamin K. Based on our results, we conclude that the effect of vitamin K is merely an increased rate of carboxylation, driving the processive carboxylation of the vitamin K-dependent protein toward completion. After all, vitamin K is the only substrate whose

Thus, the defect can be explained by a reduced rate of carboxylation, which is a result of a defective glutamate binding site and increased Km for vitamin K. This effect would be exacerbated by the apparent decreased affinity of the carboxylase for the propeptide, resulting in a reduced residence time for the substrate on the carboxylase. The combination of these two effects would result in partially carboxylated vitamin K-dependent proteins.

In summary, we have presented a plausible scenario to explain the amelioration of the patients’ symptoms in response to vitamin K therapy. Our data show that the L394R mutant γ-glutamyl carboxylase has an impaired glutamate-binding site that is more pronounced in the absence of the propeptide. We also note that, to a lesser degree, the mutation affects the propeptide and KH2 binding properties of the enzyme. These apparent defects in binding propeptide and KH2 probably arise because of linkage between different binding sites on the carboxylase.

Acknowledgments—We thank Dr. D. L. Straight and Dr. H. H. Thijssen for critically reviewing the manuscript and Jonathan D’Amore and Dr. Laura Gabiger for editorial assistance.

REFERENCES
1. Suttie, J. W. (1985) Annu. Rev. Biochem. 54, 459–477
2. Suttie, J. W. (1980) CRC Crit. Rev. Biochem. 8, 191–223
3. Price, P. A. (1988) Annu. Rev. Nutr. 8, 565–583
4. Manfioletti, G., Brancolini, C., Avanzi, G., and Schneider, C. (1993) Mol. Cell. Biol. 13, 4976–4985
5. Nelsestuen, G. L. (1976) J. Biol. Chem. 251, 5648–5656
6. Esmon, C. T., Sadowiski, J. A., and Suttie, J. W. (1975) J. Biol. Chem. 250, 4744–4748
7. Wallin, R., and Suttie, J. W. (1982) Arch. Biochem. Biophys. 214, 155–163
8. Morris, D. P., Soute, B. A., Vermeer, C., and Stafford, D. W. (1993) J. Biol. Chem. 268, 8735–8742
9. Faese, M. J., Hildebrandt, E. F., and Suttie, J. W. (1982) J. Biol. Chem. 257, 11210–11212
10. McMillan, C. W., and Roberts, H. R. (1986) N. Engl. J. Med. 274, 1313–1315
11. Chung, K. S., Bezaud, A., Goldsmith, J. C., McMillan, C. W., Menache, D., and Roberts, H. R. (1979) Blood 53, 776–787
12. Johnson, C. A., Chung, K. S., McGrath, K. M., Bean, P. E., and Roberts, H. R. (1980) Br. J. Haematol. 44, 461–469
13. Goldsmith, G. H., Jr., Pence, R. E., Ratnoff, O. D., Adelstein, D. J., and Furie, B. (1982) J. Clin. Invest. 69, 1253–1260
14. Vicente, V., Maia, R., Alberca, I., Tamagnini, G. P., and Lopez Borrasca, A. (1984) Thromb. Haemostasis 51, 343–346
15. Paul, R. M., Lian, J. B., Mosher, D. F., and Suttie, J. W. (1987) Am. J. Hum. Genet. 41, 566–583
16. Brenner, B., Tavoni, S., Zivelin, A., Keller, C. B., Suttie, J. W., Tatarsky, I., and Seligsohn, U. (1990) Br. J. Haematol. 75, 537–542
17. Pechlaner, C., Vogel, W., Erhardt, R., Pumpel, E., and Kunz, F. (1992) Thromb. Haemostasis 68, 617
18. Brenner, B., Sanchez-Vega, B., Wu, S. M., Banir, N., Stafford, D. W., and Solera, J. (1998) Blood 92, 4544–4559
19. Wu, S. M., Cheng, W. F., Frazier, D., and Stafford, D. W. (1991) Science 254, 1634–1636
20. Romer, E. E., Deo, R., Velazquez-Estades, L. J., and Roth, D. A. (1998) Biochem. Biophys. Res. Commun. 248, 783–789
21. Li, T., Yang, C. T., Jin, D., and Stafford, D. W. (2000) J. Biol. Chem. 275, 18291–18296
22. Begley, G. S., Furie, B. C., Czerwiec, E., Taylor, K. L., Furie, G. L., Bronstein, L., Stenflo, J., and Furie, B. (July 12, 2000) J. Biol. Chem. 10.1074/jbc.M003944200
23. Wu, S. M., Soute, B. A., Vermeer, C., and Stafford, D. W. (1990) J. Biol. Chem. 265, 13124–13129
24. Azrad, R., Decottignies-Le Maréchal, P., Ducrocq, C., Rigini-Tapia, A., Vidal-Cros, A., Bory, S., Dubois, J., Gaudry, M., and Marquet, A. (1988) Current Advances in Vitamin K Research (Suttie, J. W., ed) pp. 17–23, Elsevier, New York
25. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
26. Stearns, D. J., Kurosawa, S., Sims, P. J., Esmon, N. L., and Esmoun, C. T. (1988) J. Biol. Chem. 263, 826–832
27. Stanley, T. B., Jin, D. Y., Lin, P. J., and Stafford, D. W. (1999) J. Biol. Chem. 274, 16940–16944
28. Wu, S. M., Mutucumurana, V. P., and Stafford, D. W. (1997) Methods Enzymol. 282, 346–357
29. Thijssen, H. H., Janssen, C. A., and Dritti-Rejinders, M. J. (1986) Biochem. Pharmacol. 35, 3277–3282
30. Soute, B. A., Acher, F., Azrad, R., and Vermeer, C. (1990) Biochim. Biophys. Acta 1034, 11–16
31. Wu, S. M., Morris, D. P., and Stafford, D. W. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2236–2240
32. Knobloch, J. E., and Suttie, J. W. (1987) J. Biol. Chem. 262, 15334–15337
33. Cheung, A., Engelke, J. A., Sanders, C., and Suttie, J. W. (1989) Arch. Biochem. Biophys. 274, 574–581
34. Stanley, T. B., Wu, S. M., Houben, R. J., Mutucumurana, V. P., and Stafford, D. W. (1998) Biochemistry 37, 13262–13268
35. Wood, G. M., and Suttie, J. W. (1988) J. Biol. Chem. 263, 3234–3239
36. Hubbard, B. R., Jacobs, M., Ulrich, M. M., Walsh, C., Furie, B., and Furie, B. C. (1989) J. Biol. Chem. 264, 14145–14150
37. Ulrich, M. M., Furie, B., Jacobs, M. R., Vermeer, C., and Furie, B. C. (1988) J. Biol. Chem. 263, 9697–9702
38. Sugiura, I., Furie, B., Walsh, C. T., and Furie, B. C. (1996) J. Biol. Chem. 271, 17837–17844
39. Jorgensen, M. J., Cantor, A. B., Furie, B. C., Brown, C. L., Shoemaker, C. B., and Furie, B. (1987) Cell 48, 185–191
40. Morris, D. P., Stevens, R. D., Wright, D. J., and Stafford, D. W. (1995) J. Biol. Chem. 270, 30491–30498
41. Soute, B. A., Ulrich, M. M., Watson, A. D., Maddison, J. E., Ebbenkier, R. H., and Vermeer, C. (1992) Thromb. Haemostasis 68, 521–525
42. Clackson, T., Utsch, M. H., Wells, J. A., and de Vos, A. M. (1998) J. Mol. Biol. 277, 1111–1128
43. Kuliopulos, A., Nelson, N. P., Yamada, M., Walsh, C. T., Furie, B., Furie, B. C., and Roth, D. A. (1994) J. Biol. Chem. 269, 21364–21370
44. Chu, K., Wu, S. M., Stanley, T., Stafford, D. W., and High, K. A. (1996) J. Clin. Invest. 98, 1619–1625