The Propeptide of the Vitamin K-Dependent Carboxylase Substrate Accelerates Formation of the γ-Glutamyl Carbanion Intermediate†

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ABSTRACT: Vitamin K-dependent carboxylase catalyzes the post-translational γ-carboxylation of 9−12 glutamyl residues of several blood coagulation proteins. Carboxylase purified from Chinese hamster ovary (CHO) cells as a recombinant FLAG−carboxylase fusion protein [Sugiura, I., et al. (1996) *J. Biol. Chem. 271*, 17837−17844] was utilized with pentapeptide substrate FL[^{3}H-*R*,*S*]EAL with high specific radioactivity to probe the timing of glutamyl $C_\gamma-{}^3H$ cleavage relative to $C_\gamma-COO^-$ bond formation by $^{14}CO_2$ incorporation rates. Studies were conducted over a range of $NaH^{14}CO_3$ concentrations to assess uncoupling of γ-glutamyl carbanion formation and over a range of concentrations of ProPT18, the 18-residue peptide corresponding to the −18 to −1 propeptide region of prothrombin known to affect the catalytic efficiency of carboxylase. At saturation, ProPT18 accelerates $C_\gamma-{}^3H$ cleavage 11−13-fold and $C_\gamma-{}^{14}CO_2^-$ formation 6−7-fold, converting a $C_\gamma-{}^3H$ cleavage/$C_\gamma-{}^{14}CO_2^-$ formation ratio of 1.2−1.4 in the absence of ProPT18 to 2.3−2.8 in its presence, a relative increase in and uncoupling of $C_\gamma-{}^3H$ cleavage from C−C bond formation. When the HCO_3^- concentration was varied, the $V/K_{^3H^+}/V/K_{^{14}CO_2}$ ratios rose as HCO_3^- fractional saturation dropped to a ratio of 9.3−10.8/1 at low bicarbonate, indicating an uncoupling of nine out of ten γ-glutamyl carbanion formations from carboxylative capture, consistent with prior reports on microsomal enzyme [Larson, A. E., et al. (1981) *J. Biol. Chem. 256*, 11032−11035]. These results with pentapeptide substrate FLEAL validate reversible γ-glutamyl carbanion formation by pure carboxylase and indicate the ProPT18 increase in catalytic efficiency is in selective lowering of an energy barrier preceding the γ-glutamyl carbanion intermediate.

Vitamin K-dependent γ-glutamyl carboxylase is an intrinsic endoplasmic reticular membrane protein that catalyzes the post-translational modification of 9−12 glutamyl residues to γ-carboxyl glutamyl residues (Gla)[1] in a specific set of substrate proteins, including blood coagulation factor VII, factor IX, and factor X and prothrombin. These Gla-containing proteins act as proteases, and/or regulators, in the blood clotting cascade in which Ca^{2+} ligation to the bidentate side chains of Gla residues stabilizes a conformation in the Gla domains that is required for high-affinity binding to cell membranes (Furie & Furie, 1988; Mann et al., 1988). Most recently, one Gla-containing protein, Gas6, has been identified as a ligand for Axl, a receptor tyrosine kinase (Varnum et al., 1995), suggesting a role for Gla and vitamin K in signaling transduction processes. Both the bovine and human carboxylases have been cloned (Wu et al., 1991; Rehemtulla et al., 1993) and expressed in insect and mammalian cell lines, and mutational analysis on the recombinant bovine enzyme has been carried out (Roth et al., 1993, 1995; Sugiura et al., 1996). The γ-glutamyl carboxylase is unique among CO_2-fixing enzymes in several respects. In addition to its obligate requirement for vitamin K, in the form of reduced dihydronaphthoquinol, KH_2, the enzyme requires molecular O_2 for each carboxylation event. The O_2 is reductively activated and fragmented in each turnover, yielding H_2O and vitamin K 2,3-epoxide as products, a net KH_2 epoxidase activity (Scheme 1). A second unusual feature of the carboxylase is its mechanism of substrate recognition. A propeptide region N-terminal to the Gla domain in the precursor forms of substrate proteins serves as a docking element in directing efficient carboxylation (Jorgensen et al., 1987; Ulrich et al., 1988). The K_m of carboxylase with ProPT28, a synthetic substrate containing both the propeptide region (−18 to −1) and a portion of the glutamyl substrate sequence (+1 to +10) of prothrombin, is 3 orders of magnitude lower than the K_m with the 10-amino acid peptide containing only the substrate sequence (Hubbard et al., 1989). The rate of carboxylation of ProPT28 containing the prothrombin propeptide is 2−3-fold greater than that of pro-FIX28 containing the factor IX propeptide (Hubbard et al., 1989). Binding sites for both the pentapeptide substrate, FLEEL (Kuliopulos et al., 1992a), and the factor IX propeptide (Yamada et al., 1995) have been localized to the first 250 amino acids of the 758-residue enzyme, while

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[1] Abbreviations: CHO, Chinese hamster ovary; FLEEL, Phe-Leu-Glu-Glu-Leu; FLEAL, Phe-Leu-Glu-Ala-Leu; FLγAL, Phe-Leu-Gla-Ala-Leu; Gla, γ-carboxyglutamic acid; ProPT18, synthetic peptide corresponding to human proprothrombin residues −18 to −1 (His-Val-Phe-Leu-Ala-Pro-Gln-Gln-Ala-Arg-Ser-Leu-Leu-Gln-Arg-Val-Arg-Arg); KH_2, vitamin K hydroquinone; KO, vitamin K2,3-epoxide; FLAG peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys.

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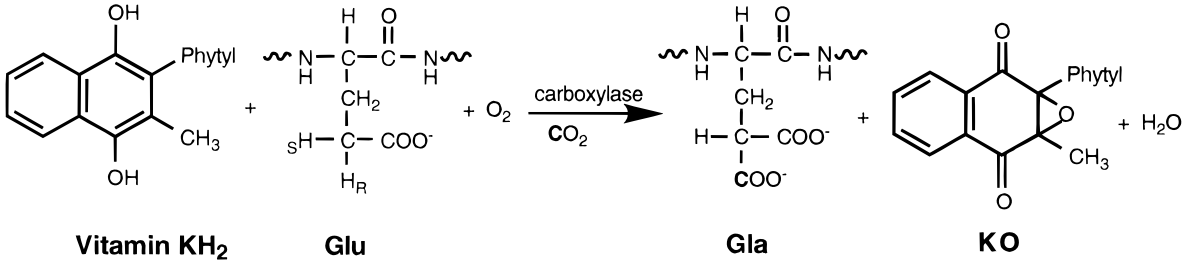
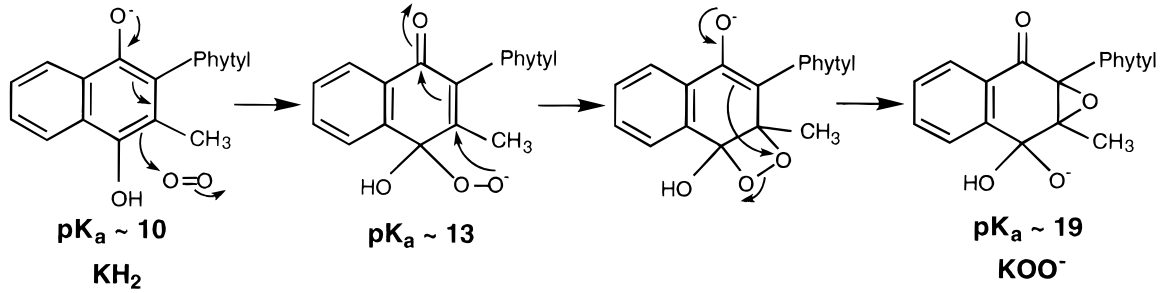
Scheme 1



Scheme 2



truncation of the C-terminal 186 residues (Roth et al., 1995) abrogates epoxidase activity.

In carrying out the iterative post-translational carboxylation of 9−12 glutamyl residues (Maréchal et al., 1984), the carboxylase must effect and coordinate several catalytic steps: the activation of O_2 and vitamin K epoxide formation, cleavage of the $C_\gamma-H_s$ bond at each glutamyl residue, and formation of the $C_\gamma-COO^-$ carbon−carbon bond in the CO_2 fixation step. Substantial insight into the role for a KH_2 epoxidation in the conversion of Glu to Gla has been provided in recent years by Dowd et al. (1991, 1995), who devised model chemistry to support a pathway for KH_2 reaction with oxygen to yield the epoxy vitamin K hydrate, KOO^-. The alkoxide ion of the KOO^- provides a base that is sufficiently strong to abstract the $C_\gamma-H_s$ hydrogen to initiate the carboxylation sequence (Scheme 2). The predications of this proposal are that the carboxylase is a KH_2 dioxygenase where some of the flux would yield KO with both atoms of O_2 retained, that the carboxylation sequence proceeds through a γ-glutamyl carbanion intermediate or transition state, and that carbanion formation occurs after the O_2-reducing step. Both the Dowd group and ours (Dowd et al., 1992; Kuliopulos et al., 1992b) have provided support for the dioxygenase pathway with $^{18}O_2$ incorporation studies. The uncoupling of KO formation from $C-COO^-$ bond formation is consistent with early data from Larson et al. (1981) that demonstrated a variation in the ratio of K epoxide formed/CO_2 fixed with sodium bicarbonate concentration. At low concentrations of sodium bicarbonate (0.35 mM), the K epoxide/CO_2 fixed ratio was 9.7/1. As sodium bicarbonate levels were increased ($\geq$6 mM), the KO/CO_2 fixed ratio approached 1/1. When CO_2 is scarce, about 10 O_2 molecules are consumed for each CO_2 covalently attached to γ-glutamyl carbon, consonant with the above mechanism where KOO^- formation must precede CO_2 fixation.

Evidence for a γ-glutamyl carbanion intermediate was sought by tritium release experiments, monitoring the loss of tritium from [β,γ-^{3}H]glutamyl-labeled FLEEL. Initial studies on carboxylase in crude microsomal membrane preparations showed washout of $^3H^+$ independent of CO_2 concentration (Friedman et al., 1979; Ducrocq et al., 1986). These results support the mechanistic route in Scheme 2 in which KOO^- can generate the peptidyl γ-Glu carbanion by $C_\gamma-H_s$ abstraction. The determination of H_s abstraction has been established in a previous stereochemistry study (Maréchal et al., 1989). At high concentrations of bicarbonate, the carbanion is quantitatively captured in C−C bond formation, while at low CO_2, the carbanion can reprotonate with a hydrogen from solvent, leading to excess tritium ion release over CO_2 fixation. Tritium ion release is stoichiometrically coupled to KO production.

With the availability of pure recombinant bovine γ-carboxylase (Sugiura et al., 1996), we have reinvestigated the kinetics of ^{3}H release from pentapeptide substrate FLEAL in which the single Glu residue has ^{3}H at both *pro-R* and *pro-S* loci at the γ-carbon to assess timing, the fate of the peptidyl glutamyl carbanion intermediate, and the effects of ProPT18 (residues of −18 to −1 of prothrombin) which increases the catalytic efficiency of the carboxylase.

EXPERIMENTAL PROCEDURES

Preparation of FLAG−Carboxylase. The vitamin K-dependent carboxylase was purified as recently described (Sugiura et al., 1996) from CHO cells as an N-terminal FLAG epitope-tagged enzyme (FLAG = DYKDDDDK) by affinity chromatography on anti-FLAG antibody resin. The FLAG−carboxylase has been shown to have steady state kinetic properties identical to those of native bovine carboxylase (Sugiura et al., 1996). Protein concentrations were determined by Bio-Rad DC Protein Assay.

Chemical Synthesis and Purification of the [^{3}H-R,S]FLEAL Peptide. FLγAL peptide (where γ is a Gla residue) was synthesized by solid phase peptide synthesis using the Fmoc-Gla(otBu)$_2$-OH precursor (Bachem). Chemical decarboxylation of FLγAL in a tritiated environment yields regiospecific but stereononselective (^{3}H at *R* and *S* loci of the γ-methylene) tritium labeling of the γ-glutamyl residue in the resultant FLEAL peptide [adapted from Hauschka (1979)]. The following procedure was conducted by the NEN Custom

Synthesis Group. FLγAL peptide (10 mg) was dissolved in 0.05 M HCl in pure T_2O which was made by oxidizing carrier-free pure tritium gas. The peptide was lyophilized to dryness, and the vial containing the peptide was sealed under vacuum and heated in an oven for 9 h at 110 °C. After cooling, the vial was opened in a fume hood and the peptide dissolved in 0.1 M ammonium bicarbonate, transferred to a clean tube, and lyophilized. The peptide was then dissolved in 0.05 M HCl and lyophilized, and this was repeated four times. HPLC analysis revealed the FLγAL peptide and FLEAL peptide. [^{3}H]FLEAL was separated from the starting peptide, FLγAL, by HPLC using a C_{18} column in 0.01 M formate/triethylamine at pH 4.4 with a linear gradient of acetonitrile from 10 to 30%. The final product was identified by mass spectroscopy. Its specific radioactivity was 8.2 Ci/mmol, providing sensitivity sufficiently high for the enzyme detritiation studies.

Measurement of the Specific Radioactivity of NaH$^{14}CO_3$ and [^{3}H]FLEAL. NaH$^{14}CO_3$ at 54 mCi/mmol from Amersham was mixed with an equal volume of 100 mM cold $NaHCO_3$ and stored at 4 °C in a vial sealed with a rubber stopper. Aliquots were withdrawn using a Hamilton syringe. The specific radioactivity was measured in two different ways. An aliquot of the solution was diluted 20-fold in 1 N NaOH to avoid any loss of ^{14}C in the form of $^{14}CO_2$, and the radioactivity of 10, 20, 40, and 80 μL of solution was measured by scintillation counting after mixing with 10 mL of Atomlight scintillation fluid (Dupont NEN), yielding a specific radioactivity of 30 900 ± 380 cpm/nmol. The amount of $NaHCO_3$ was based on the known cold $NaHCO_3$ and the labeled NaH$^{14}CO_3$ calculated from a 54 mCi/mmol specific radioactivity. The specific radioactivity was also measured by the pyruvate carboxylase and malate dehydrogenase coupled assay (Cheung & Walsh, 1976; Thampy et al., 1988) in which NADH oxidation correlates with total CO_2 fixation, while the $^{14}CO_2$ fixed was quantitated from the radioactivity of [^{14}C]malate. In a sealable cuvette with a rubber stopper, 1 mL of reaction mixture was included which contained 50 mM Tris-HCl (pH 7.75), 0.5 mM acetyl-CoA, 1.5 mM pyruvate, 2 mM ATP, 0.2 mM NADH, 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 30 units of malate dehydrogenase. The solution was degassed and flushed with argon multiple times to achieve a minimum background of CO_2. The NaH$^{14}CO_3$ (5 μL) was added, and the coupled reaction was started by adding 15 μg of pyruvate carboxylase in a 5 μL volume. NADH oxidation was measured by monitoring the decrease in absorbance at 340 nm. The reaction was stopped by adding 100 units of lactic dehydrogenase and 5 mM NADH. Trichloroacetic acid (50%, 50 μL) was added, and any free $^{14}CO_2$ was evaporated by bulb-to-bulb distillation at 70 °C. The dry pellet containing the [^{14}C]malate was dissolved in water, and the radioactivity was counted by scintillation counting after mixing with 10 mL of Atomlight scintillation fluid. The specific radioactivity was calculated to be 33 000 ± 3200 cpm/nmol, indicating that the values from two methods were consistent.

Prior to the experiment, an aliquot of [^{3}H]FLEAL stock was mixed with unlabeled 50 mM FLEAL which was synthesized by solid phase peptide synthesis and dissolved in 75% acetonitrile/0.1% trifluoroacetic acid. The solution was dried under N_2 gas. The process was repeated twice to eliminate any tritium released from decomposition of the tritiated peptide during storage. The dry peptide was dissolved to 50 mM (the labeled [^{3}H]FLEAL was <0.1%), and the radioactivity was measured in 10, 20, 40, and 80 mL quantities after 20-fold dilution.

Assay of Tritium Release and $^{14}CO_2$ Incorporation. A 12 × 75 mm polystyrene culture tube containing a 1 mL reaction mixture was sealed with a rubber stopper and flushed with 100% O_2 gas for 2 min prior to the addition of bicarbonate to reduce the background CO_2 level which will interfere with the specific radioactivity of NaH$^{14}CO_3$. The reaction mixture in a total volume of 1.25 mL contains 50 mM MOPS (pH 7.45), 500 mM NaCl, 0.1% CHAPS/PC, 8 mM DTT, 16 μM ProPT18, 800 mM $(NH_4)_2SO_4$, 4.0 mM FLEAL, 5.4 mM $NaHCO_3$, and 880 μM vitamin KH_2 prereduced with $NaBH_4$. The amount of microsomal or purified carboxylase was adjusted to give good signal to noise and linear rates of $^3H^+$ release during the 5 min assay period. The reaction was started by adding the purified carboxylase or microsomal preparation. The mixture was incubated at 25 °C, and aliquots of 125 μL were withdrawn at 0.5 or 1.0 min intervals and the reactions quenched with 7 μL of 50% TCA. In the assay for tritium release, [^{3}H]FLEAL and unlabeled bicarbonate were included, while in the assay of $^{14}CO_2$ incorporation, unlabeled FLEAL and Na$^{14}CO_3$ were included in the reaction. Tritium in the water was separated from the [^{3}H]FLEAL by bulb-to-bulb distillation, and the radioactivity of the collected water was counted in a scintillation counter. ^{14}C-labeled bicarbonate was evaporated as CO_2 under acidic conditions by bulb-to-bulb distillation. The radioactivity in the dry pellet containing [^{14}C]FLγAL was counted after dissolving in water.

RESULTS

Preparation of [γ-^{3}H]FLEAL and Calibration of Tritium Release and $^{14}CO_2$ Incorporation Rates. To carry out the tritium release assays as an index of reversible formation of a substrate γ-glutamyl carbanion intermediate, we needed a source of active carboxylase, a tritiated substrate with a high specific and regioselective label, and a quantitative release assay. The enzyme utilized was bovine γ-glutamyl carboxylase expressed in and purified from CHO cells as a FLAG epitope tagged enzyme (Sugiura et al., 1996). Enzyme was assayed both after purification to apparent homogeneity (SA = 8.7×10^8 cpm of $^{14}CO_2$ fixed per 30 min per milligram of protein) on an antibody affinity column as well as in the initial microsomal fraction (SA = 1.37×10^7 cpm per 30 min per milligram of protein). We chose FLEAL as the carboxylation substrate. It is as good a substrate as the more commonly used prototypic peptide FLEEL on the basis of our kinetic analysis on both substrates (k_{cat} = 0.4 s^{-1} and K_m = 3.3 mM for FLEAL, k_{cat} = 1 s^{-1} and K_m = 2.2 mM for FLEEL). FLEAL with only one Glu residue reduces ambiguity of interpretation in ^{3}H release assays. Tritium labeling of the FLEAL was carried out at NEN, according to the protocol of Hauschka (1979). Specific tritium labeling of the *R,S*-γ-carbon in the glutamyl residue of the peptide was introduced by chemical decarboxylation of FLγAL in pure T_2O which was oxidized from carrier-free pure tritium gas. The [^{3}H]FLEAL was purified by HPLC from any FLγAL that had not been decarboxylated to yield [^{3}H]FLEAL with a specific radioactivity of 8.2 Ci/mmol. This material is suitable for sensitive detection of ^{3}H release by carboxylase. [^{3}H]FLEAL was stored at −20 °C in small aliquots. Prior to enzymatic incubations, any

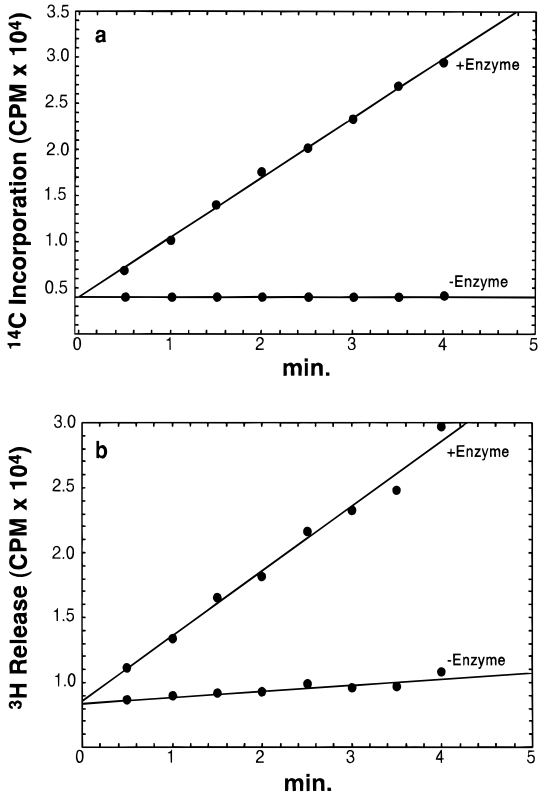


FIGURE 1: Time course of $^{14}CO_2$ incorporation into FLEAL and $^3H^+$ release from [^{3}H]FLEAL. For every time point, 125 μL of a total volume of 1.25 mL of assay mixture was withdrawn. Each aliquot contained 28 μg of microsomal protein. At 16 μM ProPT18 and 5.4 mM Na$H^{14}CO_3$, the rate of $^{14}CO_2$ incorporation was 0.30 nmol/min (a). The rate of $^3H^+$ release was 0.82 nmol/min (b). The rate of $^3H^+$ release with boiled microsomal preparation was 0.04 nmol/min. The mixtures for parts a and b were assayed at 25 °C and under 100% O_2.

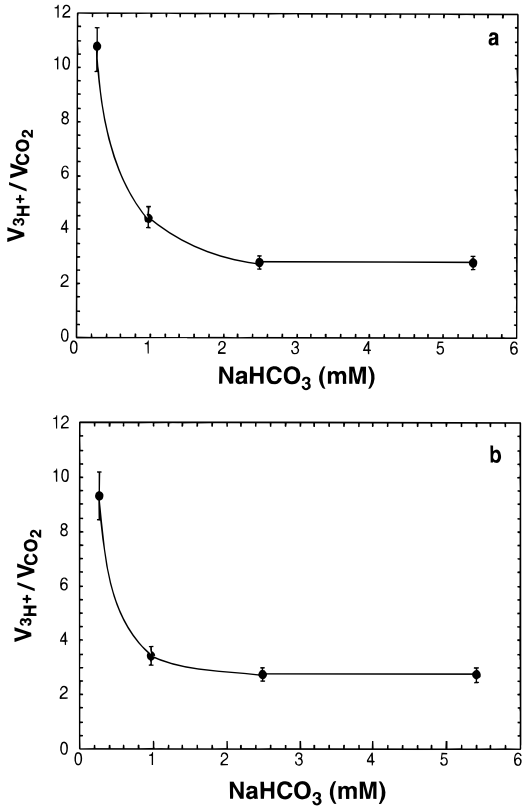


FIGURE 2: Ratio of the rate of $^3H^+$ released/CO_2 incorporation, $V_{^3H^+}/V_{^{14}CO_2}$, as a function of $NaHCO_3$ concentration. In the assay of $^{14}CO_2$ incorporation, 4.0 mM unlabeled FLEAL and Na$H^{14}CO_3$ at various concentrations were used. In the assay of $^3H^+$ release, 4.0 mM [^{3}H]FLEAL and unlabeled $NaHCO_3$ at various concentrations were used. Incubation was at 25 °C, under 100% O_2. (a) Assay with microsomal protein (28 μg/aliquot). The specific radioactivity of Na$H^{14}CO_3$ was 30 900 cpm/nmol. The specific radioactivity of [^{3}H]FLEAL was 2970 cpm/nmol. (b) Assay with purified carboxylase (3.3 μg/aliquot). The specific radioactivity of [^{3}H]FLEAL was 11 300 cpm/nmol.

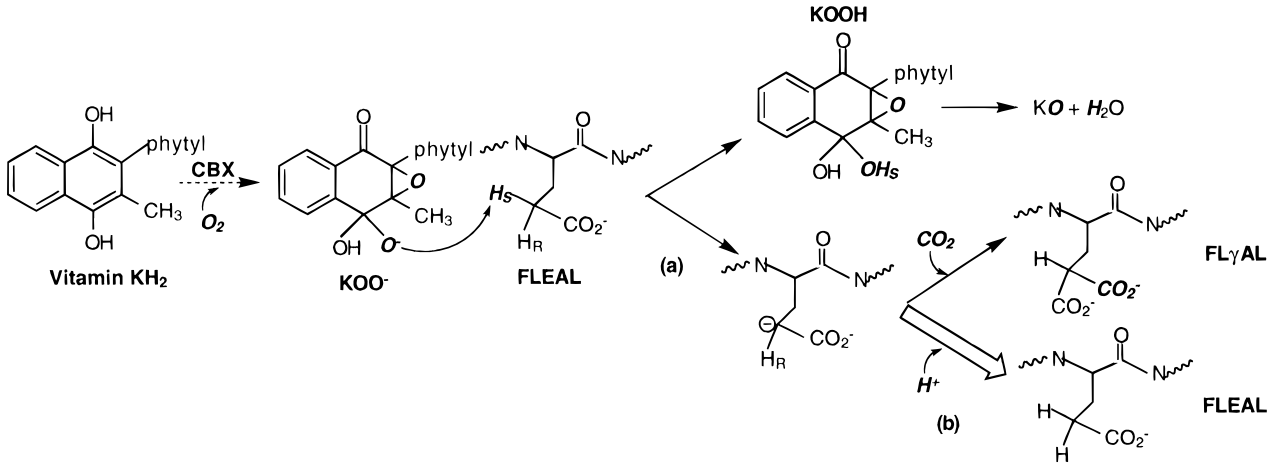
tritium released as a result of radiolysis was removed by drying under N_2, which was crucial for obtaining low background values in measuring tritium release.

Panels a and b of Figure 1 show time courses for $^{14}CO_2$ incorporation and ^{3}H release, respectively, with carboxylase activity in microsomes. Equivalent results were obtained with the affinity-purified enzyme (data not shown), and these results validate the fact that the activity is linear through the time period analyzed. Over the 5 min incubation time, both the tritium release into water and the $^{14}CO_2$ incorporation into FLEAL to yield FLγAL show excellent linearity, allowing accurate rate calculations. Absolute variation of enzyme levels gave the expected fold changes in initial rates (data not shown), confirming that assays were conducted in a linear range of enzyme concentrations.

Determination of the specific activity of the tritiated FLEAL as well as the $^{14}CO_2$ in the enzymatic incubations is critical to subsequent calculation of stoichiometry ratios of nanomoles of ^{3}H released per nanomole of $^{14}CO_2$ fixed as a measure of carbanion reprotonation/carbanion capture by C$-$COO$^-$ bond formation expressed as $V/K_{^3H^+}/V/K_{^{14}CO_2}$, because neither the ^{3}H nor the ^{14}C substrates constitute the bulk species of peptide or bicarbonate since they are present in trace quantities. The $C_\gamma-{}^3H$ cleavage rate is a surrogate for $C_\gamma-H$ cleavage, and the $C_\gamma-{}^{14}CO_2$ formation rate is a surrogate for $C_\gamma-CO_2$ formation. The specific radioactivity of Na$H^{14}CO_3$ was measured by both direct counting of the solution diluted in sodium hydroxide and quantitating the enzyme-catalyzed incorporation of $^{14}CO_2$ into pyruvate. The data from each method correlated closely, 30 900 $\pm$ 380 vs 33 000 $\pm$ 3200 cpm/nmol. The enzyme assays were conducted in a nearly CO_2-free environment to achieve minimum perturbation to the specific radioactivity of Na$H^{14}CO_3$ by degassing and refilling the reaction mixture with pure oxygen gas. The background level of CO_2 in the enzyme-free reaction mixture after degassing and refilling with oxygen was measured to be 0.1 mM by the coupled NADH assay described in Experimental Procedures.

Ratio of Tritium Release to CO_2 Fixed as a Function of $NaHCO_3$ Concentration. The ratio of tritium release to CO_2 fixation was determined as a function of $NaHCO_3$ concentration to determine the effect on partitioning of carbanion at the γ-carbon of glutamate between hydrogen ion uptake and CO_2 fixation. Panels a and b of Figure 2 respectively show the dependence of the ratio of $V/K_{^3H^+}/V/K_{^{14}CO_2}$ as a function of bicarbonate concentration in incubations with crude microsomal carboxylase and purified enzyme. Both sets of data are comparable, indicating that purification has not resulted in altered kinetic behavior. The general trend follows that previously reported by Larson et al. (1981) when they measured KO/CO_2 fixation ratios. We also see a high degree of uncoupling of C$-$H bond cleavage from $^{14}CO_2$ incorporation at low $NaHCO_3$ levels. The ratio is 10.8/1 with the microsomal preparation and 9.3/1 with pure car-

Scheme 3



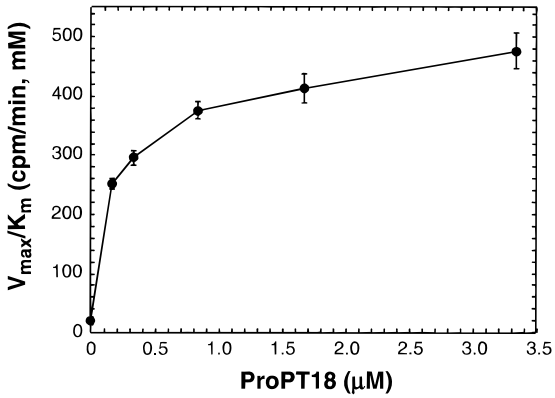


FIGURE 3: Kinetic parameters V_{max}/K_m at various concentrations of ProPT18. Initial rates (V_i) at each concentration of ProPT18 were measured with microsomal preparations, 5.4 mM $NaH^{14}CO_3$, and FLEEL at various concentrations, at 25 °C, under normal atmosphere using the same method for enzyme activity assay previously described (Kuliopulos et al., 1992a). The V_{max} and K_m values at each concentration of ProPT18 were obtained from the double-reciprocal plot of $1/V_i$ vs 1/[FLEEL]. V_{max} values are in units of counts per minute per millimolar in incubations containing 52.5 μg of microsomal protein. K_m for FLEEL is in units of millimolar (absolute concentrations of ProPT18 peptide were determined by amino acids analysis).

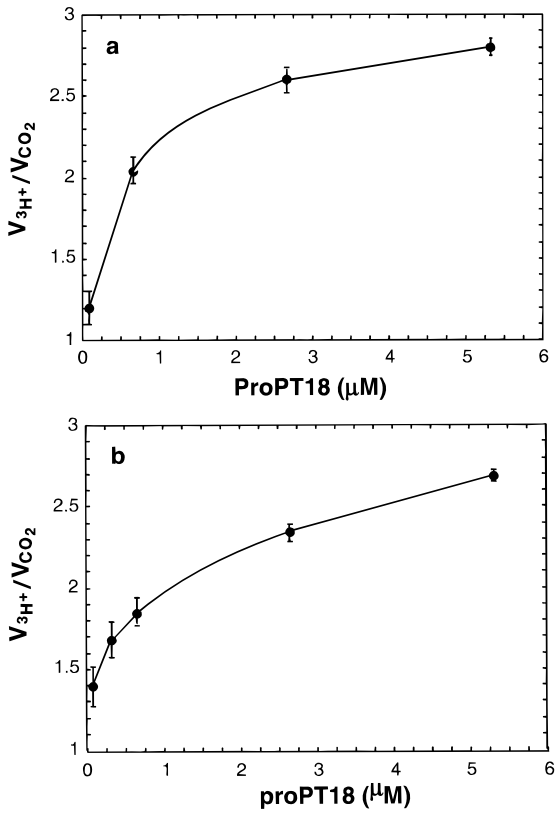
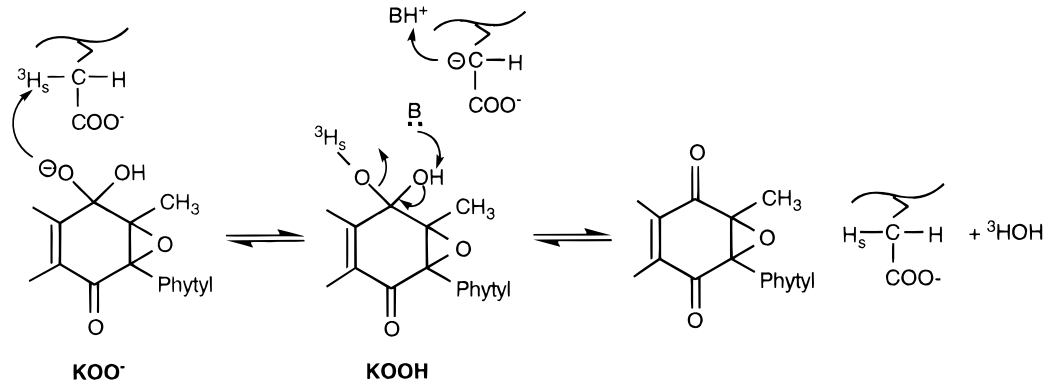


FIGURE 4: Ratio of the rate of $^3H^+$ released/CO_2 incorporation as a function of ProPT18 concentration. For the assay of CO_2 fixation, 4.0 mM cold FLEAL and 5.4 mM $NaH^{14}CO_3$ were used. In the assay of $^3H^+$ release, 4.0 mM [^{3}H]FLEAL and 5.4 mM unlabeled $NaHCO_3$ were used. Incubation was at 25 °C, under 100% O_2. (a) Assay with microsomal protein (28 μg/aliquot). The specific radioactivity of $NaH^{14}CO_3$ was 32 100 dpm/nmol. The specific radioactivity of [^{3}H]FLEAL was 7450 cpm/nmol. (b) Assay with purified carboxylase (5.3 μg/aliquot). The specific radioactivityof [^{3}H]FLEAL was 9020 cpm/nmol.

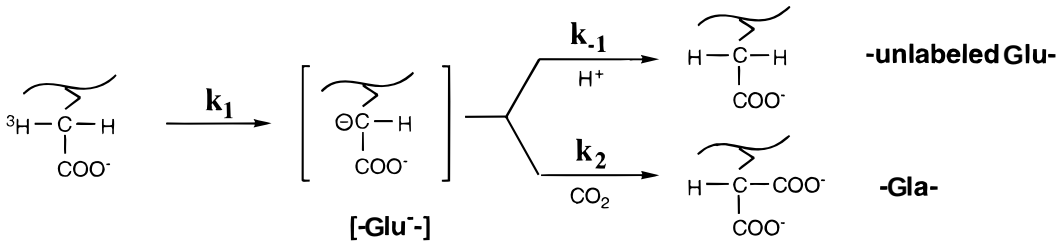
boxylase at 0.27 mM $NaHCO_3$. As the bicarbonate concentration is raised through concentrations equivalent to its K_m (0.3 mM) to saturating levels, the $V/K_{^3H^+}/V/K_{^{14}CO_2}$ ratio reaches a plateau. The plateau level is not at 1/1 but at 2.8/1 for microsomal enzyme and 2.3/1 for purified carboxylase. Thus, even at saturating bicarbonate levels, the C−H cleavage is 2−3-fold faster than $C_\gamma-COO^-$ bond formation. The good correlation of our ratio of $V/K_{^3H^+}/V/K_{^{14}CO_2}$ of 9.3−10.8/1 with the ratio of K epoxide/CO_2 fixed of 9.7/1 (Larson et al., 1981) at low CO_2 concentrations indicates that $C_\gamma-H_s$ bond cleavage is stoichiometrically coupled to KO production.

Effect of Propeptide in γ-Carboxylase Catalysis. The above studies with FL[^{3}H]EAL were all conducted with saturating (5.3 μM) ProPT18 present. In accord with prior observations, ProPT18 increases catalytic efficiency (Knoblock & Suttie, 1987; Sugiura et al., 1996), expressed as V_{max}/K_m for peptide substrates, as shown for unlabeled FLEEL in Figure 3. The K_m for the propeptide was determined to be 0.2 μM. To dissect what role ProPT18 may play in the dynamics of glutamyl $C_\gamma-H_s$ bond cleavage and $C_\gamma-COO^-$ bond formation, we repeated the tritium release and [^{14}C]CO_2 fixation initial rate ratio measurements over a range of ProPT18 concentrations from 0.1 to 5.3 μM (Figure 3) for both microsomal carboxylase (Figure 4a) and purified enzyme (Figure 4b). As ProPT18 levels increase, both the $C_\gamma-^3H$ cleavage rate and the $C_\gamma-^{14}CO_2$ formation rate increase but differentially. The ProPT18 accelerates $C_\gamma-^3H$ cleavage 11−13-fold but $C_\gamma-^{14}CO_2$ formation only 6−7-fold. The net effect is to convert a $V/K_{^3H^+}/V/K_{^{14}CO_2}$ ratio of 1.2−1.4/1 (Figure 2) at low ProPT18 to a ratio of 2.3−

Scheme 4



Scheme 5



2.8/1 at saturating ProPT18, a selective acceleration of the peptide γ-glutamyl carbanion intermediate formation with a net uncoupling from carboxylation.

DISCUSSION

The vitamin K-dependent carboxylase/epoxygenase is a remarkable catalyst, both in its mode of recognition of the 9−12 glutamyl residues for iterative carboxylation directed by a propeptide region in protein substrates and in its ability to carry out post-translational modification at the inactivated methylene carbon of the glutamyl residues.

To achieve glutamyl $C_\gamma-H_s$ cleavage to produce the carbanion center as a carbon nucleophile for attacking CO_2, the carboxylase has recruited and coupled a naphthoquinol dioxygenase catalytic apparatus to the task at hand (Scheme 4). It appears that the problem of generating a base in the active site strong enough to abstract the $C_\gamma-H_s$ at an appreciable rate is solved by the "base enhancement" mechanism (Dowd et al., 1992) in which the KH_2 dioxygenation process yields a KOO^- alkoxide in the active site microenvironment as a species of high proton affinity. Prior studies had established that KO epoxide formation could be uncoupled from CO_2 fixation at low HCO_3^-/CO_2 concentrations so that 90% of the KH_2 epoxygenase flux was uncoupled from $C_\gamma-COO^-$ bond formation. If every KO-forming turnover involves $C_\gamma-H_s$ bond cleavage as a source of protonation of KOO^-, then one would also expect up to 10 $C_\gamma-H_s$ cleavage events/CO_2 fixation under low HCO_3^-/CO_2 conditions as well. This is the stoichiometry we observe here with both pure carboxylase and enzyme in microsomes, consistent with the base enhancement mechanism and $C_\gamma-H_s$ being a source for quantitative KOO^- protonation.

On the basis of the proposed reaction mechanism and the kinetic studies reported here, the peptidyl γ-glutamyl carbanion intermediate generated from proton abstraction followed by vitamin K epoxidation (k_1) has two fates as noted in Scheme 5, carboxylation yielding the Gla product (k_2) or reprotonation giving back the Glu substrate, now unlabeled (k_{-1}). Flux of intermediate back through k_{-1} is measured as tritium release to water and counted as uncoupling of the intermediate formation from product generation. The ratio of $V/K^{3}_{H^+}/V/K^{14}_{CO_2}$ is a measure of k_{-1}/k_2 and thereby the degree of uncoupling of the intermediate formation from product generation. At high CO_2 levels, the ratios of 2.3−2.8/1 in the presence of saturating ProPT18 reveal that about 60% of the γ-glutamyl carbanion intermediate in FLEAL partitions back to reprotonation rather than carboxylation.

In order to elucidate the effects of ProPT18 on the kinetics of intermediate partitioning, we first analyzed its effects on the catalytic efficiency (V_{max}/K_m) of the FLEEL substrate which is commercially available and processed in a manner similar to that of FLEAL ($k_{cat} = 1\ s^{-1}$ and $K_m = 2.2$ mM for FLEEL, $k_{cat} = 0.4\ s^{-1}$ and $K_m = 3.3$ mM for FLEAL). ProPT18 increased the catalytic efficiency over 20-fold with a K_m of 0.2 μM. As ProPT18 was varied from 0.1 to 5.3 μM, it increased the rate of FLEAL γ-glutamyl carbanion intermediate formation 11−13-fold and the rate of carboxylation 6−7-fold. The uncoupling was increased from 1.2−1.4 to 2.3−2.8. ProPT18 peptide interacts with carboxylase and selectively lowers the energy barrier in one or more steps, leading to formation of the γ-glutamyl carbanion intermediate. This increases the flux through the k_1 step and gives rise to a higher steady state concentration of the γ-glutamyl carbanion intermediate ([-Glu⁻-]), resulting in both an observed increased rate of product generation (k_2[-Glu⁻-]) and a shift in partition to favor reprotonation (k_{-1}[-Glu⁻-]) as detected by the higher uncoupling rate (k_{-1}). In effect, the carboxylation step has become more rate-limiting and the carbanion formation step selectively increased.

The enzymatic detritiation of FL[^{3}H]EAL involves tritium at trace levels and even at saturating levels of pentapeptide substrate yields a V/K tritium effect and not a V^{3}_{H} effect. Similar arguments apply to the [^{14}C]bicarbonate substrate. Converting tritium release rates ($C_\gamma-{}^3H$ cleavage) to protium release rates ($C_\gamma-{}^1H$ cleavage) requires correction by the tritium kinetic isotope effect. While not measured directly,

it can be estimated when the deuterium kinetic isotope effect is known. Maréchel et al. (1984) have measured $V_{max}(H)/V_{max}(^2H)$ effects with deuterated peptides (Boc[^{2}H]EEV and FL[^{2}H]EEV) and found very low values of 1.2. If the $V_{max}(^2H)$ is approximated by an equivalently small $V/K(^2H)$ effect, then one can calculate $V/K(^3H)$ from the relationship $V/K(^1H)/V/K(^3H) = [V/K(^1H)/V/K(^2H)]^{1.44}$ (Walsh, 1979), leading to a $V/K(^1H)/V/K(^3H)$ of 1.3. Thus, we expect the direct measure of the $C_\gamma-^3H$ cleavage rates is a good marker for the flux of the bulk $C_\gamma-^1H$ species. Because the $^{12}C/^{14}C$ isotope effect is likely to be very close to unity, one typically assumes that $V/K(^{12}C)/V/K(^{14}C)$ is also unity within the limits of such initial velocity steady state analysis, and the measurements and conclusions about the ^{14}C trace substrates are also mirrored by the bulk population of unlabeled ^{12}C substrate molecules.

In protein substrates, the obligate directing role of the propeptide domain may be alignment of downstream glutamyl residues for placement adjacent to the site of KOO^- generation to enhance the rate of glutamyl $C_\gamma-H_s$ cleavage. Another possible mechanism of the propeptide enhancement is the accelerated formation of KOO^- species by lowering the energy of oxygen activation. Since it has been shown that ProIX28 containing Asp_6-Asp_7 is not carboxylated (Hubbard et al., 1989), measurement of substoichiometric KOO^- formation in the presence and absence of propeptide-linked noncarboxylatable substrate such as FLDAL will be an interesting experiment for distinguishing if the propeptide selectively lowers the energy barrier in proton abstraction or in KOO^- formation. In conclusion, these studies validate reversible formation of peptidyl glutamyl γ-carbanion intermediates, support the KOO^- base enhancement mechanism, and indicate a molecular role for propeptide in enabling substrate carbanion formation in the action of vitamin K-dependent carboxylase.

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