Insight into the Coupling Mechanism of the Vitamin K-Dependent Carboxylase: Mutation of Histidine 160 Disrupts Glutamic Acid Carbanion Formation and Efficient Coupling of Vitamin K Epoxidation to Glutamic Acid Carboxylation[†]

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ABSTRACT: Vitamin K-dependent (VKD) proteins become activated by the VKD carboxylase, which converts Glu's to carboxylated Glu's (Gla's) in their Gla domains. The carboxylase uses vitamin K epoxidation to drive Glu carboxylation, and the two half-reactions are coupled in 1:1 stoichiometry by an unknown mechanism. We now report the first identification of a residue, His160, required for coupling. A H160A mutant showed wild-type levels of epoxidation but substantially less carboxylation. Monitoring proton abstraction using a peptide with Glu tritiated at the γ-carbon position revealed that poor coupling was due to impaired carbanion formation. H160A showed a 10-fold lower ratio of tritium release to vitamin K epoxidation than wild-type enzyme (i.e., 0.12 versus 1.14, respectively), which could fully account for the fold decrease in coupling efficiency. The Ala substitution in His160 did not affect the K_m for vitamin K and caused only a 2-fold increase in the K_m for Glu and 2-fold decrease in the activation of vitamin K epoxidation by Glu. The H160A K_m for CO_2 was 5-fold higher than the wild-type enzyme. However, the k_{cat} for H160A carboxylation was 8−9-fold lower than the wild-type enzyme with all three substrates (i.e., Glu, CO_2, and vitamin K), suggesting a catalytic role for His160 in carbanion formation. We propose that His160 facilitates the formation of the transition state for carbanion formation. His160 is highly conserved in metazoan VKD carboxylases but not in some bacterial orthologues (acquired by horizontal gene transfer), which has implications for how bacteria have adapted the carboxylase for novel functions.

Vitamin K-dependent (VKD)[1] carboxylation occurs in the endoplasmic reticulum during the secretion of VKD proteins and results in the conversion of clusters of Glu's to carboxylated Glu's (Gla's) in the Gla domains of these proteins (*1*). The VKD proteins contain a specific recognition sequence, which is usually a propeptide, that allows selective binding by the VKD carboxylase (*2*). The propeptide facilitates processive carboxylation of the multiple Glu's and also regulates carboxylase binding to the Glu substrate and vitamin K cofactor (*3–6*). Full carboxylation transforms the Gla domain into a calcium-binding module that targets VKD proteins either to cell surfaces where negatively charged phospholipids are exposed or to hydroxyapatite in the extracellular matrix. Carboxylation is thus required for the activity of VKD proteins, which have a diverse range of functions that include hemostasis, apoptosis, calcium homeostasis, growth control, and signal transduction. Naturally occurring carboxylase mutations are rare, consistent with the broad physiological impact of the carboxylase. The first mutations were identified in patients with a bleeding disorder characterized by decreased activity in several hemostatic VKD proteins (*7–10*). More recently, however, several mutations have also been found to cause a disease unrelated to hemostasis, i.e., pseudoxanthoma elasticum (*11*), but how the carboxylase mutations cause this disease remains to be determined.

The carboxylase was originally discovered in mammals; however, orthologues have since been identified in other multicellular organisms, i.e., fish, *Conus*, *Drosophila*, and tunicates (*12–18*). Fish contain hemostatic VKD proteins (*12*), and *Conus* use VKD peptides for predation (*19*), but the function of VKD carboxylation in *Drosophila* and tunicates is not known. Until recently, VKD carboxylation was thought to be restricted to the animal kingdom; however, bacterial proteins with extensive homology to the VKD carboxylase have now been identified in genome sequencing projects. Characterization has been performed on only one of these VKD carboxylase orthologues, which is from the pathogen *Leptospira borgpetersenii* that causes leptospirosis. The properties of the VKD orthologue suggest that *L.*

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[1] Abbreviations: VKD, vitamin K-dependent; Glu, glutamic acid; Gla, γ-carboxylated glutamic acid; KH_2, vitamin K hydroquinone; K25, a phylloquinone analogue with five isoprenyl groups; KO, vitamin K epoxide; FLEEL, the pentapeptide Phe-Leu-Glu-Glu-Leu; FLγEL, the pentapeptide Phe-Leu-γ-carboxylated Glu-Glu-Leu; FL(*R*,*S*-^{3}H)EEL, the pentapeptide Phe-Leu-[*R*,*S*-^{3}H]Glu-Glu-Leu, which contains tritium at the γ-carbon position in Glu; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; BCA, bicinchoninic acid; BSA, bovine serum albumin.

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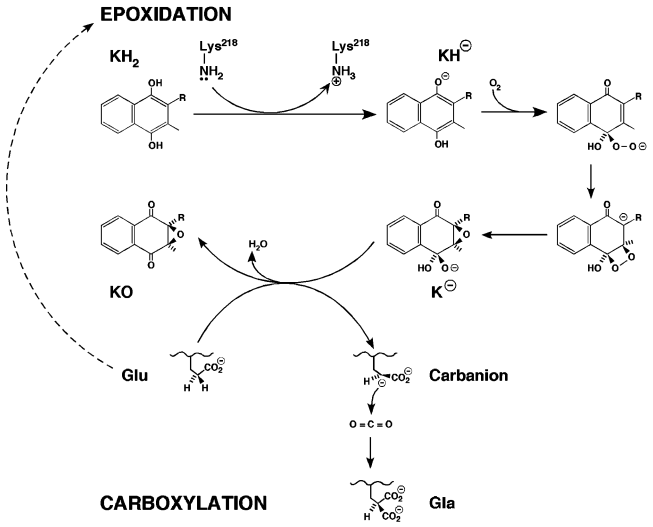


FIGURE 1: The carboxylase is a bifunctional enzyme. The carboxylase is both an epoxidase that converts reduced vitamin K (KH_2) to vitamin K epoxide (KO) and a carboxylase that generates carboxylated Glu (Gla) from Glu. Lys218 in the carboxylase deprotonates KH_2 to form KH^- (*26*), which reacts with O_2 to generate a high-energy vitamin K base (K^-). K^- then deprotonates Glu to produce a carbanion intermediate that reacts with CO_2 to form Gla, while K^- collapses to the KO product. The carboxylase is regulated so that epoxidation is efficiently coupled to carboxylation and is also regulated so that very little epoxidation occurs in the absence of the VKD protein substrate (represented by the dashed arrow) (*37, 38*). The feedback pathway prevents the unfettered production of a vitamin K base that could react with other molecules if Glu substrate is not available for carboxylation.

borgpetersenii may have adapted the VKD carboxylase for a role other than Glu carboxylation (*20*).

The carboxylase is a bifunctional enzyme that uses the energy of vitamin K epoxidation to drive Glu carboxylation. A "base amplification" mechanism has been proposed (*21*) in which a carboxylase active site weak base deprotonates reduced vitamin K (KH_2), which is then oxygenated to generate a vitamin K intermediate sufficiently basic for abstracting a hydrogen from the γ-position of Glu to form a carbanion (Figure 1). Subsequent reaction of the carbanion intermediate with CO_2 then produces Gla while the vitamin K base forms a vitamin K epoxide product that is recycled back to KH_2 by a separate enzyme, the vitamin K epoxide reductase (*22*). Evidence for the carbanion intermediate includes tritium incorporation into Glu substrate in the presence of tritiated water (*23*) and fluoride elimination from (2*R*,3*S*)-3-fluoroglutamate (*24*). How the carboxylase facilitates the carboxylation and epoxidation reactions is poorly understood, as it is a large integral membrane protein and the only structural information available is a 2D crystal that does not reveal functional residues (*25*). An important advance in defining the mechanism has been the identification of Lys218 as the catalytic base that initiates the reaction by deprotonating KH_2 to allow reaction with O_2 and formation of the high-energy vitamin K intermediate (Figure 1) (*26*).

Epoxidation is efficiently coupled to carboxylation such that stoichiometric amounts of each reaction are observed (*27*). However, how the carboxylase is regulated to achieve efficient coupling is unknown as residues that facilitate this process have previously not been identified. We now report that His160 is important in the coupling of epoxidation to carboxylation. We previously demonstrated that substitution of this residue by Ala resulted in a mutant with wild-type levels of epoxidase activity (*26*). Surprisingly, the H160A mutant was inefficient for carboxylation, for unknown reasons, and studies were therefore undertaken to determine why carboxylation was uncoupled from epoxidation. As described below, these studies reveal that His160 is critical for the generation of the carbanion intermediate.

EXPERIMENTAL PROCEDURES

Expression and Isolation of Carboxylase. A carboxylase variant with His160 substituted by Ala was generated and subcloned into baculovirus for expression in SF21 insect cells, as previously described (*26*). The H160A mutant and wild-type carboxylase both contained a C-terminal extension of AAADYKDDDDK, comprising an Ala linker and the eight amino acid FLAG epitope. Solubilized microsomes were prepared from baculovirus-infected insect cells, and the carboxylases were affinity-purified using anti-FLAG antibody coupled to agarose (Sigma), as before (*26*), except that propeptide was not used in the present isolation. All of the experiments described in the studies below used purified material.

Activity Assays. Vitamin K epoxidation was measured in reaction cocktails containing final concentrations of 0.8 M ammonium sulfate, 0.16% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.16% phosphatidylcholine, 2.5 mM Phe-Leu-Glu-Glu-Leu (FLEEL; AnaSpec), 10 μM factor X propeptide, 2.5 mM DTT, 1.3 mM sodium bicarbonate, 50 mM BES, pH 6.9, and 200 μM KH_2. The KH_2 was prepared by reduction of phytonadione (Hospira, Inc.) using 150 mM DTT in 20 mM Tris-HCl, pH 8.6. The phosphatidylcholine was dispersed by resuspension in CHAPS and vortexing before addition to the remainder of the reaction cocktail. Following incubation for various times at 20 °C, the reactions were quenched by adding a 1:1 mixture of ethanol and hexane, at a 3:1 v:v ratio over that of the reaction mixture. A vitamin K standard (K25, 2 nmol; GL Synthesis) was then immediately added to correct for recovery in the subsequent analysis. The samples were vortexed, the organic phase was then dried under nitrogen, and vitamin K epoxidation was quantitated by HPLC analysis as previously described (*20*). All assays were performed in duplicate and included a negative control, which was a sample obtained by performing the anti-FLAG affinity purification on mock-infected insect cells that lack carboxylase. To determine the specific activity of epoxidation, the amount of carboxylase protein was measured in a quantitative Western using an antibody-linked fluorescence assay, as previously described (*26*).

Carboxylase activity was measured in duplicate using the same reaction mixture as described above for the epoxidase assay, except that [^{14}C]$NaHCO_3$ was used instead of the sodium bicarbonate. The reactions were quenched by the addition of trichloroacetic acid (10% final concentration), and the samples were then quantitated for [^{14}C]CO_2 incorporation into FLEEL by scintillation counting, as before (*28*).

The carboxylation of full-length, propeptide-containing factor IX was performed using the carboxylase reaction conditions described above but with FLEEL and propeptide replaced by the factor IX substrate. The propeptide-containing form of factor IX was generated in cells expressing high

levels of factor IX that saturate propeptide processing. The factor IX was purified using an antibody against the factor IX propeptide, as previously described (*3*), and the amount of propeptide-containing factor IX was quantitated by a BCA assay (Pierce).

Determination of Endogenous CO_2 in Reaction Cocktails. The amount of endogenous CO_2 in the reaction mixture was measured by determining the specific activity of ^{14}C-carboxylated Glu generated in the reaction. Carboxylation was performed as described above except that [^{14}C]$NaHCO_3$ concentrations of 0.1, 0.5, 1, or 5 mM were used. [^{14}C]CO_2 carboxylated FLEEL was chromatographed on a P-2 column (11 × 150 mm, fine mesh; Bio-Rad) in 25 mM ammonium bicarbonate, pH 7.4, to remove reagents that would interfere with the subsequent HPLC analysis. The samples were lyophilized and then resuspended in 100 μL of 2.5 M KOH, and the peptide was then digested for 20 h at 110 °C. The hydrolysates were chromatographed on ion-exchange columns, as before (*28*), and then lyophilized and resuspended in 100 μL of H_2O. Aliquots containing ~400 nmol of total amino acid were derivatized in an 85 μL mixture containing 65 mM *o*-phthalaldehyde, 600 mM borate, pH 10.7, and 120 mM 2-mercaptoethanol. Immediately after derivatization, the samples were analyzed by HPLC using a C18 column (ODS Hypersil, 100 × 4.6 mm, 5 μm; Thermo Electron Corp.) and gradient elution from 5% to 50% acetonitrile with 50 mM citrate, pH 7.4, and 0.08% trifluoroacetic acid. The derivatized amino acids were detected by fluorescence (excitation at 323 nm and emission at 430 nm), and the Gla peak was quantitated using a control Gla standard (Sigma) as well as an amino acid mixture of known concentration (AAH; Pierce). The Gla peak was collected and quantitated radioactivity was by scintillation counting, revealing specific activities of 25, 75, 84, and 125 cpm/pmol when 0.1, 0.5, 1, or 5 mM [^{14}C]$NaHCO_3$ was used in the original carboxylation reaction, respectively. These values were used to calculate the amount of endogenous CO_2 in the reaction, which was 0.4 mM.

In subsequent studies, the measured amount of [^{14}C]CO_2 incorporated into FLEEL was adjusted for the dilution by endogenous CO_2 to determine the extent of FLEEL carboxylation. The equation

$$\frac{[^{14}\text{C-CO}_2]}{[^{14}\text{C-CO}_2] + 0.4} \times 120 \text{ cpm/pmol} \qquad (1)$$

(where 120 cpm/pmol is the specific activity of commerically available [^{14}C]$NaHCO_3$ and the remaining values shown are in millimolar) was used to convert the observed [^{14}C]CO_2 counts per min into picomole values.

A Method for Assaying Low Levels of Carboxylase Activity. A background is observed in the carboxylase assay due to the nonenzymatic generation of an unknown [^{14}C]CO_2 side product. This background limits detection of activity at low carboxylase concentrations, and to improve the sensitivity of the assay, we carried out a procedure that removed this contaminant. At the end of the reaction, the samples were quenched by the addition of 1 mL of H_2O and then boiled to ~200 μL, which eliminated unreacted [^{14}C]CO_2. The samples were then cooled to 4 °C, followed by the addition of 800 μL of ice-cold acetone, vortexing, and incubation on ice for 1 h. After centrifugation at 10000*g*, the samples were evaporated to ~200 μL with a stream of dry nitrogen and then loaded on P-2 columns. Carboxylated FLEEL was eluted in 25 mM ammonium bicarbonate, and the fractions containing the peptide were pooled, lyophilized, and then taken up in 100 μL of H_2O for scintillation counting. The FLEEL was well resolved from the contaminant, with excellent reproducibility in recovery.

This method was applied to the determination of the H160A kinetic constant for KH_2. As described in the Results, this analysis used low concentrations of KH_2. The low concentrations of KH_2 required that low levels of carboxylase activity be used so that the amount of KH_2 turnover was not too high to prevent initial rate determinations. H160A epoxidation is much higher than carboxylation, and so the use of low amounts of H160A reduced the carboxylase activity levels close to background in the normal assay. However, the elimination of the contaminant background considerably improved the signal-to-noise ratio for H160A, and this method was therefore valuable in the analysis of H160A.

Synthesis of Tritiated FLEEL. A peptide with Glu substituted by Gla, FLγEL, was synthesized (AnaSpec) and then purified and analyzed by HPLC and mass spectrometry, which showed that a homogeneous preparation of peptide of the appropriate size had been generated. FLγEL (5 mg) was taken up in 50 μL of 50 mM HCl, lyophilized to dryness, and then taken up in 50 μL of 25 mM tritiated HCl (25 mCi; Moravec Biochemicals Inc.) and again lyophilized. The dried sample was immediately transferred to a hydrolysis vial (Waters) which was then repeatedly evacuated and flushed with dry nitrogen using a PicoTag workstation. The evacuated vial was heated at 110 °C for 9 h, and the sample was neutralized with 100 μL of 100 mM ammonium bicarbonate, pH 7.4. Following lyophilization to dryness, the sample was repeatedly (four times) taken up in 100 μL of 50 mM HCl and lyophilized to dryness. After the final lyophilization, the sample was dissolved in 150 μL of 50 mM ammonium bicarbonate, pH 7.4, and then purified by HPLC using a Hypersil C18 column and isocratic elution with 16% acetonitrile and 50 mM ammonium bicarbonate, pH 7.4. Fractions containing FLEEL were identified by absorbance at 258 nm, pooled, and then lyophilized to dryness. The sample was taken up in 300 μL of 50 mM sodium acetate, pH 5.5, and then purified by HPLC using the same C18 column and isocratic elution with 16% acetonitrile and 50 mM sodium acetate, pH 5.5. Fractions containing the tritiated FLEEL (i.e., FL[*R*,*S*-^{3}H]EEL) were pooled, and scintillation counting of an aliquot was used to determine the amount of tritium incorporated into the peptide. Quantitation of the peptide by absorbance at 258 and reference to FLEEL standards was then used to calculate the specific activity of FL[*R*,*S*-^{3}H]EEL, and values of ~2000 cpm/nmol were obtained. The peptide was lyophilized to dryness for storage.

RESULTS

His160 Facilitates the Coupling of Epoxidation to Carboxylation. Analysis of an Ala-substituted mutant in the carboxylase (H160A) implicated a role for His160 in the coupling of vitamin K epoxidation to Glu carboxylation. Thus, H160A expressed in insect cells (Figure 2) that lack endogenous carboxylase (*29*) showed wild-type epoxidase

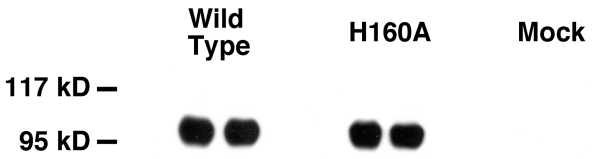


FIGURE 2: Expression of carboxylase variants in insect cells. Microsomes prepared from SF21 cells mock-infected or infected with baculovirus containing either wild-type or the H160A carboxylase were analyzed in duplicate in a Western using antibody against the C-terminal FLAG epitope.

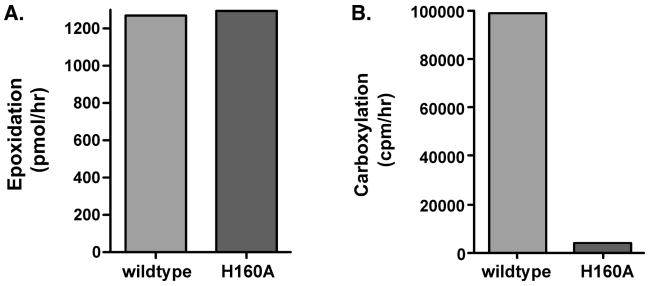


FIGURE 3: Coupling of epoxidation to carboxylation is impaired in the H160A carboxylase mutant. Wild-type and H160A carboxylases were purified from solubilized microsomes from baculovirus-infected insect cells (Figure 2), and equivalent amounts of enzyme were then assayed for epoxidation (part A) or carboxylation of FLEEL (part B), as described in the Experimental Procedures.

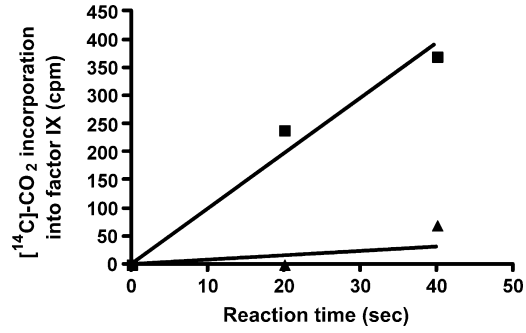


FIGURE 4: H160A is impaired in VKD protein carboxylation. Wild-type (squares) and H160A (triangles) carboxylases were purified in the absence of propeptide and then reacted with propeptide-containing full-length factor IX, which was prepared as described in the Experimental Procedures. The carboxylase was in excess of factor IX so that only a single turnover of factor IX could occur, which allowed a measurement of the rate of carboxylation independent of the rate of release. The H160A and wild-type carboxylases were quantitated by assaying for epoxidase activity, and equivalent amounts of each enzyme (4.2 pmol) were then incubated for 1 h at 20 °C with factor IX (1.4 pmol) in reaction cocktails (190 μL) that lacked KH_2. The samples were then cooled to 10 °C, and the reactions were initiated by the addition of KH_2. Aliquots were removed at 20 s intervals and quenched by the addition of gel electrophoresis dye. The samples were then gel-electrophoresed along with [^{14}C]BSA standards, followed by transfer to nitrocellulose and quantitation of [^{14}C]CO_2 incorporation into factor IX by PhosphorImager analysis, as before (*3*).

activity (Figure 3A) but substantially less carboxylase activity than the wild-type enzyme (Figure 3B). This assay measures the carboxylation of a small Glu-containing peptide derived from the Gla domain of a VKD protein. Protein carboxylation was also measured using a propeptide-containing VKD protein substrate (factor IX) in a single turnover experiment, which similarly showed less carboxylation with H160A than with the wild-type enzyme (Figure 4). His160 is one of three His residues conserved between metazoan and *Leptospira* VKD carboxylases (*20*); however, mutants with Ala substitutions for the remaining two residues (i.e., H287A and H381A) showed wild-type levels in both epoxidation (*26*) and carboxylation (data not shown). The results for H160A therefore implicated His160 as important in the coupling of epoxidation to carboxylation.

The His160 Mutation Causes a Small Effect on Glu Regulation of Epoxidation and a Large Effect on Propeptide Stimulation of Activity. The carboxylase reaction is also coupled such that epoxidation does not occur when VKD substrate is not available for carboxylation (Figure 1), and we therefore tested whether the mutation in His160 affected this reverse coupling mechanism. The wild-type and H160A carboxylases expressed in insect cells are not exposed to VKD proteins, and these enzymes were purified and then tested for epoxidation in the presence or absence of propeptide and/or Glu-containing substrate (i.e., FLEEL). In the absence of propeptide, wild-type epoxidation was stimulated ~5-fold more by FLEEL than H160A (i.e., 16-fold versus 3-fold stimulation, Table 1); however, in the presence of propeptide only a 2-fold difference was observed (i.e., 11-fold for the wild-type enzyme versus 5-fold for H160A; compare the epoxidase specific activities with propeptide versus propeptide plus Glu in Table 1). This 2-fold difference in the regulation of wild-type and H160A carboxylase was much smaller than the degree of uncoupling of epoxidation from carboxylation in H160A (Figure 3).

Carboxylase activity was also measured in this experiment so that the coupling ratio between carboxylation and epoxidation could be determined in the presence or absence of propeptide. A comparison of these two reactions required a determination of the specific activity of the [^{14}C]CO_2 in order to convert the observed counts per minute of [^{14}C]CO_2 incorporation into picomoles of carboxylation. The [^{14}C]CO_2 specific activity depends upon the amount of endogenous CO_2 in the reaction mixture, and we therefore established a method that directly measures this value in the reaction. As described in the Experimental Procedures, FLEEL carboxylated in a [^{14}C]CO_2-containing cocktail was isolated and base-hydrolyzed, and Gla fractionated by HPLC was quantitated for both the amount of material and radioactivity. The specific activity of Gla measured at several [^{14}C]CO_2 concentrations allowed a determination of the amount of nonradioactive CO_2 in the reaction, which was then used to convert counts per minute of [^{14}C]CO_2 incorporation into picomoles of carboxylation (Table 1). A comparison of carboxylation and epoxidation revealed a coupling ratio of 0.62 for the wild-type enzyme and 0.04 for H160A in the presence of propeptide and 0.44 and 0.03, respectively, in the absence of propeptide (Table 1). Thus, the propeptide did not have a large effect on the coupling efficiency of the H160A enzyme.

H160A carboxylation and epoxidation were both stimulated ~10-fold more by propeptide than the wild-type enzyme (Table 1), and we therefore tested how stimulation responded to propeptide concentration since this response reflects the affinity of propeptide binding to the carboxylase. H160A and wild-type carboxylases were purified in the absence of propeptide and then assayed for activity in the presence of varying amounts of the factor X propeptide. As shown in Figure 5, carboxylation and epoxidation both

Table 1: His160 Substitution Alters Glu and Propeptide Stimulation of Epoxidation[a]

carboxylase variant	addition	epoxidase specific activity (pmol of KO h^{-1} μg^{-1})	fold increase in epoxidation	propeptide stimulation	carboxylase specific activity (pmol of Gla h^{-1} μg^{-1})	propeptide stimulation	ratio of carboxylation to epoxidation
wild type	none	23	1		ND		
	Glu	376	16		164		0.44
	propeptide	240	10		ND		
	Glu + propeptide	2700	117	7	1671	10	0.62
H160A	none	11	1		ND		
	Glu	37	3		1		0.03
	propeptide	514	47		ND		
	Glu + propeptide	2458	223	66	108	108	0.04

[a] Wild-type and H160A carboxylases were expressed in insect cells that lack endogenous VKD proteins and purified without exposure to these proteins. The effect of Glu-containing substrate (FLEEL) and propeptide on vitamin K epoxidation and Glu carboxylation was then monitored by permuting the reaction mixtures described in the Experimental Procedures so that the reactions either contained or lacked FLEEL and/or propeptide. The fold increase in epoxidation is the ratio of the amount of epoxidation observed with the addition of FLEEL and/or propeptide to that observed with no addition (i.e., none). The propeptide stimulation compares the amount of epoxidation or carboxylation observed when both propeptide and FLEEL are present versus that observed when only FLEEL is present. The concentration of H160A used (90 nM) was higher than that of wild-type carboxylase (13 nM) so that the carboxylase activity of H160A would be detectable even in the absence of propeptide. As described in the text, the carboxylase activities were calculated after determining the specific activity of $[^{14}C]CO_2$ in the reaction. ND is not detected.

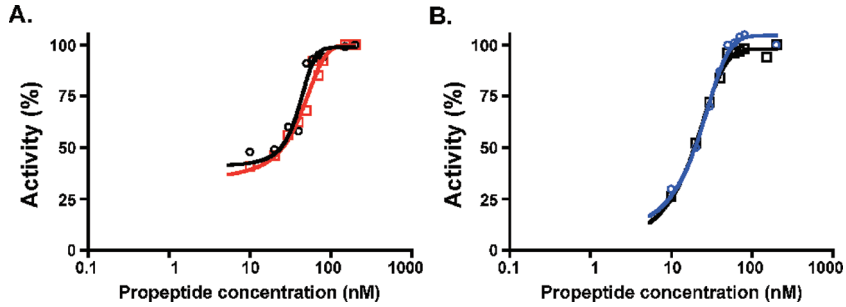


FIGURE 5: Activation of wild-type and H160A carboxylation and epoxidation occurs at similar propeptide concentrations. Wild-type (A) and H160A (B) carboxylases purified in the absence of propeptide were tested for FLEEL carboxylation (squares) or vitamin K epoxidation (circles) as described in the Experimental Procedures but with a range of concentrations of factor X propeptide (10−200 nM).

Table 2: Kinetic Analyses Reveal Large Decreases in k_{cat} but Minimal Changes in K_m of H160A for FLEEL and Vitamin K[a]

carboxylase variant	carboxylation				epoxidation (vitamin K)	
	FLEEL		vitamin K			
	K_m (mM)	k_{cat} (h^{-1})	K_m (μM)	k_{cat} (h^{-1})	K_m (μM)	k_{cat} (h^{-1})
wild type	0.9	243	9.0	222	8.9	294
H160A	1.4	31	7.8	24	9.3	275

[a] To determine the kinetic constants for Glu carboxylation, reactions (170 μL) were performed for 2 h using the reaction conditions described in the Experimental Procedures except that the CO_2 concentration was 8 mM and the FLEEL concentration was varied between 0.25 and 4 mM. To determine the kinetic constants for KH_2, FLEEL carboxylation and epoxidation reactions (600 μL) were performed for 20 min using KH_2 concentrations that ranged between 1 and 100 μM and a CO_2 concentration of 3.5 mM (for the wild-type enzyme) or 8 mM (for H160A). As described in the Experimental Procedures, a method was developed that significantly improved the sensitivity of the carboxylase assay, allowing initial rate determinations for H160A in analyzing the effect of KH_2 concentration on carboxylation.

responded the same to propeptide concentration for each enzyme, and the propeptide concentration for 50% activity was identical for wild-type carboxylase and H160A (~20 nM). These data suggest that differential stimulation is not due to altered propeptide binding by H160A.

The H160A and Wild-Type Enzymes Interact Similarly with the KH_2 and Glu Substrates. Vitamin K and Glu must be appropriately positioned to achieve efficient coupling, and we therefore tested whether carboxylase interaction with either of these substrates was disrupted in H160A. Analysis of carboxylation using varying concentrations of KH_2 showed a similar K_m for H160A and wild-type carboxylase but a 9-fold decrease in k_{cat} for H160A (Table 2). In contrast, when the effect of KH_2 concentration on epoxidation was analyzed, the K_m and k_{cat} of H160A were both the same as that of the wild-type enzyme, clearly indicating that the effect of mutating His160 is entirely on the carboxylation half of the reaction. Carboxylation was also tested over a range of Glu substrate concentrations, which revealed an 8-fold lower k_{cat} for H160A than for the wild-type enzyme but only a small increase in K_m (i.e., 1.6-fold, Table 2). The results suggest that impaired Glu or vitamin K binding is not responsible for the reduced coupling observed in H160A.

H160A Shows Impaired Interaction with CO_2. Coupling of epoxidation to carboxylation could also be disrupted if the interaction between Glu and CO_2 is impaired, and we therefore determined how H160A and wild-type carboxylases responded to changes in CO_2 concentration. Substrate inhibition was observed at CO_2 concentrations above 10 mM (data not shown), and so the experiments were performed below this level. As shown in Figure 6, H160A and the wild-type enzyme showed significantly different responses to changes in CO_2 concentrations. H160A exhibited a 5-fold increase in K_m and 8-fold decrease in k_{cat} compared to wild-

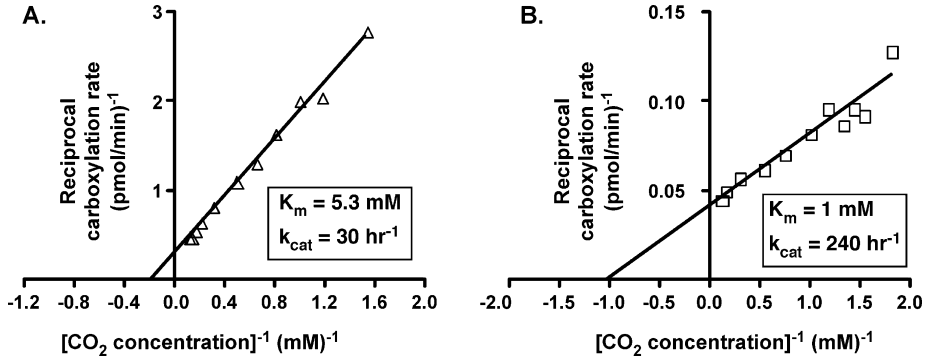


FIGURE 6: The H160A mutant shows impaired CO_2 interaction. Carboxylase reactions (100 μL) containing H160A (A) or the wild-type (B) enzyme were incubated for 1 h using the reaction cocktail described in the Experimental Procedures except that a range of [^{14}C]CO_2 concentrations (0.2−9.7 mM) was used. Equivalent amounts of the wild-type and H160A enzyme were used (6 pmol), as determined by both a quantitative Western and an epoxidase assay. As described in the Experimental Procedures, a nonenzymatic background is observed in the carboxylase assay, and the background would be different at varying [^{14}C]CO_2 concentrations. A background correction was therefore made by performing the carboxylase assays in the absence of enzyme, i.e., using anti-FLAG affinity-purified preparations from mock-infected cells, and four different concentrations of [^{14}C]CO_2 (1, 2.5, 5, and 9.4 mM). A linear relationship between background levels and [^{14}C]CO_2 concentration was observed, and a linear curve was used to correct for the background at each concentration of CO_2 in the experiment shown in the figure. The total concentration of CO_2, i.e., [^{14}C]CO_2 plus endogenous CO_2, was used for the determination of the kinetic parameters, and the pmol/min carboxylation rate was determined from [^{14}C]CO_2 cpm incorporation as described in the Experimental Procedures.

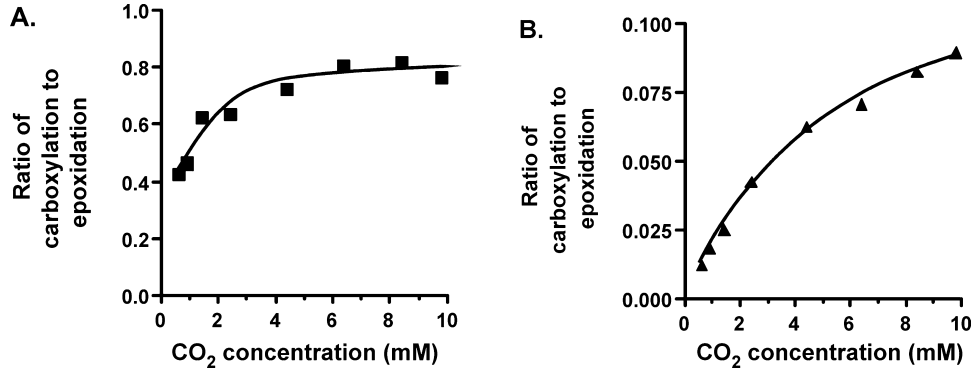
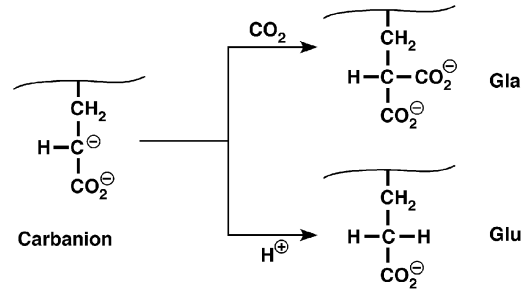


FIGURE 7: H160A and wild-type enzyme coupling efficiencies respond differently to CO_2 concentration. Wild-type (A) and H160A (B) carboxylases were assayed for epoxidation and FLEEL carboxylation as described in the Experimental Procedures except that the total CO_2 concentration (i.e., [^{14}C]CO_2 plus endogenous CO_2) varied between 0.4 and 9.8 mM. The background in the carboxylase assay was corrected as described in the legend to Figure 6. The difference in coupling ratios for the wild-type and H160A enzymes was due only to changes in carboxylation, as epoxidation did not change with varying CO_2 concentration.

type carboxylase, indicating an overall 40-fold decrease in catalytic efficiency.

Low CO_2 concentrations uncouple carboxylation from epoxidation in the wild-type enzyme (*27*), raising the question of whether the coupling efficiency of H160A and the wild-type enzyme is affected the same by changes in CO_2. When the ratio of epoxidation to carboxylation was measured at varying concentrations of CO_2, both enzymes showed increased coupling at higher levels of CO_2 (Figure 7), which was due solely to changes in carboxylation because epoxidation was the same at all concentrations of CO_2. However, H160A and the wild-type enzyme showed differences in their response to changes in CO_2 levels. First, the coupling ratio in the wild-type enzyme only increased 1.7-fold over the range of CO_2 concentrations analyzed versus a 9-fold increase for H160A. Second, H160A showed a continuous increase in the coupling ratio over the range tested while the wild-type enzyme plateaued at the higher levels of CO_2 (Figure 7). Therefore, the comparative coupling efficiencies between the wild-type and H160A enzymes were 45-fold at the lowest CO_2 concentration and 9-fold at the highest CO_2 concentration tested. Thus, substitution of

Scheme 1



His160 by Ala disrupted the utilization of CO_2 by the carboxylase.

His160 Facilitates Carbanion Formation. Impaired carboxylase interaction with CO_2 raised the question of the fate of the carbanion in H160A. Thus, in addition to carboxylation, the carbanion has the potential for protonation (Scheme 1), which might occur more frequently in H160A than the wild-type enzyme due to impaired CO_2 interaction. To probe the fate of the carbanion, we exploited a previous approach which showed that tritium from solvent can be incorporated into Glu-containing substrate (*23, 30*). Carboxylation was performed in tritiated water, and epoxidation, carboxylation,

and tritium incorporation into the Glu substrate (FLEEL) were measured. Carboxylation was measured using HPLC to fractionate FLEEL from the carboxylated FLEEL product, FLγEL, and absorbance to quantitate the amount of substrate and product. This assay detected large amounts of FLγEL generated by the wild-type enzyme but only trace amounts produced by H160A (Figure 8), consistent with the results obtained using the assay that measures [^{14}C]CO_2 incorporation into FLEEL (Figure 7). In the case of the wild-type enzyme, the amount of carboxylation (80 nmol) and epoxidation (81 nmol) indicated a coupling ratio of 1:1, as expected because the concentration of CO_2 in the reaction (8 mM) should have resulted in maximal coupling (Figure 7). Only trace amounts of tritium incorporation into FLEEL were observed in the wild-type enzyme-mediated reaction (~50 cpm, Figure 8A). This result was as expected since most of the epoxidation was coupled to carboxylation rather than protonation (Scheme 1). Surprisingly, however, the H160A-mediated reaction also showed very little tritium incorporation (Figure 8B). On the basis of the coupling ratios determined at different CO_2 concentrations (Figure 7), epoxidation in H160A should have resulted in approximately 10% carboxylation at the 8 mM CO_2 concentration used, which is consistent with the poor level of carboxylation that was detected (Figure 8B). If the remaining 90% of the carbanion was protonated (Scheme 1), then ~3200 cpm of tritium incorporation into FLEEL would be predicted for the amount of vitamin K epoxidation that was observed for H60A (29 nmol). However, the observed number was only ~2% of this value, which is much less than expected even if tritium incorporation is reduced by isotope effects.

The results from the tritium incorporation experiment suggested that impaired carboxylation in H160A is not due to an altered fate of the carbanion (Scheme 1) but rather to defective carbanion formation. We therefore monitored carbanion formation by measuring hydrogen abstraction from the γ-carbon. This test was based on previous methods demonstrating that thermal decarboxylation of Gla-containing proteins dried from tritiated solvent results in regiospecific exchange of tritium for the departing CO_2 group in Glu (*31*). These tritiated substrates have been valuable for studying carbanion formation (*27, 32*), as the released tritium generates tritiated water (Scheme 2) that can be quantitated. As carboxylation has been shown to occur only at the first position in FLEEL (*33, 34*), we used the peptide FLγEL to generate the tritiated FLEEL. FLγEL was heat decarboxylated after lyophilization from tritiated water, resulting in efficient conversion of FLγEL to FL[*R*,*S*-^{3}H]EEL (Figure 9), which had a specific activity of ~2000 cpm/nmol. FL[*R*,*S*-^{3}H]EEL was purified by HPLC and then used to monitor carbanion formation: H160A and wild-type carboxylase were incubated with FL[*R*,*S*-^{3}H]EEL and tritium release and vitamin K epoxidation were then measured. In the case of the wild-type enzyme, the ratio of tritium release to epoxidation was 1.14 (Table 3), and the stoichiometry of these two reactions was therefore similar to that of carboxylation to epoxidation. In contrast, H160A showed a 0.12 ratio of tritium release to epoxidation (Table 3), which was a 10-fold lower ratio than that of the wild-type enzyme. These results strongly support a role for His160 in carbanion formation.

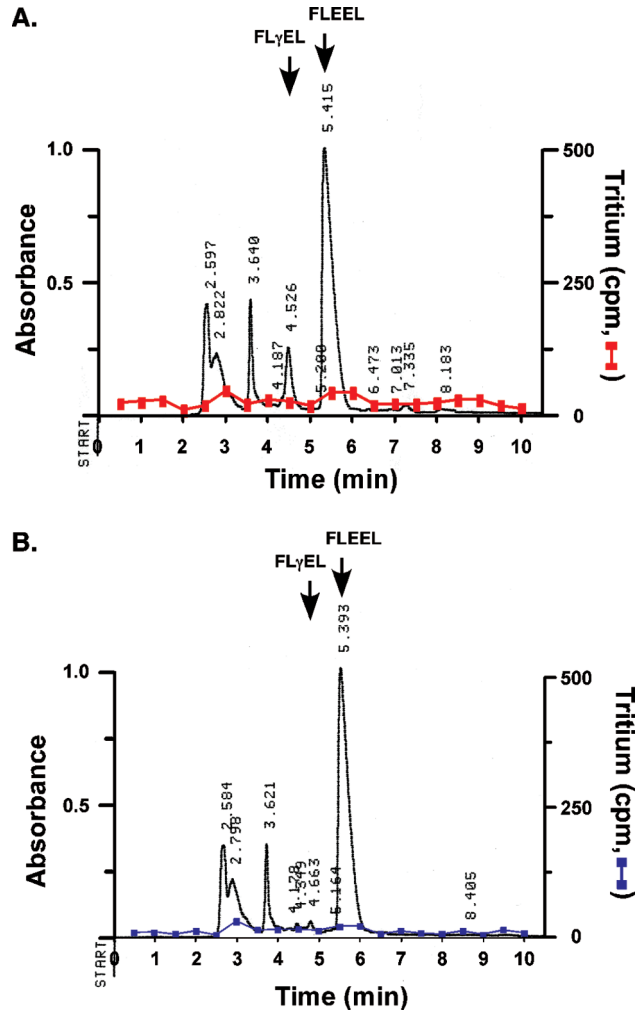


FIGURE 8: H160A shows low levels of carbanion reprotonation. Tritium exchange from solvent into FLEEL was used to measure reprotonation of Glu carbanion. Wild-type (A) and H160A (B) carboxylation was performed (170 μL) using the carboxylation reaction conditions described in the Experimental Procedures except that 1.3 mM [^{14}C]$NaHCO_3$ was replaced by 8 mM sodium bicarbonate, the cocktail contained 9.2 mCi/mL tritiated water (Moravec Biochemicals Inc.), and the KH_2 concentration was 260 μM. A parallel reaction that differed only in the absence of tritium was also performed to measure epoxidase activity. The reactions were incubated for 20 h (over which time both wild-type and H160A carboxylases were found to be stable; data not shown), with two additions (at 5 and 10 h) of 260 μM KH_2. The epoxidase reactions were processed as described in the Experimental Procedures. The carboxylase reactions were boiled and chromatographed on P-2 columns as described in the Experimental Procedures, and after lyophilization, the purified FLEEL was taken up in 25 μL of water. The entire sample was analyzed by reverse-phase HPLC (C18 ODS Hypersil, 250 × 4.6 mm, 5 μm) using isocratic elution with 15% acetonitrile and 50 mM citrate, pH 7.4. The FLEEL substrate and carboxylated FLEEL product (FLγEL) peaks (5.4 and 4.5 min, respectively, in part A) were identified by absorbance at 258 nm and reference to known standards, and both peaks were quantitated by standardization with authentic FLEEL. The amount of tritium incorporation was determined by collecting 500 μL fractions and quantitating by scintillation counting. The chromatograms shown are from duplicate reactions that were analyzed, and the entire experiment was performed twice, giving the same results in both experiments.

DISCUSSION

Vitamin K epoxidation drives Glu carboxylation, and the two reactions are coupled with a 1:1 stoichiometry when

Scheme 2

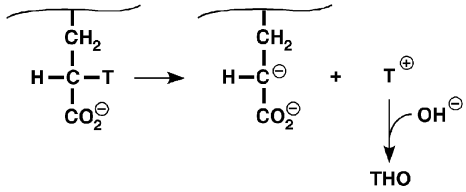


FIGURE 9: Generation of tritiated FLEEL. (A) The FLEEL and carboxylated FLEEL (FLγEL) standards used in the HPLC analysis are shown. (B) FLγEL was heat decarboxylated after lyophilization from tritiated water, and the FLγEL and tritiated FLEEL (i.e., FL[*R*,*S*-^{3}H]EEL) peptides were resolved on a C18 column using HPLC, as detailed in the Experimental Procedures.

Table 3: Substitution of Ala for His160 Impairs Carbanion Formation[a]

carboxylase variant	epoxidation (nmol)	tritium release (nmol)		ratio of tritium release to epoxidation
		absolute	corrected[b]	
wild type	37.8	46.7	43	1.14
H160A	31.9	7.4	3.7	0.12
mock	ND	3.7		

[a] Affinity-purified preparations from insect cells that were either mock-infected or infected with baculovirus containing H160A or wild-type carboxylase were incubated in reaction mixtures (170 μL) whose composition is as described in the Experimental Procedures except for the use of 8 mM sodium bicarbonate, 500 μM KH_2, and 5 mM FLEEL, which was a mixture of FL[*R*,*S*-^{3}H]EEL and unlabeled FLEEL that had a final specific activity of 267 cpm/nmol. The preparation of FL[*R*,*S*-^{3}H]EEL is described in the Experimental Procedures. After 4 h incubation, an aliquot (10 μL) was withdrawn, diluted with 150 μL of H_2O, and then quenched in ethanol/hexane and analyzed for vitamin K epoxidation. The remainder of the reaction was quenched by the addition of 320 μL of 10% perchloric acid, and the samples were frozen, followed by bulb-to-bulb distillation in evacuated Thunberg tubes (Kontes). The residue remaining after one round of distillation was taken up in 250 μL of H_2O and redistilled, and pilot studies that included additional distillations showed that two rounds were sufficient for full recovery of tritium released from FL[*R*,*S*-^{3}H]EEL (data not shown). The distillates for each sample were pooled and quantitated by scintillation counting. ND is not detected. [b] The corrected value has been adjusted for the amount of tritium release observed with the mock-infected control.

substrates are saturating; however, the mechanism for achieving efficient coupling is unknown as residues that regulate coupling have previously not been identified. We now report that His160 is important in the coupling of epoxidation to carboxylation. A variant with His160 substituted by Ala had wild-type levels of epoxidation but impaired carboxylation, i.e., 6% using normal assay conditions (Figure 3). Coupling efficiency is dependent on CO_2 concentration (*27*), and H160A showed a greater degree of uncoupling than the wild-type enzyme at low CO_2 concentrations (Figure 7), as well as impaired CO_2 binding (Figure 6). However, poor CO_2 binding cannot fully account for the inefficient coupling in H160A, as the k_{cat} for carboxylation was substantially decreased in the H160A mutant. Moreover, the decreased CO_2 binding in H160A did not result in appreciable carbanion reprotonation, as assessed by quantitating tritium exchange from solvent into the Glu substrate (Figure 8). This observation raised the question of whether carbanion formation was impaired in H160A, and this possibility was directly tested by measuring hydrogen abstraction from the γ-carbon. When a peptide containing tritium at this position was used as a substrate, H160A showed a 10-fold decrease in tritium release compared to the wild-type enzyme, which was equivalent to the fold decrease in coupling efficiency observed using the same assay conditions (Table 3, Figure 7). These results strongly implicate His160 in having a role in carbanion formation that is ultimately important for the generation of the carboxylated Glu.

A comparison with previously characterized mutant carboxylases shows that impaired carbanion formation in H160A is not explained by poor binding to a Glu-containing substrate, because the K_m of H160A for Glu substrate was only slightly higher than that of the wild-type enzyme (1.6-fold, Table 2). In contrast, significantly higher (7−24-fold) increases in K_m are associated with impaired carboxylase activity when residues that are part of the Glu binding site (i.e., Leu394, Tyr395, and Trp399) are mutated (*35, 36*). Furthermore, Glu binding increases the affinity of the carboxylase for vitamin K (*6*), and substitution of Leu394, Tyr395, or Trp399 results in an increased K_m (~5-fold) for vitamin K (*35, 36*). H160A, however, showed a K_m for vitamin K that was identical to that of the wild-type enzyme (Table 2), which also indicates that His160 is not simply required for binding to Glu. In addition to having an effect on the K_m for vitamin K, Glu binding also activates vitamin K epoxidation by a reverse coupling mechanism that is not yet defined (Figure 1) (*37, 38*). The impaired carbanion formation in H160A was accompanied by only a small effect on this regulatory mechanism: H160A showed only 2-fold less Glu stimulation of epoxidation versus 16-fold less coupling of epoxidation to carboxylation than the wild-type enzyme, when the two types of reactions were assayed under identical conditions (i.e., the same concentration of CO_2 and the presence of propeptide; Table 1). The 2-fold difference in the stimulation of epoxidase activity parallels the small change in K_m for Glu that was observed (Table 2) and further indicates that His160 does not play a dominant role in binding to Glu in the ground state. An important point is that the k_{cat} for carboxylation by the H160A enzyme was much lower than that of the wild-type enzyme in the analyses of all three substrates (i.e., Glu, KH_2, and CO_2, Figure 6 and Table 2), which implicates His160 as playing a catalytic role in Glu carbanion formation. Thus, residues in the ~390−400 amino acid region of the carboxylase position

the Glu for reactivity with His160, which in turn facilitates the formation of the Glu carbanion.

An attractive hypothesis for the mechanistic role of His160 is that its ionic nature facilitates the formation of the transition state for generating the carbanion. As the vitamin K base abstracts a proton from the glutamyl γ-carbon, an aci-carboxylate structure is formed that allows electron redistribution to occur between the γ-carbon and carboxyl group oxygens. Interaction of His160 with the increasing negative charge on a carboxyl group oxygen could stabilize the aci-carboxylate, facilitating carbanion formation by making the γ-carbon more labile for hydrogen abstraction. Loss of the tetrahedral structure at the γ-carbon once deprotonation begins could move the position of the carboxyl group within the active site, thereby resulting in His160 interaction with Glu only after proton abstraction from the γ-carbon is initiated. His160 would therefore selectively stabilize the carbanion and help to draw Glu into the transition state. Hydrogen abstraction from the γ-carbon is stereospecific (*39*), and the configuration of the carboxyl group on the γ-carbon is inverted during carboxylation (*40*). The addition of a second carboxyl group accompanies the inversion and restores the tetrahedral geometry of the γ-carbon, which would terminate the interaction between the original carboxyl group and His160.

An alternative and intriguing possibility for how His160 facilitates the formation of the transition state for the carbanion is that it forms a low barrier hydrogen bond with an oxygen in the aci-carboxylate. These bonds, which are characterized by being short and high energy, are formed when the proton affinities of the acceptor and donor heteroatoms involved in hydrogen bonding are similar (*41–43*). In the case of the carboxylase and Glu substrate, the formation of the aci-carboxylate resonance structure would result in a substantial increase in the pK_a of the carboxyl oxygens, and as the pK_a approached that of a neutral His side chain (pK_a ~14), a low barrier hydrogen bond could form. Strong binding of His160 to the aci-carboxylate versus weaker binding to Glu would therefore selectively stabilize the carbanion and promote the formation of the transition state. Subsequent reaction of the carbanion with CO_2 would eliminate the aci-carboxylate, and the carboxyl groups in the Gla product would now have pK_a's (~4−5) substantially lower than that of His160, resulting in loss of the low barrier hydrogen bond.

The reason why defective carbanion formation in H160A was accompanied by impaired CO_2 binding (Figures 6 and 7) is not obvious. Presumably, the effect on CO_2 binding in H160A is indirect, since the reaction has been reported to be unconcerted, i.e., showing γ-carbon−hydrogen cleavage that is independent of CO_2 concentration (*32*). One possibility is that CO_2 interaction is impaired because the binding site for CO_2, which has not been identified, is impacted by the formation of the carbanion. Thus, the change from a tetrahedral to planar γ-carbon creates space that may allow CO_2 entry, which could explain kinetic analysis showing that CO_2 is likely the last substrate to bind (*44*). In the case of H160A, then, impaired carbanion formation would indirectly block CO_2 access. Alternative explanations are also possible, and further studies will be necessary to determine why substitution of His160 impacts CO_2 interaction, as well as to define the mechanism by which His160 facilitates carbanion formation.

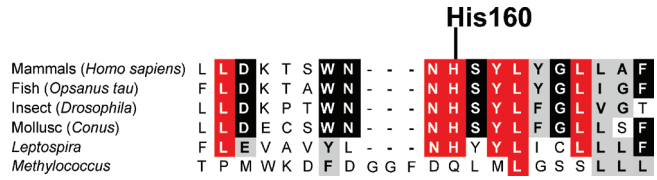


FIGURE 10: His160 is conserved in metazoan and some bacterial VKD carboxylase orthologues. The sequence of the carboxylase region containing His160 is shown for four evolutionarily distinct metazoans and two bacteria, *L. borgpetersenii* and *Methylococcus capsulatus*. Alignment of the sequences was performed as previously described (*20, 47*). The red and black highlighting indicates residues that are identical in at least five or four proteins, respectively. The gray highlighting indicates chemically similar residues present in at least four proteins.

Propeptide stimulates both epoxidase and carboxylase activity through an unknown mechanism, and this effect was much larger (~10-fold) in H160A than in the wild-type enzyme (Table 1). The increased propeptide effect was due to the fact that the basal activity of the wild-type enzyme in the absence of propeptide (and presence of Glu substrate) was much higher than that of the H160A mutant. The difference in propeptide activation does not appear to be a direct effect due to impaired propeptide binding in H160A because the propeptide concentration for 50% activity was similar for both enzymes (Figure 5). In contrast, mutants with substitutions in a region that has been proposed as the propeptide-binding site require substantially higher concentrations of propeptide than the wild-type enzyme to achieve the same level of propeptide activation (*45*). The H160A response is similar to what has been observed with mutants substituted in the residues discussed above that are part of the Glu-binding site (*35, 36*). Those mutants may show larger responses to propeptide than the wild-type enzyme because of the correlation between propeptide and Glu binding (*5, 46*); however, the small change in K_m for Glu in H160A makes a similar explanation for this mutant unlikely. A mechanism in which propeptide stimulation of activity occurs through an activating conformational change could explain the results with H160A. Thus, the substitution of His160 by Ala may cause a structural perturbation in the active site that is partially inactivating in the absence of propeptide. The presence of propeptide and consequent conformational change, then, would rescue this perturbation and cause the much larger increase in activity for H160A than the wild-type enzyme that was observed. As new active site residues are revealed, it will be of interest to determine whether differences in response to propeptide becomes a general theme.

The evolutionary conservation of His160 is of interest with regard to the function of the VKD carboxylase in different organisms. His160 is present in all VKD orthologues identified in metazoans (Figure 10), where the function of the orthologues has clearly been established as carboxylation either by the demonstration of carboxylase activity or by the identification of VKD proteins in the organisms. VKD carboxylase orthologues have also been detected in bacteria, which are thought to have acquired the orthologues by horizontal gene transfer from metazoans (*47*). Only a subset of these orthologues has retained His160. An example is an orthologue in the pathogen *L. borgpetersenii*, which is the

only bacterial VKD carboxylase orthologue analyzed so far for activity. This orthologue exhibits efficient vitamin K epoxidation but no detectable Glu carboxylation and has a divergent structure that lacks the Glu-binding site conserved in all known metazoan VKD carboxylases (*20*). These observations suggested that *Leptospira* has adapted the VKD orthologue for a function other than Glu carboxylation, which led to the proposal that vitamin K epoxidation is used to drive a new reaction or to cause oxidative damage or to deplete vitamin K to indirectly inhibit VKD protein carboxylation in the infected host (*20*). The retention of His160 in the VKD orthologue supports the possibility of a new reaction: thus, vitamin K epoxidation still appears to be used to generate a negatively charged intermediate which is stabilized by interaction with His160, but in a reaction that is distinct from Glu carboxylation. The VKD carboxylase orthologues acquired by most bacterial organisms have actually lost His160 (e.g., *Methylococcus*, Figure 10) (*47*). While these orthologues have not been analyzed, they likely have epoxidase activity because they have all retained Lys218, the residue that initiates epoxidation by deprotonating reduced vitamin K (*26*). The lack of His160 in this class of bacterial VKD orthologues, however, would suggest a role for vitamin K epoxidation different from that used by *Leptospira*. Thus, bacteria may have adapted the VKD carboxylase for at least two novel functions.

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