Brønsted Analysis Reveals Lys218 as the Carboxylase Active Site Base That Deprotonates Vitamin K Hydroquinone To Initiate Vitamin K-Dependent Protein Carboxylation†

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ABSTRACT: The vitamin K-dependent (VKD) carboxylase converts Glu's to carboxylated Glu's in VKD proteins to render them functional in a broad range of physiologies. The carboxylase uses vitamin K hydroquinone (KH_2) epoxidation to drive Glu carboxylation, and one of its critical roles is to provide a catalytic base that deprotonates KH_2 to allow epoxidation. A long-standing model invoked Cys as the catalytic base but was ruled out by activity retention in a mutant where every Cys is substituted by Ala. Inhibitor analysis of the cysteine-less mutant suggested that the base is an activated amine [Rishavy et al. (2004) *Proc. Natl. Acad. Sci. U.S.A. 101*, 13732−13737], and in the present study, we used an evolutionary approach to identify candidate amines, which revealed His160, His287, His381, and Lys218. When mutational analysis was performed using an expression system lacking endogenous carboxylase, the His to Ala mutants all showed full epoxidase activity but K218A activity was not detectable. The addition of exogenous amines restored K218A activity while having little effect on wild type carboxylase, and pH studies indicated that rescue was dependent upon the basic form of the amine. Importantly, Brønsted analysis that measured the effect of amines with different pK_a values showed that K218A activity rescue depended upon the basicity of the amine. The combined results provide strong evidence that Lys218 is the essential base that deprotonates KH_2 to initiate the reaction. The identification of this base is an important advance in defining the carboxylase active site and has implications regarding carboxylase membrane topology and the feedback mechanism by which the Glu substrate regulates KH_2 oxygenation.

The vitamin K-dependent (VKD)[1] carboxylase is a resident endoplasmic reticulum enzyme that converts specific Glu's to carboxylated Glu's (Gla's) in VKD proteins during their secretion (*1*). The VKD proteins contain a domain, usually a propeptide, that mediates their high-affinity binding to the carboxylase and that also modulates carboxylase binding to Glu's and vitamin K (*2*−*4*). The propeptide facilitates the processive carboxylation of clusters of Glu's in VKD proteins (*5*, *6*), which generates a calcium-binding module that allows these proteins to bind either to hydroxyapatite in the extracellular matrices or to cell surfaces where anionic phospholipids become exposed. Carboxylation allows mammalian VKD proteins to participate in diverse functions that include hemostasis, apoptosis, growth control, bone morphogenesis, calcium homeostasis, and signal transduction (*7*). The broad physiological impact of the carboxylase therefore highlights the importance of understanding its mechanism.

VKD carboxylation is also important to nonmammalian organisms, because the VKD carboxylase has been identified in fish, *Conus*, and *Drosophila* (*8*−*11*). In fish, a role for carboxylation in hemostasis has been indicated by the identification of hemostatic VKD proteins (*12*), while in *Conus*, the function is predation, as demonstrated by the ability of VKD peptides to antagonize neurotransmission when injected into prey (*13*). The function of VKD carboxylation in *Drosophila* is not known, because VKD proteins have not yet been identified. Our recent studies show that a VKD carboxylase orthologue is also present in an organism outside of the animal kingdom, i.e., the bacterial pathogen *Leptospira interrogans*, which most likely acquired the carboxylase by horizontal gene transfer during the infection of an animal host (*14*). Interestingly, the *Leptospira* VKD orthologue shows altered enzymatic activity from that of VKD carboxylases in the animal kingdom. Thus, in metazoans, the carboxylase is a bifunctional enzyme that uses the energy of vitamin K hydroquinone (KH_2) epoxidation to drive Glu carboxylation. The *Leptospira* VKD orthologue, however, performs KH_2 epoxidation but not Glu carboxylation (*14*), and *Leptospira* therefore appears to have adapted KH_2 epoxidation for some other function that is not yet known.

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[1] Abbreviations: VKD, vitamin K-dependent; KH_2, vitamin K hydroquinone; KO, vitamin K epoxide; Gla, γ-carboxylated Glu; NEM, *N*-ethylmaleimide; DEPC, diethylpyrocarbonate.

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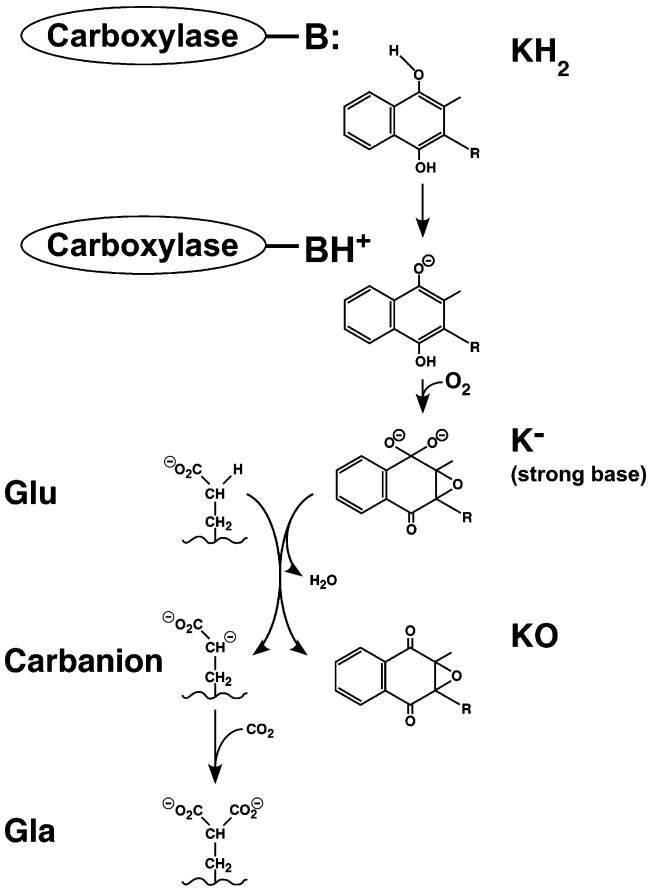

FIGURE 1: Base amplification model for VKD protein carboxylation. A carboxylase active site basic residue (B:) deprotonates reduced vitamin K (KH_2), and the deprotonated form then reacts with O_2 to form a highly reactive strong base (e.g., the dialkoxide form shown, K^-). Deprotonation of glutamyl residues (Glu) by this strong base results in the formation of a carbanion, which reacts with CO_2 to form a γ-carboxylated Glu (Gla), while the vitamin K base is protonated to form vitamin K epoxide (KO).

Determining how KH_2 epoxidation drives Glu carboxylation has been challenging, because both half-reactions are unique and therefore without enzymatic precedence, and a model for the overall reaction has been proposed on the basis of combined biochemical- and chemical-modeling studies. Biochemical experiments indicated that Glu carboxylation involves the formation of a γ-glutamyl carbanion, which attacks CO_2 to form Gla (Figure 1). Evidence for the carbanion included the observations that tritium exchange into Glu is detected in the presence of tritiated water (*15*) and that fluoride is eliminated from (2*R*,3*S*)-3-fluoroglutamate (*16*). The formation of the γ-glutamyl carbanion, which has a pK_a of around 25−28, would require a strong base, far stronger than could be provided by any protein side chain. A potential mechanism for generating the carbanion through the coupling of KH_2 epoxidation to Glu carboxylation was uncovered by chemical modeling experiments. Those experiments (*17*) showed that the reaction of KH_2 with O_2, a required substrate of the carboxylase, led to the formation of a vitamin K intermediate (K^- in Figure 1) with sufficient basicity to abstract the hydrogen from Glu to generate the γ-glutamyl carbanion. However, the KH_2 would first require deprotonation to react with O_2. Therefore, one critical role of the carboxylase is to provide an active site base that initiates the reaction by deprotonating KH_2. This mechanism proposed by Dowd et al. (*17*), in which a weak active site base is used to generate a strong vitamin K base, has been termed the "base amplification model" (Figure 1).

The identity of the carboxylase catalytic base that deprotonates KH_2 was previously unknown. Only a few functional residues have been identified, because the carboxylase is a large integral-membrane enzyme without structural determinations from X-ray crystallography or nuclear magnetic resonance (NMR) and related families of enzymes that might suggest functional residues have not been identified. Further, the formation of covalent intermediates with substrates, which can often identify critical residues, is not predicted by the base amplification model (Figure 1) and has not been observed. Studies showing that the carboxylase is strongly inactivated by thiol-specific reagents like *N*-ethylmaleimide (NEM) led to the long-standing acceptance of a Cys as the critical active site base (*18*−*23*). We performed biochemical mapping, which indicated that the carboxylase is multiply modified by NEM (*24*). In a series of experiments that ultimately led to the generation of a cysteine-less carboxylase mutant with all 10 Cys residues substituted by Ala, we showed that Cys does not act as the catalytic base (*25*). Thus, if the base was a Cys residue, then its substitution by Ala, which cannot ionize to act as a base, would mean that in the cysteine-less mutant KH_2 deprotonation would depend solely upon the pK_a of KH_2. This value (pK_a $\sim$ 12 or higher if modulated by a hydrophobic active site) is substantially above the carboxylase pH optimum (pH $\sim$ 7), and therefore, the activity in the cysteine-less mutant would be predicted to be several orders of magnitude lower than that of wild type carboxylase. The mutant, however, retained 7% activity (*25*), indicating that Cys does not function as the active site base that deprotonates KH_2. Chemical inactivation of the cysteine-less mutant suggested that, instead, the base was an activated amine (*25*).

To identify the carboxylase active site base, we used chemical rescue of activity in combination with evolutionary comparison and mutagenesis. Chemical rescue has been a powerful approach for identifying functional residues in several enzymes (*26*−*36*). The ability to replace an enzyme side chain with a series of exogenous reagents allows for a direct comparison between the physical properties of the reagents and their effect on catalysis in a quantitative manner, which permits hypotheses about the mechanistic role of the side chain to be tested and is therefore highly diagnostic. As indicated below, the combined studies provide compelling and specific evidence that Lys218 acts as a general base in the mechanism of the carboxylase.

EXPERIMENTAL PROCEDURES

Construction of Baculoviruses Containing FLAG-Tagged Carboxylase Mutants and Expression in Infected SF21 Cells. All carboxylase mutants were constructed in wild type carboxylase-FLAG in pBacPAK1 (*25*), which expresses the 758 amino acid human VKD carboxylase bearing a C-terminal extension of AAADYKDDDDK, where the last eight amino acids are the FLAG epitope.

H160A and H287A. Single-overlap polymerase chain reaction (PCR) was used to generate a plasmid that contained both the H160A and H287A mutations, and each individual mutation was then subcloned into the remainder of the full-

length carboxylase cDNA to generate carboxylases bearing either the H160A or H287A mutation. PCR was performed using the primers GC430S and HA1AS or HA1S and HA2AS (see Table S1 in the Supporting Information) with wild type carboxylase-FLAG in pBacPAK1 as the template. Products were isolated, and the fragments were then fused by PCR using the GC430S and HA2AS primers. The product was gel-isolated and then cloned into pCR2.1-TOPO, followed by sequencing of both strands of the plasmid. A 0.37 kb *Bst*X I−*Nco* I fragment bearing only the H160A mutation and a 0.19 kb *Nco* I−*Eco*R I fragment containing only the H287A mutation were then isolated, and each fragment was subcloned to generate individual mutations in full-length carboxylase-FLAG in pBacPAK1.

H381A. The H381A mutant was also generated using overlap PCR. The first two PCR reactions used primers GC10A978S and HA3AS or HA3S and GC1309AS, and the overlap PCR to generate the fused product used the primers GC10A978S and GC1309AS. The final product was cloned into pCR2.1-TOPO, followed by sequencing of both strands of the plasmid. The 0.35 kb *Eco*R I−*Pml* I fragment containing the H381A mutation was isolated and subcloned into carboxylase-FLAG in pBacPAK1.

K218A. Overlap PCR was used to generate K218A. The first two PCR reactions used primers GC430S and GCK218A2 or GCK218A1 and GC1309AS, and the overlap PCR to generate the fused product used the primers GC430S and GC1309AS. The final product was cloned into pCR2.1-TOPO and then sequenced. A 0.53 kb *Bst*X I−*Eco*R I fragment was isolated and subcloned into carboxylase-FLAG in pBacPAK1.

To generate baculoviruses containing the mutant carboxylases, the individual plasmids were cotransfected with BacPAK6 into SF21 cells as before (*37*), followed by plaque purification and screening by a Western using anti-FLAG antibody (*14*). Positively identified viral plaques were then used to generate preparative stocks of each virus. For each mutant, two independent plasmids were transfected and analyzed, yielding identical results.

Carboxylase Isolation. Microsomes were prepared from SF21 cells (6×10^8) as previously described (*25*). Samples were adjusted to a final protein concentration of 4 mg/mL, as determined by a BCA (Pierce) and then solubilized with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and phosphatidyl choline (0.5 and 0.1% respective final concentrations), followed by centrifugation at 100000*g* for 1 h, all at 4 °C. In one experiment, the solubilization of wild type carboxylase and the mutant K218A was performed in the presence or absence of factor X propeptide (5 μM) to test the effect of propeptide on K218A stability during isolation. In another experiment, each carboxylase form was coexpressed with baculovirus containing factor IX (at multiplicities of infection of 5 and 1, respectively), as before (*25*), to determine if factor IX influenced the stability and consequent activity of K218A.

Purified carboxylase was isolated by affinity purification using anti-FLAG antibody. Solubilized microsomes (500 μL) were nutated overnight with 100 μL anti-FLAG agarose (Sigma), and the resin was then washed by four successive rounds of centrifugation (10000*g*, 15 s) and incubation for 1 min in 1 mL of 25 mM Tris·HCl, 0.25% CHAPS, 0.25% phosphatidyl choline, and 500 mM NaCl at pH 7.4, all at 4 °C. The enzyme was eluted by incubating the resin with 400 μL of the same buffer but containing FLAG peptide (100 μg/mL, Sigma) and factor X propeptide (5 μM, added to stabilize the enzyme) for 1 h at 20 °C, followed by centrifugation (10000*g*, 15 s) to recover the free carboxylase.

Activity Assays. Epoxidase activity was measured in duplicate in reaction mixtures containing final concentrations of 0.5 M NaCl, 0.06% CHAPS, 0.06% phosphatidyl choline, 0.06% sodium cholate, 2.5 mM Boc-Glu-Glu-Leu-OMe (Bachem), 10 μM factor X propeptide, 2.5 mM dithiothreitol (DTT), 1.3 mM sodium bicarbonate, 200 μM KH_2, and either 50 mM BES at pH 6.6 or a tripartite buffer that comprised 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)/25 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)/25 mM TRICINE, which was titrated to the desired pH with NaOH. The KH_2 was prepared by reduction of phylloquinone (Sigma) (*38*). Reactions were incubated at 20 °C for 1 h and then quenched by the addition of 3:2 (vol/vol) 2-propanol/hexane. An internal standard (2 nmol K25, GL Synthesis) was added immediately after quenching to correct for the recovery of vitamin K. Samples were vortexed, and the organic phase was then dried under N_2 and analyzed by high-performance liquid chromatography (HPLC) as previously described (*14*). The epoxidase specific activity was determined by processing epoxidase activity assays in parallel with a quantitative Western of the samples using an antibody-linked fluorescence assay. Protein samples were gel-electrophoresed along with a standard FLAG-BAP fusion protein (1.5 pmol, Sigma), and the gel was processed in a Western using anti-FLAG antibody (0.4 μg/mL), doubly purified anti-rabbit alkaline phosphatase conjugate (Bio-Rad), and AttoPhos substrate (Promega, used according to the instructions of the manufacturer) followed by quantitation on a StormImager.

Carboxylase activity was assayed in duplicate using the same reaction mixture as described above for the epoxidase assay, except that sodium bicarbonate was replaced by [^{14}C]-$NaHCO_3$. Incubation was for 1 h, followed by quenching with trichloroacetic acid (10% final concentration). The samples were then processed for scintillation counting as described (*37*).

The pH dependence of the carboxylase was determined by assaying solubilized microsomes for epoxidase activity in the MES/MOPS/TRICINE tripartite buffer ranging in pH from 5.5 to 8.5. To test for carboxylase stability over this pH range, samples were first incubated for 1 h at 20 °C in 32 mM buffer at pH 5.5, 6.5, 7.5, or 8.5. The samples were then assayed for carboxylase activity in a reaction cocktail containing 150 mM *N*,*N*-bis(2-hydroxyethyl)-2-amino ethanesulfonic acid (BES) buffer at pH 7.0. The variation in activities was ∼10−15%, indicating that the carboxylase was stable over the pH range tested.

Amine Rescue of K218A. Wild type and K218A carboxylases were tested for ethylamine rescue by incubation in the carboxylase or epoxidase reaction mixtures in the absence or presence of 60 mM ethylamine. The reaction was performed using the MES/MOPS/TRICINE tripartite buffer adjusted to pH 8.5, and incubation was for 1 h. To test the pH dependence of ethylamine rescue, K218A epoxidase activity was measured in the presence of 60 mM ethylamine using the tripartite buffer adjusted to several different pH values, as indicated in the Results.

Table 1: Physical Constants of Amines Used for Rescue of K218A Activity[a]

amine	pK_a	molecular volume (Å^{-3})
methyl-	10.6	42.1
ethyl-	10.6	60.9
propyl-	10.5	79.8
butyl-	10.6	98.7
ethanol-	9.5	71.5
2-fluoroethyl-	9.0	64.4
2-cyanoethyl-	7.7	70.5
2,2,2-trifluoroethyl-	5.7	71.6

[a] Values were taken from Toney and Kirsch (*26*).

Brønsted Analysis of Amine-Rescued K218A. The effect of a series of amines (Table 1) on epoxidase activity was assayed by incubation for 1 h using the tripartite buffer adjusted to pH 8.5 and a range (20−80 mM) of amine concentrations. The observed epoxidation rate constants, k_{obs} (h^{-1}), were determined for each amine from the rate of epoxide formation, v (nM h^{-1}) and enzyme concentration (measured by a quantitative Western), using eq 1.

$$v = k_{obs}[\text{enzyme}] \quad (1)$$

All carboxylase substrates were present at saturating concentrations, so that the rate of epoxide formation is given by eq 2,

$$v = k_B[\text{amine}][\text{enzyme}] \quad (2)$$

where k_B (M^{-1} h^{-1}) is the second-order rate constant for amine rescue. The correction for the amount of each amine present in the basic form at pH 8.5 and the determination of the second-order rate constant for each amine were accomplished by performing linear regression analysis using eq 3, where K_a is the acid dissociation constant for the amine and k_{sol} is the rate constant in the absence of amine (which was 0 throughout).

$$k_{obs} = \frac{k_B[\text{amine}]_{total}}{1 + ([H^+]/K_a)} + k_{sol} \quad (3)$$

For Brønsted analysis, the logarithm of the resulting k_B values for a set of amines was used as the dependent variable along with the molecular volume and pK_a of the amines (Table 1) as independent variables in multiple linear regression analysis using the LINEST function within Excel.

RESULTS

A Bioinformatic Approach for Identifying Candidate Active Site Base Residues. Carboxylase sequences from evolutionarily distinct organisms were compared to identify conserved residues that might potentially be the active site base. The *L. interrogans* VKD orthologue was particularly valuable in this comparison because it is significantly divergent from the metazoan carboxylases but has retained KH_2 epoxidase activity, with a specific activity similar to that of human carboxylase (*14*). Activity retention indicates the conservation of active site side chains, like the catalytic base, which are required for KH_2 epoxidation, and therefore, the sequence alignment of the *Leptospira* VKD orthologue with the metazoan carboxylases could reveal candidate active site base residues. The aligned sequences were first examined for His residues, because we observed rapid inactivation of the carboxylase by diethylpyrocarbonate (DEPC) (see Figure S1 in the Supporting Information), which is usually highly specific for His (*39*). Alignment of VKD carboxylases from the animal kingdom (i.e., mammals, fish, insect, and mollusc) with that of the VKD *L. interrogans* orthologue revealed three conserved His residues: His160, His287, and His381 (Figure 2A). Only one mammalian VKD carboxylase is shown in the alignment in Figure 2, to avoid biasing one class of metazoan carboxylases over another; however, multiple mammalian VKD carboxylases have been identified (*40*−*42*) and examination of these orthologues showed that they all contain the three His residues. Functional analysis was therefore performed on these residues.

The Active Site Base Is Not a His Residue. His160, His287, and His381 in the human carboxylase were individually mutated to Ala, and the mutants were then tested for activity. Substitution to Ala was chosen because this residue cannot ionize to act as a base and therefore would result in inactivation if the corresponding His functioned as the catalytic base. The mutants were expressed in SF21 cells, which do not contain endogenous carboxylase activity but can synthesize active enzyme that is exogenously introduced (*43*). All three mutants exhibited robust expression, as indicated by Western analysis (Figure 3A). The specific activities of the mutants were determined by performing the vitamin K epoxidase assay in parallel with a quantitative Western, which showed that all three mutants exhibited substantial activity similar to that of wild type enzyme (Table 2). These results indicate that a His residue does not act as the active site base.

A Carboxylase with Lys218 Substituted by Ala Lacks Detectable Activity. The activity of the His mutants (Table 2) raises the question of the mechanism of DEPC inactivation of the carboxylase (see Figure S1 in the Supporting Information). Modification of a noncatalytic histidine could cause inactivation through steric hindrance, or alternatively, cross-reactivity of DEPC with a residue other than histidine could cause inactivation. Reactivity of active site lysine residues with DEPC has previously been observed (*44*−*46*). Because of these considerations, we searched the aligned carboxylase sequences for conserved Lys residues, which revealed only one such amino acid, i.e., Lys218 (Figure 2B). Interestingly, a previous experiment by others (*47*) showed that a bovine carboxylase mutant with Lys218 substituted by Ala had reduced activity. In that analysis, the mutant was expressed in mammalian cells that contain endogenous wild type enzyme, and therefore, it was not possible to distinguish whether the K218A mutant had a low level of activity or was completely inactive, as would be expected for a mutant substituted in the active site base. We therefore generated K218A and expressed this mutant in SF21 cells to determine the activity level without interference from endogenous wild type carboxylase.

K218A was expressed at levels similar to that of the wild type enzyme (Figure 3B) but did not exhibit detectable epoxidase activity (Table 3). We previously found that the stability and consequent activity of some carboxylase mutants depends upon the presence of either VKD propeptide or VKD protein during isolation (*25*). K218A was therefore isolated in the presence or absence of propeptide and was also isolated from SF21 cells coexpressing both K218A and

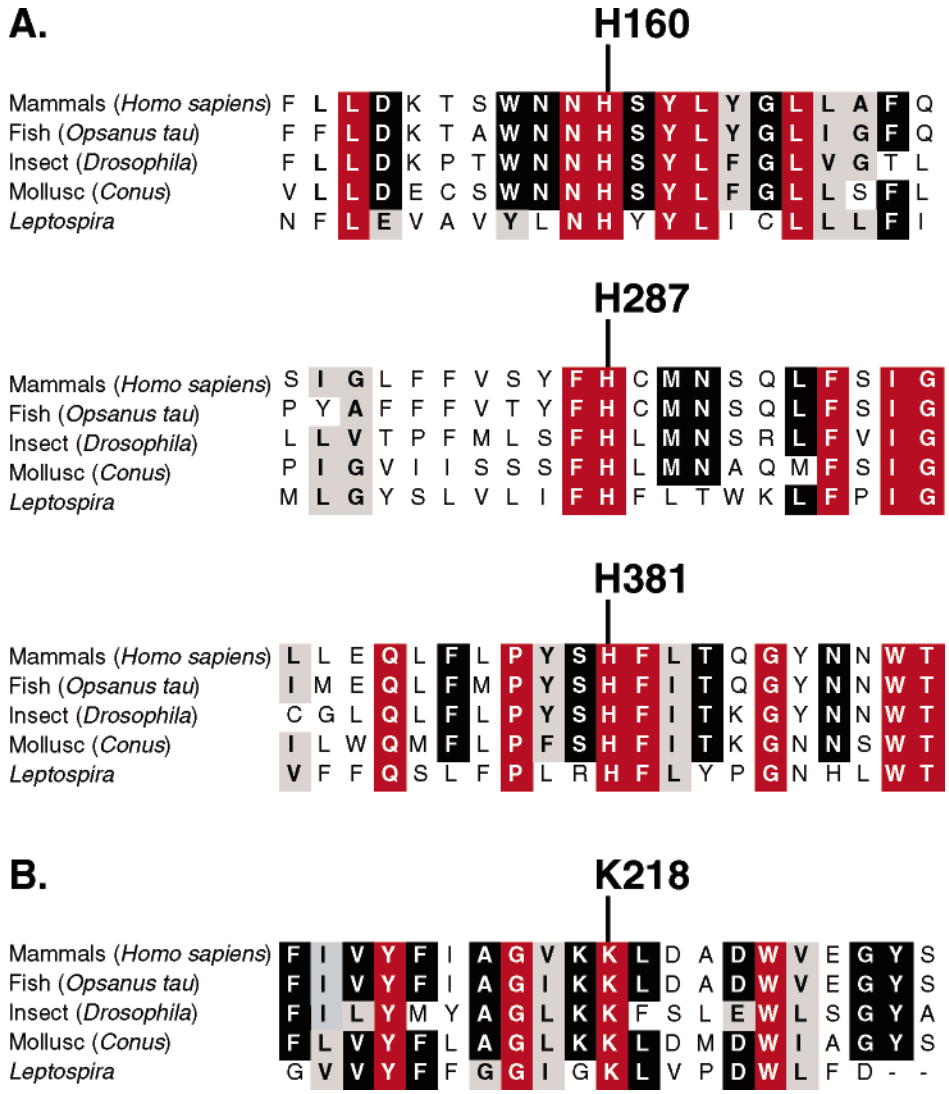

FIGURE 2: Regions of the VKD carboxylase orthologues containing conserved amine residues. Carboxylase protein sequences from four evolutionarily distinct metazoans (two vertebrates, a mollusc, and an insect) and one bacterium that has an orthologue with VKD epoxidase activity (*Leptospira*) were aligned using the ClustalW algorithm as previously described (*14*). Residues that are identical in all five proteins are highlighted in red; those identical in four of five proteins are highlighted in black; and chemically similar residues present in at least four proteins are highlighted in gray. The only conserved His (A) or Lys (B) residues present in all five VKD carboxylase orthologues are shown, along with neighboring residues and the position of the conserved residue in the human VKD carboxylase.

factor IX. However, neither manipulation resulted in detectable K218A activity (data not shown). The results, then, suggested that Lys218 could be the catalytic base.

K218A Activity Is Restored by Exogenous Amines. The structural difference between Lys and Ala made it possible to determine if the Ala substitution resulted in the loss of activity because of chemical deficiency or because of some indirect effect, for example, structural perturbation of the enzyme. Thus, the substitution of an Ala for Lys leaves space in the active site into which small amines can diffuse. Exogenous amines have been shown to rescue the activity of Lys-substituted mutants in other enzymes (*26*−*33*), where the characteristics of amine rescue clarified the catalytic role of the missing Lys side chain. When wild type and K218A carboxylases were incubated in the presence or absence of ethylamine, we observed substantial epoxidase activity recovery in K218A but only a small (1.2-fold) effect of the amine on the wild type enzyme (Figure 4). The apparent specific activity of rescued K218A was only 2% of that of wild type carboxylase, as determined by combined epoxidase activity and quantitative Western analysis. This value, however, was condition-dependent, and the actual specific activity may be much higher, because ethylamine rescue was not saturable (Figure 5A). Nonsaturability indicates weak amine binding and is consistent with the results obtained with other rescued enzymes that have been studied (*26*, *28*−*31*, *33*, *34*, *36*).

If ethylamine rescues K218A activity through restoration of a catalytic base, then only the basic form of ethylamine would be predicted to restore activity. We therefore tested how changes in pH and the consequent amount of the basic form affected activity rescue. This analysis revealed that ethylamine, which has a pK_a of 10.6 (Table 1), resulted in enhanced rescue of K218A epoxidase activity as the pH increased (Figure 5B). A different amine, 2-cyanoethylamine, also showed enhanced activity restoration with an increase in pH (data not shown), showing that the effect was not specific to ethylamine. The effect of the increase in pH was on the amine and not the carboxylase, because wild type carboxylase showed a decrease in activity over the same pH

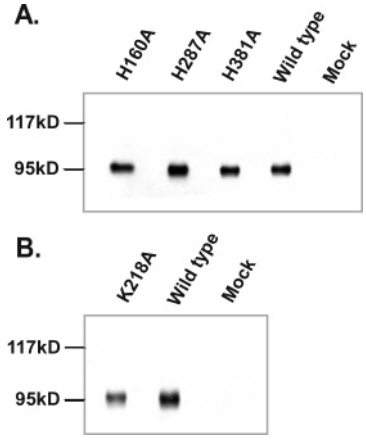

FIGURE 3: Expression of carboxylase mutants in SF21 cells. Solubilized microsomes were prepared from SF21 cells that were mock-infected or infected with baculoviruses containing wild type carboxylase or carboxylase mutants with Ala substituted for evolutionarily conserved His (A) or Lys (B) residues. Western analysis was then performed using an anti-FLAG antibody that reacts with the FLAG epitope appended to the carboxylase C terminus.

Table 2: Carboxylases Mutated in Evolutionarily Conserved His Residues Are Active[a]

carboxylase variant	specific activity (pmol of KO μg^{-1} h^{-1})
wild type	10 000
H160A	9400
H287A	16 300
H381A	14 200
mock	0

[a] Solubilized microsomes prepared from SF21 cells that were mock-infected or infected with baculoviruses containing the indicated carboxylases were assayed for protein by a quantitative Western and for activity by an epoxidase assay that measures the conversion of KH_2 to KO.

Table 3: K218A Mutant Lacks Detectable Epoxidase Activity[a]

carboxylase variant	specific activity (pmol of KO μg^{-1} h^{-1})
wild type	10 000
K218A	0
mock	0

[a] The specific epoxidase activity was determined as described in the footnote of Table 2.

range (Figure 6). The pH dependence of amine rescue of K218A activity therefore indicated that the restoration of activity was due to the basic form of the amine. These results provide strong evidence that Lys218 acts as a general base catalyst for vitamin K epoxidation.

Glu carboxylation is driven by vitamin K epoxidation, and we therefore tested whether ethylamine could also rescue this activity in K218A. As shown in Figure 7, ethylamine restored Glu carboxylation activity in K218A while having no effect on the wild type enzyme. In wild type carboxylase, the epoxidation and carboxylation reactions are coupled such that an approximate 1:1 ratio of each half-reaction is usually observed (*48*). When the ratio of epoxidation to carboxylation for amine-rescued K218A and wild type carboxylase was measured in a single experiment, K218A showed coupling that was the same as that of the wild type enzyme (data not shown), indicating that amine-rescued K218A was similarly regulated.

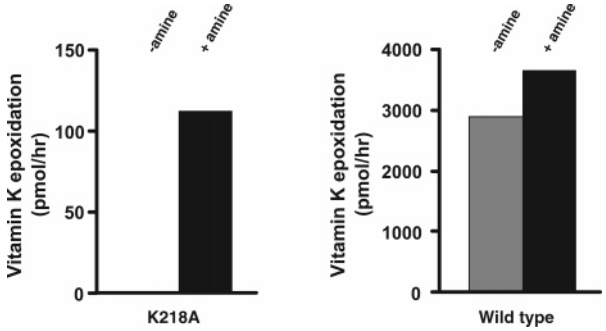

FIGURE 4: Amine rescue of K218A epoxidase activity. Similar levels of affinity-purified wild type or K218A carboxylase protein were assayed for KH_2 epoxidation at pH 8.5 in the absence or presence of 60 mM ethylamine.

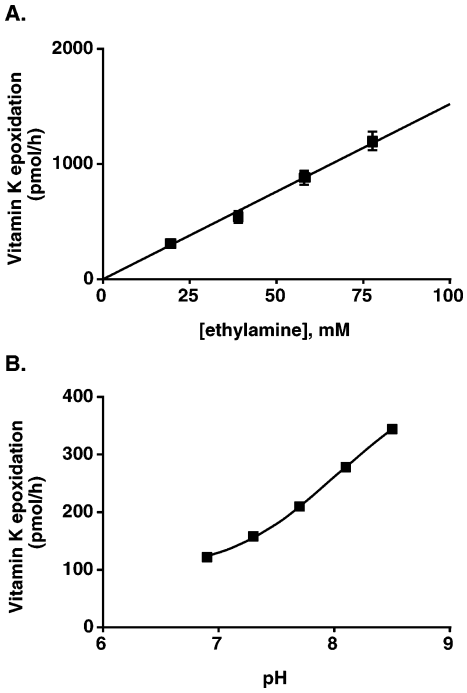

FIGURE 5: Amine rescue of K218A activity is dose-dependent and requires the free base form. (A) The effect of the amine concentration on K218A activity rescue was tested by performing the vitamin K epoxidase assay with K218A carboxylase (250 nM) at pH 8.5 in reaction mixtures that were identical except for the amount of ethylamine. (B) The pH dependence of amine rescue was determined by performing the epoxidase assay using 60 mM ethylamine in reaction mixtures that were identical except for the pH. A tripartite buffer system comprising MES, MOPS, and TRICINE was used to control for variations as a result of the buffer.

Brønsted Analysis Reveals That Amine Rescue of K218A Activity Is Dependent upon the Basicity of the Amine. If Lys218 acts as a general base catalyst during KH_2 epoxidation, then amine rescue of K218A should depend upon the basicity of the amine, such that a stronger base should restore more activity. In this case, the logarithm of the second-order rate constant k_B for the amine rescue of K218A should be linearly related to the pK_a of the rescuing amine. We therefore performed Brønsted analysis for the amine rescue of K218A to determine if the Lys218 amine is the base that initiates KH_2 epoxidation.

One additional factor that must also be considered in Brønsted analysis of an enzyme-catalyzed reaction is the molecular volume of the amine, because access of the amine into the active site may have steric constraints (*26*).

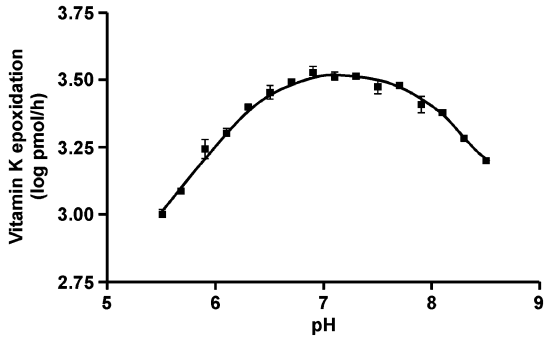

FIGURE 6: Wild type carboxylase activity decreases above pH 7.5. Wild type carboxylase was assayed for vitamin K epoxidase activity in a reaction mixture containing the MES/MOPS/TRICINE tripartite buffer system, which was adjusted to the indicated pH values. As described in the Experimental Procedures, the stability of enzymatic activity over this pH range was also tested by first incubating the carboxylase at different pH values and then assaying for activity at a single pH. This test showed that the carboxylase was stable over the 5.5−8.5 pH range studied and that the change in activity shown here is due to enzymatic rather than stability differences.

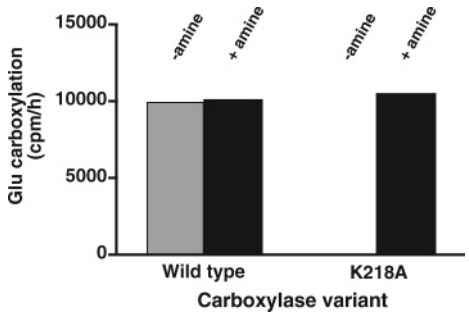

FIGURE 7: Amine rescue of K218A carboxylase activity. Amine rescue was performed as in Figure 4, except that similar amounts of carboxylase activity rather than protein were used and carboxylation of a Glu-containing substrate was assayed.

Consequently, a correction for the dependence of k_B on the molecular volume of the amine must also be determined. We therefore first measured the molecular volume dependence by testing the ability of a series of amines with identical pK_a values (i.e., 10.6) but with different molecular volumes (42−99 Å^{-3}, Table 1) to rescue K218A activity. Incubation of K218A with increasing concentrations of methyl-, ethyl-, propyl-, or butylamine all resulted in a linear dose response (e.g., as in Figure 5A), from which k_B was determined (eqs 2 and 3 in the Experimental Procedures). A plot of log k_B versus the molecular volume for the amines revealed a linear dependence of rescue on molecular volume, with a slope of −0.018 Å^{-3} (Figure 8).

To determine the dependence of rescue on basicity, a series of amines with differing pK_a values and molecular volumes (Table 1) were then assayed for the rescue of K218A activity. log k_B was determined for each amine (eq 3 in the Experimental Procedures), and these determinations were used with the molecular volume and pK_a values of each amine in multiple linear regression analysis, according to eq 4 (*26*)

$$\log k_B = (\beta)(pK_a) + (V)(\text{molecular volume}) + C \quad (4)$$

where β is the Brønsted coefficient for amine rescue, which reflects the degree of proton transfer in the transition state, and V is the molecular volume dependence (*26*). C is a

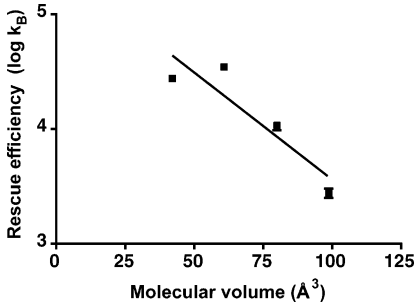

FIGURE 8: Molecular volume dependence of amine rescue of K218A activity. The K218A carboxylase was assayed for activity in the presence of four different amines (the first four shown in Table 1), having the same pK_a but different molecular volumes. In each case, a range of amine concentrations (20−80 mM) was used to determine the second-order rate constant k_B for the rescue of K218A epoxidase activity, as described in the Experimental Procedures. The inverse proportionality of rescue efficiency to the size of the rescuing amine indicates steric constraints upon the binding of the amine in the active site.

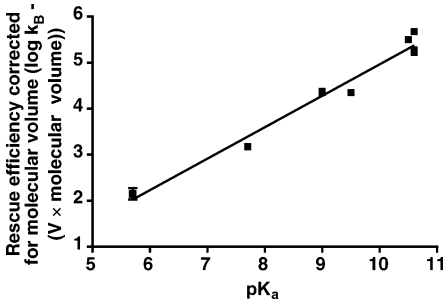

FIGURE 9: Brønsted analysis implicates Lys218 as a general base catalyst. A series of amines with different pK_a and molecular volumes (Table 1) was used to determine the second-order rate constant k_B for the rescue of K218A epoxidase activity. As described in the text, multiple linear regression was used to correct for the effect of molecular volume. The logarithms of the corrected second-order rate constants show an excellent linear correlation with the pK_a of the rescuing amine, and this enhancement in the rate with increased basicity establishes the role of Lys218 as a general base in the mechanism of the carboxylase.

constant that indicates the catalytic efficiency but is a condition-dependent value that therefore does not have mechanistic meaning. The values obtained from multiple linear regression were $\beta = 0.68 \pm 0.05$, $V = (-0.0191 \pm 0.006)$ Å^{-3}, and $C = -1.8 \pm 0.7$. The molecular volume dependence value was in excellent agreement with that determined using the subset of amines with identical pK_a values. A plot of the volume-corrected rate constant [i.e., log k_B − (V × molecular volume)] versus pK_a for the amines was then constructed to illustrate the isolated effect of amine basicity on the rescue of K218A epoxidation (Figure 9), which clearly indicated that amine rescue of K218A activity depends upon the pK_a of the amine. This interpretation was consistent with the analysis of a subset of amines with nearly constant molecular volumes (70.5−71.6 Å^{-3}, Table 1). Utilization of this subset of amines eliminates the effect of the molecular volume and isolates the effect of amine basicity. The resulting plot yielded a Brønsted coefficient (β) of 0.57 ± 0.04, in good agreement with the value derived from the full set of amines after volume correction. The observation of a Brønsted relationship of increasing basicity with increasing amine rescue of K218A activity confirms that Lys218 acts as a general base in the epoxidation of vitamin K.

DISCUSSION

Our experiments provide, for the first time, direct chemical evidence for the identification of the critical active site base in the VKD carboxylase that deprotonates KH_2 to initiate carboxylation (Figure 1). The application of Brønsted analysis to an enzyme-catalyzed reaction is a powerful tool that has been used to elucidate the role of lysine side chains in several proteins (*26*, *28*−*30*, *33*). In the case of the carboxylase, Lys218 was identified as a candidate for the general base: a comparison of VKD carboxylase orthologues from different species, including the evolutionarily divergent active VKD orthologue from *L. interrogans*, revealed four residues as candidates for the base (Figure 2), and substitution of only one of these residues (Lys218) resulted in the complete loss of epoxidase activity when replaced by an amino acid (Ala) that cannot act as a base (Tables 2 and 3). The activity of the K218A mutant was rescued by amines that had only a marginal effect on wild type carboxylase activity (Figures 4 and 7). Importantly, K218A activity restoration depended upon the basicity of the rescuing amine (Figure 9), revealing that Lys218 acts as a general base that is indispensable to the function of the carboxylase. This analysis also showed that the molecular volume dependence of amine rescue of the carboxylase is the smallest dependence observed among enzymes that have undergone this determination (*26*, *28*−*31*, *33*, *34*, *36*), indicating a relatively low degree of steric constraint in the region of interaction between Lys218 and vitamin K. A low steric constraint may be significant because the carboxylase uses several different vitamin K isoforms for carboxylation (*7*), and therefore, its interaction with vitamin K may require steric flexibility to accommodate the different shapes of these isoforms.

The chemical rescue approach was particularly valuable for analyzing carboxylase function because an alternative approach using residue-selective chemical modification has actually been misleading, as in the implication of Cys as the active site base by thiol-selective reagents (see the Introduction) or His by the histidyl-selective reagent DEPC (see Figure S1 in the Supporting Information). Interpreting chemical modification experiments can be confounded by the issue of reagent specificity, as illustrated by the inhibition of the cysteine-less carboxylase by NEM (*25*), and is also complicated by the issue of whether modification causes a loss in chemical reactivity or has some nonspecific effect (e.g., blocking substrate access to the active site). In the case of the carboxylase, the main value in the chemical modification approach has been to eliminate candidates, which ultimately led to the identification of Lys218 as the active site base.

Identification of Lys218 as a residue critical to catalysis is a significant advance in defining the active site of the carboxylase. Because options like structural determination or enzymatic precedents have not been available for the carboxylase, major advances for identifying functional residues have largely been made by analyzing naturally occurring mutants or using evolutionary comparisons. Naturally occurring carboxylase mutations are rare, as expected, because of the indispensable function of the carboxylase, and the properties of the mutated amino acids indicate that none would be capable of serving as a general base catalyst. One naturally occurring mutant, L394R, implicated a region of the carboxylase as being critical to Glu interaction (*49*−*51*), consistent with evolutionary comparisons that showed that this region is highly conserved in metazoan VKD carboxylases (*40*). Analysis of another naturally occurring mutant, W157R, has suggested that the vitamin K-interacting region of the carboxylase, which has not yet been identified, may be near the N terminus (*52*). This possibility would be in line with the N-terminal location of Lys218, because the active site base must be closely juxtaposed to vitamin K for the reaction to occur (Figure 1).

Vitamin K must also be in close proximity to the Glu substrate for reactivity, and the location of Lys218 in the carboxylase, an integral-membrane protein in the endoplasmic reticulum, is of interest with regard to how vitamin K accesses the carboxylase active site. Thus, vitamin K is hydrophobic and almost certainly located in the membrane, while the VKD proteins and the carboxylase Glu-interacting region are lumenal (*49*−*51*, *53*), but how vitamin K in the membrane interfaces with Glu in the lumenal active site is unknown. The carboxylase N terminus is hydrophobic, and several membrane-spanning sequences have been proposed throughout the N-terminal 300 amino acids (*54*, *55*). However, an alternative possibility is that some of these sequences are monotopic, i.e., they insert into only one leaflet of the membrane. This motif has been observed in other enzymes with known crystal structures that share the same requirement as the carboxylase, i.e., access of a hydrophobic substrate from the membrane into a lumenal (or cytoplasmic) active site (*56*). The monotopic sequences form a tight junction between the membrane and aqueous environment and generate an entryway that allows the hydrophobic substrate in the membrane to access the active site. In the case of the carboxylase, two sequences (197−217 and 252−272) were suggested by hydropathy analysis to span the membrane, but they did not act as transmembrane domains when functionally tested (*55*). Lys218 resides just beyond the C terminus of one of these sequences. This location may be significant, because the positioning of Lys218 at the end of a monotopic sequence would poise it perfectly for interfacing both vitamin K in the membrane and the lumenal active site.

Lys218 must be unprotonated for function (Figure 1), and the use of Lys as a general base at physiological pH, when it would typically be protonated, means that the carboxylase must provide a mechanism to maintain it in an unprotonated state. One mechanism observed in other enzymes that use Lys as a base is a hydrophobic active site environment, which disfavors protonation of the ϵ-amino group and results in a lowered pK_a, and the location of Lys218 near the vitamin K binding site, which is likely hydrophobic, suggests a similar mechanism in the carboxylase. An additional mechanism, however, may be the presence of a neighboring Lys residue that modulates basicity. The placement of two Lys residues in close proximity results in the unstable localization of two positively charged side chains, and consequently, the affinity of one Lys for a proton is greatly decreased to the point that it becomes unprotonated at physiological pH. The carboxylase, then, may be reminiscent of acetoacetate decarboxylase, which contains a Lys−Lys motif comprising the unprotonated Lys base and the protonated Lys that modulates basicity (*57*). Metazoan VKD carboxylases contain a Lys adjacent

to Lys218, at position 217, which could potentially modulate the pK_a of Lys218.

A mechanism in which a neighboring Lys residue modulates protonation of Lys218 is attractive in explaining the observed regulation of KH_2 epoxidation by the Glu substrate. Thus, mammalian carboxylases are regulated by an unknown mechanism such that KH_2 epoxidation does not occur in the absence of the Glu substrate (*58*), and this regulation prevents the unfettered production of an undesirable strong vitamin K base (Figure 1) that could react with other molecules if the Glu substrate is not available for carboxylation. The interaction of the negatively charged Glu substrate with the modulating Lys would neutralize its charge and therefore remove its influence on Lys218, restoring the inherent basicity of Lys218 for deprotonation of KH_2, but only when the Glu substrate is present. In this regard, it is interesting to note that the *L. interrogans* VKD orthologue differs from the mammalian carboxylases in showing unregulated KH_2 epoxidation in the absence of the Glu substrate (*14*). The *L. interrogans* VKD orthologue also differs from all of the metazoan VKD carboxylases in having lost the conserved Lys217 residue (Figure 2B), which may explain the unregulated epoxidase activity in this orthologue. Studies that test whether the Lys217 residue or some other neighboring Lys residue does in fact modulate Lys218 activity will clearly be of interest.

In summary, our application of Brønsted analysis to the VKD carboxylase has provided the first direct chemical evidence for the identity of the critical active site base that initiates carboxylation. The carboxylase employs Lys218 as a general base, possibly by modulating the pK_a of the ϵ-amino group by proximity to that of Lys217. Thus, these experiments have begun to define the active site for KH_2 epoxidation and have raised interesting possibilities that could explain the poorly understood mechanism of regulation of vitamin K epoxidation by the Glu substrate and that address the impact of carboxylase topology on function. Future experiments along these lines have excellent potential to unravel the complexity of this important enzyme.

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SUPPORTING INFORMATION AVAILABLE

Table S1, oligonucleotides used to generate Lys- and His-substituted mutants; Figure S1, carboxylase activity inhibited by DEPC. This material is available free of charge via the Internet at http://pubs.acs.org.

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