The Conversion of Vitamin K Epoxide to Vitamin K Quinone and Vitamin K Quinone to Vitamin K Hydroquinone Uses the Same Active Site Cysteines†

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ABSTRACT: Vitamin K epoxide (or oxido) reductase (VKOR) is the target of warfarin and provides vitamin K hydroquinone for the carboxylation of select glutamic acid residues of the vitamin K-dependent proteins which are important for coagulation, signaling, and bone metabolism. It has been known for at least 20 years that cysteines are required for VKOR function. To investigate their importance, we mutated each of the seven cysteines in VKOR. In addition, we made VKOR with both C43 and C51 mutated to alanine (C43A/C51A), as well as a VKOR with residues C43−C51 deleted. Each mutated enzyme was purified and characterized. We report here that C132 and C135 of the CXXC motif are essential for both the conversion of vitamin K epoxide to vitamin K and the conversion of vitamin K to vitamin K hydroquinone. Surprisingly, conserved cysteines, 43 and 51, appear not to be important for either reaction. For the in vitro reaction driven by dithiothreitol, the 43−51 deletion mutation retained 85% and C43A/C51A 112% of the wild-type activity. The facile purification of the nine different mutations reported here illustrates the ease and reproducibility of VKOR purification by the method reported in our recent publication [Chu, P.-H., Huang, T.-Y., Williams, J., and Stafford, D. W. (2006) *Proc. Natl. Acad. Sci. U S A. 103*, 19308−19313].

Vitamin K epoxide reductase (VKOR)[1] is a 163-amino acid integral membrane enzyme of the endoplasmic reticulum (ER) (*1*, *2*). Our data predict that it has three transmembrane domains with the N-terminus in the ER lumen and the C-terminus in the cytoplasm (*3*). The function of VKOR is to regenerate vitamin K and vitamin K hydroquinone (K and KH_2) from vitamin K 2,3-epoxide (KO), a byproduct of the vitamin K-dependent carboxylation reaction. Inhibition of VKOR by warfarin limits the amount of KH_2 available for the carboxylation reaction and results in partially carboxylated vitamin K-dependent proteins (*4*). VKOR is the direct target of warfarin which in the United States alone has more than 21 million (*5*) prescriptions written annually.

Silverman published a theoretical mechanism in 1981 (*6*) in which he postulated that two cysteines were involved in the catalytic mechanism of VKOR. After the sequence of VKOR was revealed by cloning (*1*, *2*), it was noticed that it had a CXXC motif characteristic of the redox center of the thioredoxin families (*7*). The presence of this motif, at cysteines 132 and 135, supports the reaction mechanism proposed by Silverman. It has also been shown that the mutation of either of the two cysteines in the CXXC motif results in the loss of VKOR's KO to K activity (*8*, *9*). On the basis of these studies, an expanded quantum chemical calculation has been published (*10*, *11*). The results of Deerfield et al. are consistent with Silverman's hypothesis and also with cysteines 132 and 135 being active site residues.

We have devised a method for purifying the recombinant VKOR to near homogeneity and have shown that VKOR can both convert KO to K and K to KH_2 (*12*). Therefore, we revisit the role of the cysteines in VKOR for both reactions using purified VKOR. We mutated each of the cysteines individually, as well as in some combinations, and expressed the mutant enzymes in insect cells. The VKOR activities of the purified enzyme were compared with those of the wild-type enzyme.

Our results with purified VKOR provide additional evidence that C132 and C135 function as active site residues, not only for the conversion of KO to K but also for the conversion of K to KH_2.

EXPERIMENTAL PROCEDURES

Materials. The following materials were used: Sf9 insect cells (Lineburger Cancer Center, University of North Carolina at Chapel Hill), protease inhibitors aprotinin, leupeptin, and pepstatin (Roche Applied Science, Indianapolis, IN), dithiothreitol (DTT) (Research Products International Corp., Prospect, IL), 1,2-dihexanoyl-*sn*-glycero-3′-phosphocholine (DHPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids, Alabaster, AL), *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAP), bovine serum albumin (BSA), and anti-HPC4 monoclonal antibody (Oklahoma Medical Research Foundation, Oklahoma City, OK), warfarin, deoxycholate, and β-hydroxytoluene (BHT) (Sigma, St. Louis, MO), tris(hydroxypropyl)phosphine (THP)

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[1] Abbreviations: VKOR, vitamin K epoxide reductase; K, vitamin K; KO, vitamin K 2,3-epoxide; KH_2, vitamin K hydroquinone; ER, endoplasmic reticulum; SD, standard deviation.

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(Calbiochem, San Diego, CA), SnakeSkin dialysis tubing (Pierce, Rockford, IL), SYPRO ruby protein gel stain reagent (Molecular Probes, Eugene, OR), 2-methyl-3-(3,7,11,15,19-pentamethyl-2-eicosenyl)-1,4-naphthalenedione, $K_1(25)$ (GL Synthesis Inc., Worcester, MA), vitamin K_1 (Abbot Labs, Chicago, IL), vitamin K epoxide (KO) (a gift from P. Dowd, University of Pittsburgh, deceased), tris(2-carboxyethyl)-phosphine (TCEP) hydrochloride (Molecular Probes), sequencing-grade modified chymotrypsin (Roche Applied Science), Coomassie Blue R-250, protein standard markers, and SDS−PAGE ready gel (Bio-Rad, Hercules, CA), and pFastBacI vector and *Escherichia coli* strain DH10Bac (Invitrogen, Carlsbad, CA).

Site-Directed Mutagenesis. Human wild-type VKOR cDNA with sequence encoding the HPC4 antibody tag (EDQVD-PRLIDGK) at its carboxyl terminus was used as a PCR template for creating all VKOR mutants. Various VKOR mutants were generated by the "Megaprimer" method of PCR mutagenesis (*13*, *14*). The cDNA encoding VKOR mutants was subcloned into the *Eco*RI restriction site of the pFastBacI vector. All VKOR mutants were verified by sequencing the entire cDNA at the Sequencing Facility of the University of North Carolina.

Expression of Cysteine Mutants in Sf9 Insect Cells. pFastBacI vectors containing various mutant VKOR cDNAs were transformed into DH10Bac cells to produce recombinant bacmid DNA. The VKOR-expressing recombinant baculovirus was generated by transfecting the recombinant bacmid DNA into Sf9 insect cells by following to the instructions of the manufacturer (Invitrogen, San Diego, CA). Expression of VKOR was done by infecting 1 L of Sf9 cells with the recombinant virus for 48−52 h. Cells were collected by centrifugation and stored at −80 °C.

Purification of VKOR from Insect Cells. Microsomes were prepared as described previously (*15*), and VKOR was purified according to the method described by Chu et al. (*12*).

VKOR Quantitation. Purified wild-type and mutant VKOR enzymes were subjected to a 10% reducing SDS−NuPAGE Bis-Tris gel. The gel was stained with SYPRO ruby gel stain and visualized by a transilluminator at 302 nm. A serial dilution of BSA samples was run in parallel to construct a standard curve. The concentrations of both the VKOR samples and the BSA standard were adjusted so that there was a linear response over the range of analysis. The density of the protein bands was determined using Spot Density Tools on an AlphaImager according to the manufacturer's instructions (Alpha Innotech, San Leandro, CA). The concentration of purified VKOR enzymes was calculated by comparison with the accompanying standards.

VKOR Activity Assays. VKOR activity of KO to K was measured as described previously (*12*), with the exception of a reaction time of 30 min. The activity assay of VKOR converting K to KH_2 was as described previously (*12*), with the exception that equivalent volumes of purified VKOR mutants were taken for the assay. The incubation time was also 30 min.

Warfarin Inhibition. Equivalent amounts of purified VKOR diluted with 25 mM TAPS (pH 8.6), 150 mM NaCl, and 30% glycerol to 200 μL were used for each reaction. Final warfarin concentrations varying from 0 to 64 μM were added to the purified VKOR variants and the mixtures incubated for 5 min on ice. VKOR activity from KO to K was then assayed as described above.

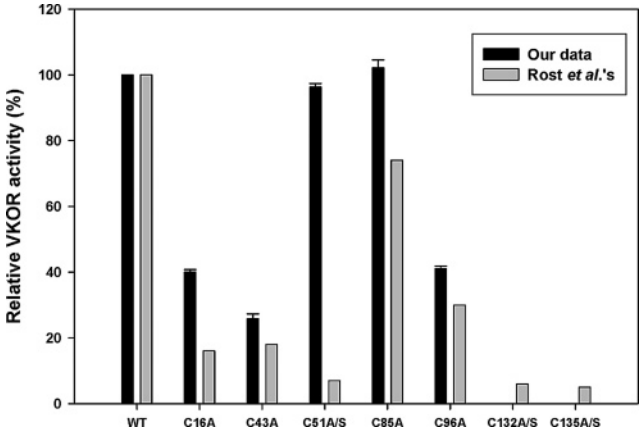

FIGURE 1: Relative activity of conversion of KO to K by purified VKOR. The assays were performed in 200 μM KO and 5 mM DTT with 25 mM TAPS (pH 8.6), 150 mM NaCl, and 30% glycerol. All assays were incubated at 30 °C for 30 min. The VKOR activity of purified enzymes was normalized to the concentration of VKOR. No data are shown for the C132A or C135A mutation because the activity was zero. Data are shown as means ± SD ($n = 3$).

In-Gel Protease Digestion. Purified VKOR samples were loaded onto 10% nonreducing SDS−PAGE gels. After separation, the gel was fixed with 25% isopropyl alcohol and 10% acetic acid for 20 min and stained with 0.01% Coomassie Brilliant Blue R250 in 10% acetic acid for 1 h. It was destained with 10% acetic acid and the VKOR band excised. The gel pieces were completely destained with 50 mM NH_4HCO_3 in 40% ethanol and then washed three times (15 min each) with intermittent vortexing, with 1 mL of 25 mM NH_4HCO_3. The excised VKOR band was then cut into 1 mm^3 pieces. Subsequently, the gel pieces were dehydrated with 1 mL of acetonitrile three times for 10 min, again with intermittent vortexing, and completely dried with a Speed-Vac. Protease digestion was started with the addition of sufficient 10 ng/μL sequencing-grade modified chymotrypsin in 25 mM NH_4HCO_3 buffer to immerse the dried gel pieces. After rehydration on ice for 30 min, the gel pieces were covered with an overlay of 20 μL of 25 mM NH_4HCO_3 buffer so they would remain immersed throughout the digestion. The protein was digested with chymotrypsin for 5 h at 30 °C without agitation. Digestion reaction supernatants were pooled for MALDI-TOF mass spectra. Samples were reduced via addition of TCEP to a concentration of 2 mM.

RESULTS

Conversion of Vitamin K Epoxide to Vitamin K by VKOR and Its Mutants. We examined the ability of purified VKOR mutants to convert KO to K. We diluted each purified VKOR preparation until the activity was proportional to enzyme concentration. The relative activities of the cysteine mutants are shown in Figure 1; the approximate results of Rost et al. (*9*) (gray columns) are shown for comparison. Except for cysteine 51, our results are similar to those of Rost et al.; C51A retains essentially wild-type activity in our experiments, while in their experiments, C51S was indistinguishable from the background.

Conversion of Vitamin K to Vitamin K Hydroquinone by VKOR. To determine if the same mutations that affect the

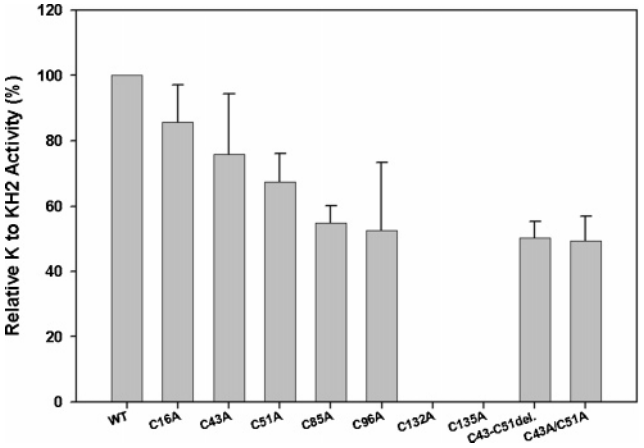

FIGURE 2: Relative activity of conversion of K to KH_2 by purified cysteine-mutated VKORs. Assays were performed by using 0.22 mM K and 5 mM DTT with 25 mM TAPS (pH 8.6), 150 mM NaCl, and 30% glycerol. Incubation was carried out at 30 °C for 30 min. The activity was normalized to the concentration of VKOR. No data are shown for the C132A or C135A mutation because the activity was zero. Data are shown as means ± SD ($n = 3$).

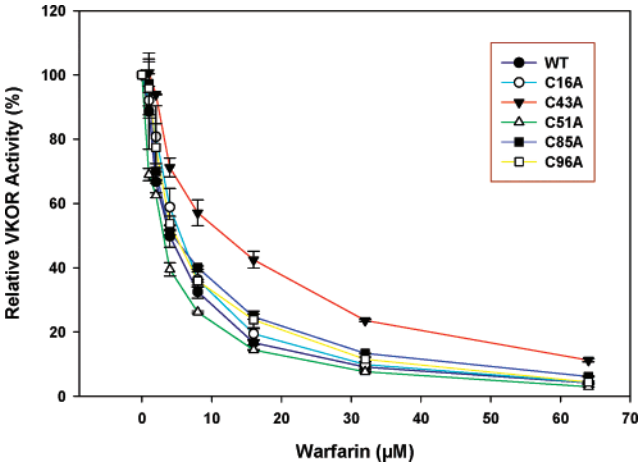

FIGURE 3: Warfarin inhibition of purified cysteine-mutated VKORs. Equivalent amounts of purified VKOR were assayed for 30 min at 30 °C in the presence of varying concentrations of warfarin (0−64 μM) with 200 μM KO and 5 mM DTT. Data are presented as means ± SD ($n = 3$).

activity of KO to K conversion also affect the activity of conversion of K to KH_2, we used each of the purified cysteine mutants to assay for the ability of VKOR to convert K to KH_2 (Figure 2). Because DTT contributes to the much slower nonenzymatic conversion of K to KH2, parallel mock reactions were run as controls. A C132A or C135A mutation eliminates K to KH_2 activity; however, all other mutations retain at least 50% of the wild-type activity.

Warfarin Inhibition. VKOR is the therapeutic target of warfarin. Therefore, warfarin inhibition studies were conducted for the purified mutants for the KO to K reaction. Figure 3 shows that only cysteine 43 has a measurable effect on the dosage of warfarin required for 50% inhibition; C43A requires 12 μM warfarin for 50% inhibition, and wild-type VKOR requires 4 μM warfarin for 50% inhibition.

C43 and C51 Can Form a Disulfide Bond. MS-Fit in Protein Prospector was used to search for possible peptide peaks of disulfide-linked peptides in chymotrypsin digestions of nonreduced VKOR samples from MALDI-TOF spectra. There are two candidate peptides at *m*/*z* 1355.54 and 1695.74 which correspond to residues 43−55 and 40−55, respectively, with an intramolecule disulfide between cysteines 43 and 51 (Figure 4, left). The mass of these two peptides increases 2 Da when the sample is reduced by TCEP, indicating that the disulfide is reduced (Figure 4, right).

If a disulfide bond is required for structure, we might be able to remove the loop between cysteines 43 and 51 without having a major effect on the structure or function of VKOR. To test this hypothesis, we joined residue 42 to residue 52 of VKOR, eliminating the intervening sequences. Purified VKOR with this deletion retained 85% of wild-type activity for the conversion of KO to K (Figure 5). To test if a disulfide bond is required for structure or function, we created the C43A/C51A double mutation. This mutation does not affect the KO to K VKOR activity (Figure 5), and the K to KH_2 activity is ~50% of that of the wild type.

DISCUSSION

The main purpose of our experiments was to examine the role of VKOR's four conserved cysteines (43, 51, 132, and 135) in the conversion of KO to K and K to KH_2.

There are previous suggestions that VKOR can convert both KO to K and K to KH_2 (*16*); however, all previous work was done with solubilized microsomes. We also previously suggested that VKOR converts both KO to K and K to KH_2 because we observed that coexpression of VKOR is effective in increasing the extent of carboxylation of vitamin K-dependent proteins in HEK 293 cells (*17*). Recently, we demonstrated that purified VKOR can convert K to KH_2 (*12*); this observation is confirmed by the work presented here.

As mentioned in the introductory section, there is substantial circumstantial evidence that cysteines 132 and 135 participate in the KO to K reaction. However, there are four cysteines in VKOR that are completely conserved throughout evolution (*7*). Because two of the conserved cysteine residues, 132 and 135, are part of the CXXC motif, we hypothesized that the other conserved cysteines, 43 and 51, might be involved in the conversion of K to KH_2. This would be consistent with the suggestion of Lee and Fasco that two different cysteine sites were required for these two reactions (*18*). However, we found that mutating either residue 132 or 135 eliminates both KO to K and K to KH_2 activity, while mutating both cysteines 43 and 51 to alanine resulted in an enzyme with no loss of in vitro activity.

Since four cysteine residues are conserved across a wide range of species, it was reasonable to postulate that they are functionally or structurally important (*7*). Rost et al. (*9*) mutated each of the cysteines in VKOR and reported that "VKOR activities of Cys51Ser, Cys132Ser and Cys135Ser were almost not detectable by our assays, whereas proteins mutated at Cys16Ser, Cys43Ser, Cys85Ser and Cys96Ser showed 15−30% of wild-type activity. The latter four cysteine residues were also substituted by ... alanine. Except for Cys85Ala, which exhibited 70% of VKOR activity, all other alanine variants ... did not differ significantly from the serine variants."

Many of our results are similar to those of Rost et al., even though there were major differences in the way that our experiments were conducted. Their mutant enzymes were made in HEK 293 cells and were characterized using whole

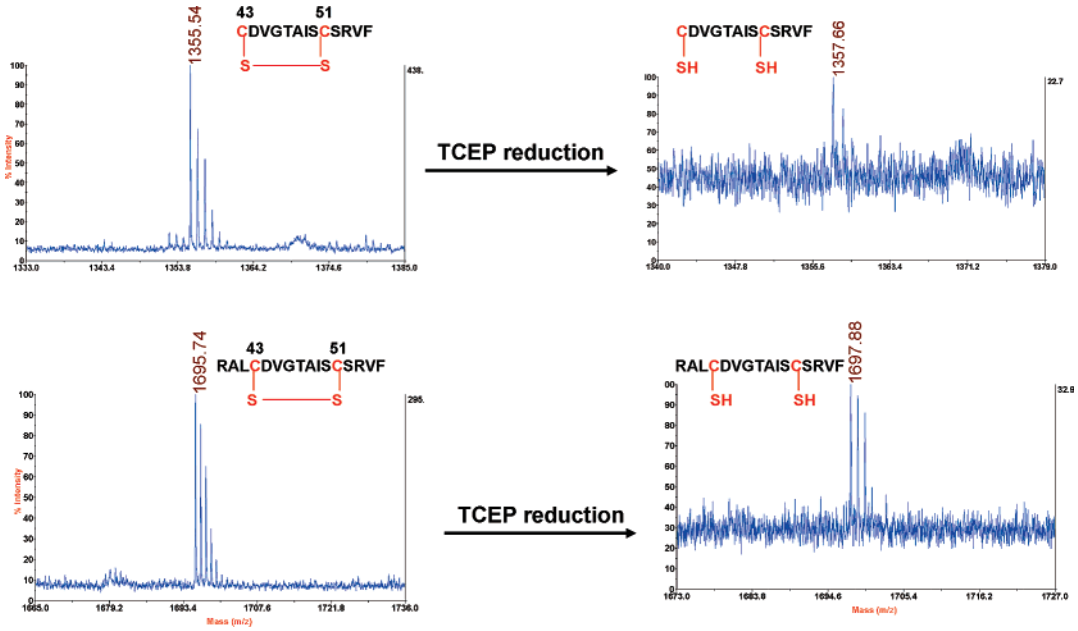

FIGURE 4: Evidence that cysteines 43 and 51 form a disulfide bond. MALDI-TOF MS spectrum of in-gel chymotrypsin-digested purified VKOR under reduced conditions (right) and nonreduced conditions (left).

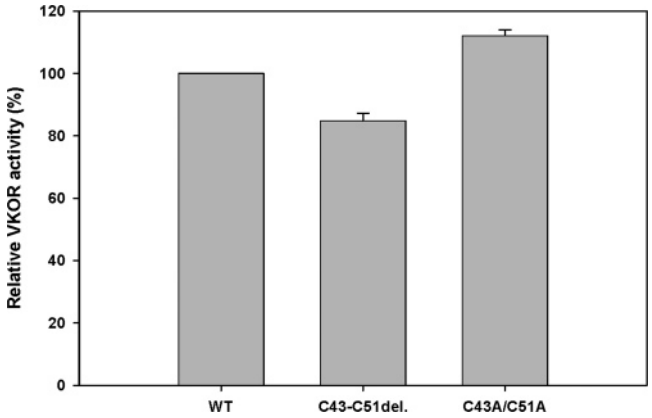

FIGURE 5: Relative VKOR activity of C43−C51 loop deletion, double mutants, and the wild type. Purified enzymes were used for the KO to K assay. Data are presented as means ± SD ($n = 3$).

cell extracts, while our data are derived from purified enzymes produced in insect cells. The one difference in the conclusions drawn from our work and theirs concerns the role of cysteine 51. They found no activity above background for the C51S mutation, while we found that C51A had activity similar to that of the wild type. Although the mutations are different, ours to alanine and theirs to the structurally more similar serine, our major conclusion that C51 is not essential for enzyme activity is not affected by our choice of amino acid substitution. Further evidence that cysteines 43 and 51 are not important for activity is that deletion of the entire loop from residue 43 to 51 resulted in a VKOR molecule with 85% activity, while VKOR with both cysteines 43 and 51 mutated to alanine had slightly greater than wild-type activity.

The rationale for deleting amino acids 43−51 is that while characterizing VKOR, we observed a disulfide bond that formed between C43 and C51. This means that these two residues are sufficiently close to allow the disulfide to form and suggests that one might be able to join residue 42 to residue 52 and maintain function if a disulfide bond between cysteines 43 and 51 is an important structural element. This assumes that the additional residues in the loop are not structurally or functionally important. In fact, our results were consistent with this prediction because the C43−C51 deletion retains 85% of the wild-type activity. However, the disulfide bond is apparently not important for structure or activity because the C43A/C51A double mutation also had 100% activity. Therefore, the disulfide bond is not necessary for activity, and cysteine appears not to be required for catalysis. Thus, even though the conservation of amino acid residues across such a broad range of species implies a functional role for cysteines 43 and 51, they appear not to be critical for structure or function.

A significant caveat to the conclusion that cysteines 43 and 51 are not required for enzymatic activity is that in the KO to K reaction, a disulfide is thought to form between cysteines 132 and 135 for each reaction cycle. In our assay, this disulfide bond is reduced by dithiothreitol. Thus, the in vivo reaction could require the conserved cysteines 43 and 51 even though they appear not to be important in our experiments. Presumably, in vivo, this function is provided by one or more enzymes. Indeed, thioredoxin (*19*) and thioredoxin with PDI (*20*) have been reported to function as reducing agents for VKOR in vitro, the implication being that they fulfill the in vivo function. Preusch, however, presented evidence that thioredoxin was not involved (*21*). According to a recent paper by Wajij et al. (*22*), the reaction is driven by protein disulfide isomerase (PDI) together with the folding of proteins within the ER. A naive hypothesis could invoke an interaction that allowed the disulfide bond between residues 132 and 135 to be reduced by cysteines 43 and 51. However, these residues are on opposite sides of the membrane, and it would be necessary to invoke a Grotthus (relatively free proton transport) mechanism for this reaction to occur. Nevertheless, no matter what enzymes are used for reducing VKOR, we could miss the importance of the mutations of cysteines 43 and 51 because we conducted our experiments with dithiothreitol instead of with the in vivo enzymes.

It is interesting to note that C43A is less sensitive to warfarin than any of the other mutations. C43A requires 12 μM for half-maximal inhibition, while wild-type VKOR and all of the other cysteine mutations are half-maximally inhibited at ~4 μM warfarin. It is also interesting that the loop deletion mutation (not shown) has a minimal effect on warfarin resistance or sensitivity. V45A, which is within the deleted loop, has been identified in a patient with warfarin resistance (*2*).

In summary, our results show that the same residues, cysteines 132 and 135, are required for both the conversion of vitamin K epoxide to vitamin K quinone and the conversion of vitamin K quinone to vitamin K hydroquinone. We also show that conserved cysteine residues 43 and 51 appear not to be important for either reaction, at least for the in vitro reaction driven by DTT. It is also significant that a second individual in our laboratory has reproduced the purification of VKOR for a number of different mutants and has also verified the conversion of vitamin K to reduced vitamin K by the purified enzyme.

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