

The Vitamin K Oxidoreductase Is a Multimer That Efficiently Reduces Vitamin K Epoxide to Hydroquinone to Allow Vitamin K-dependent Protein Carboxylation*

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Background: How the vitamin K oxidoreductase (VKORC1) supports vitamin K-dependent protein carboxylation is poorly understood.

Results: VKORC1 multimers efficiently perform both reactions that reduce vitamin K, and the inactive monomer in wild type mutant heteromers suppresses reduction.

Conclusion: VKORC1 fully reduces vitamin K required for carboxylation.

Significance: Multimers are important in VKORC1 mechanism and wild type mutant heteromers impact patients with warfarin resistance.

The vitamin K oxidoreductase (VKORC1) recycles vitamin K to support the activation of vitamin K-dependent (VKD) proteins, which have diverse functions that include hemostasis and calcification. VKD proteins are activated by Glu carboxylation, which depends upon the oxygenation of vitamin K hydroquinone (KH₂). The vitamin K epoxide (KO) product is recycled by two reactions, *i.e.* KO reduction to vitamin K quinone (K) and then to KH₂, and recent studies have called into question whether VKORC1 reduces K to KH₂. Analysis in insect cells lacking endogenous carboxylation components showed that r-VKORC1 reduces KO to efficiently drive carboxylation, indicating KH₂ production. Direct detection of the vitamin K reaction products is confounded by KH₂ oxidation, and we therefore developed a new assay that stabilized KH₂ and allowed quantitation. Purified VKORC1 analyzed in this assay showed efficient KO to KH₂ reduction. Studies in 293 cells expressing tagged r-VKORC1 revealed that VKORC1 is a multimer, most likely a dimer. A monomer can only perform one reaction, and a dimer is therefore interesting in explaining how VKORC1 accomplishes both reactions. An inactive mutant (VKORC1(C132A/C135A)) was dominant negative in heterodimers with wild type VKORC1, resulting in decreased KO reduction in cells and carboxylation *in vitro*. The results are significant regarding human VKORC1 mutations, as warfarin-resistant patients have mutant and wild type VKORC1 alleles. A VKORC1 dimer indicates a mixed population of homodimers and heterodimers that may have different functional properties, and VKORC1 reduction may therefore be more complex in these patients than appreciated previously.

The vitamin K oxidoreductase (VKORC1)² and γ -glutamyl carboxylase are integral membrane proteins that reside in the endoplasmic reticulum and together activate vitamin K-dependent (VKD) proteins during their secretion (1). The carboxylase uses reduced vitamin K to drive the conversion of clusters of Glu to carboxylated Glu (Gla). This modification generates a calcium-binding module required for the activities of VKD proteins, which have diverse functions that include hemostasis, calcium homeostasis, growth control, signal transduction, and apoptosis (2). Carboxylation of VKD proteins results in the production of an epoxide form of vitamin K, which is reduced by VKORC1 (Fig. 1). VKORC1 is the target of warfarin, which is used by millions of people to control hemostasis (3). The role of VKORC1 in VKD protein carboxylation and its manipulation during warfarin therapy highlight the importance of understanding how this enzyme reduces vitamin K. VKD protein carboxylation only occurs in metazoans (2); however, VKOR homologs have also been identified in bacteria (4–7). These homologs appear to be quinone, rather than epoxide, reductases, and have a role in protein folding (4, 7, 8).

Early studies indicated that VKORC1 uses thiols to reduce vitamin K (9, 10), and this mechanism was confirmed following the identification of the gene for VKORC1 and mutational analysis (11–15). Four Cys residues are evolutionarily conserved in all organisms containing VKOR (5). Two Cys residues (Cys-132 and Cys-135 in human VKORC1) are part of a thioredoxin motif that reduces vitamin K. Reduction results in the formation of a disulfide bond and enzyme inactivation, and thiol regeneration is required for subsequent VKOR activity. The Cys-132–Cys-135 disulfide bond is membrane-embedded, and reduction of the disulfide bond occurs through an electron relay pathway that has been demonstrated in both bacterial and human VKORs (8, 16). This pathway involves the transfer of

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² The abbreviations used are: VKORC1, vitamin K oxidoreductase; r-VKORC1, recombinant VKORC1; KO, vitamin K epoxide; KH₂, vitamin K hydroquinone; K, vitamin K quinone; VKD, vitamin K-dependent; Gla, carboxylated Glu.

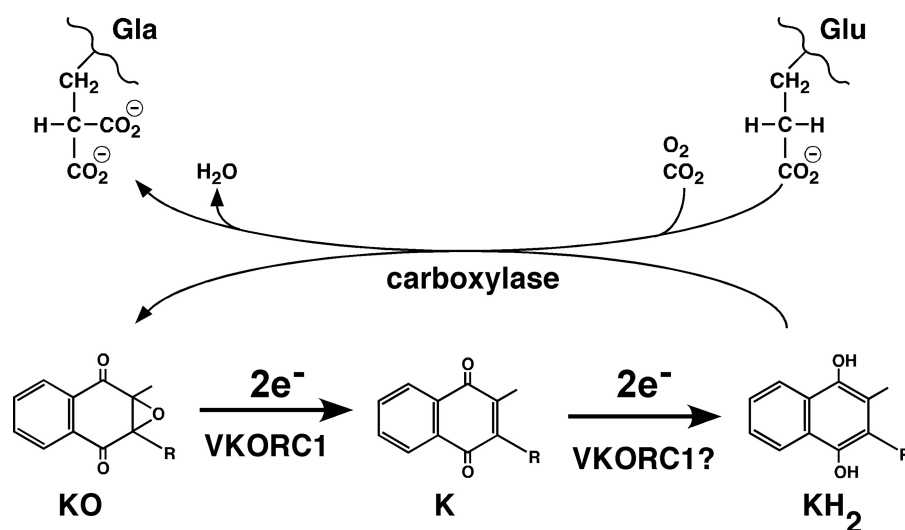


FIGURE 1. **The vitamin K cycle.** Carboxylation of glutamyl residues (Glu) to carboxylated Glu (Gla) in VKD proteins results in the oxygenation of vitamin K hydroquinone (KH_2) to vitamin K epoxide (KO). The KO product is then recycled to KH_2 by two reactions. VKORC1 is known to perform the first reaction, *i.e.* KO to vitamin K quinone (K), and this study tests whether it can perform both reactions to fully reduce KO.

reducing equivalents from a redox protein to two evolutionarily conserved Cys residues that reside in a loop outside of the membrane, which then transfer the electrons to Cys-132–Cys-135 to regenerate thiols. The redox protein that provides electrons to the loop Cys residues in human VKORC1 has not yet been identified.

The identification of VKORC1 is recent, and currently, there are many unknowns in the mechanism of vitamin K epoxide (KO) reduction. One important question is whether VKORC1 contributes to the full reduction of KO to vitamin K hydroquinone (KH_2). Reduction requires the transfer of four electrons, which occurs in two reactions (see Fig. 1), and VKORC1 can only provide two electrons for a single reaction. Although it is well established that VKORC1 is responsible for KO reduction to the vitamin K quinone (K) intermediate, it has recently been proposed that an enzyme other than VKORC1 performs the reduction of K to KH_2 (17, 18). A non-VKORC1 vitamin K quinone reductase exists, as bleeding defects in patients undergoing warfarin therapy can be corrected by the administration of K. Treatment involves large amounts of vitamin K, and *in vitro* studies suggest a high K_m for the quinone reductase (19), which has not yet been identified. Dietary levels of vitamin K are much lower than those used in pharmacological intervention, and it is therefore unknown whether the quinone reductase is important to carboxylation under normal physiological conditions. Defining whether KO to KH_2 reduction involves only VKORC1 or two different enzymes is important, as the mechanistic implications would be quite different in each case. For example, VKD protein carboxylation in different tissues could vary significantly if KH_2 production requires the expression of two different enzymes *versus* only VKORC1. Assessing the role of VKORC1 in KO to KH_2 reduction has been problematic because KH_2 is rapidly oxidized and VKORC1 has been difficult to purify. In the one reported purification of VKORC1, the efficiency of K to KH_2 production was \sim 50-fold lower than that of KO to K (20), which led to the proposal that VKORC1 only reduces KO to K (17). Our previous results suggested that VKORC1 performs both reactions (16), and we therefore

developed new approaches to examine VKORC1 reactivity. As described below, the studies revealed that VKORC1 exists as a multimer that can perform both reactions to efficiently reduce KO to KH_2 .

EXPERIMENTAL PROCEDURES

Construction and Expression of r-VKORC1 Variants—Wild type human r-VKORC1 was tagged at either the N or C terminus with the FLAG epitope. FLAG-VKORC1 had the N-terminal sequence MDYKDDDDKAAA and $\text{VKORC1}_{\text{FLAG}}$ had the C-terminal sequence AAAGSGSGSGSGSGSDYKDDDDK. A mutant with Cys residues 132 and 135 substituted by Ala, $\text{VKORC1(C132A/C135A)}$, was also generated and contained the same C terminus as $\text{VKORC1}_{\text{FLAG}}$. All three constructs were subcloned into pCMV6-AC (Origene) for expression in mammalian cells. Sequencing was performed on both strands of the VKORC1 ORFs to ensure that the only changes were those intentionally introduced. $\text{VKORC1}_{\text{FLAG}}$ was also subcloned into BacPak8 (Clontech) for expression in SF21 insect cells.

r-VKORC1/pCMV6-AC variants were stably expressed in 293 cells by using selection with Geneticin (0.4 mg/ml, Invitrogen). In each case, \sim 20 clones were screened by Western analysis using an anti-VKORC1 antibody (21) to identify a cell line with the desired level of expression. Recombinant and endogenous VKORC1 were distinguishable on SDS-PAGE, and quantitation of each form on a LICOR Odyssey scanner was used to determine the level of VKORC1 overexpression. An activity assay that measures KO to K reduction (16) was also performed on 293 cells and r-VKORC1 $_{\text{FLAG}}$ 293 cells, to determine whether activity was proportional to the amount of VKORC1 protein expression. r-VKORC1 $_{\text{FLAG}}$ was also expressed in insect cells by baculovirus infection. Baculovirus was generated by cotransfecting r-VKORC1 $_{\text{FLAG}}$ /BacPak8 with BacPAK6 DNA, which results in recombination that generates the virus. Plaques were screened by Western analysis using anti-VKORC1 antibody, and the virus was then scaled up for analysis. Baculoviruses containing untagged VKORC1 or carboxyl-

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ase were also used in these studies, and virus generation was described previously (16, 22).

Immunoprecipitation of VKORC1 from 293 Cells—293 cells and wild type r-VKORC1_{FLAG} and r-VKORC1(C132A/C135A)_{FLAG} 293 cells (10^7 cells each) were lysed in 3 ml of buffer A (25 mM sodium phosphate, 25 mM KCl, and 20% glycerol, pH 7.9) containing 0.75% CHAPS. Samples were dounced, centrifuged ($3000 \times g$, 15 min), and the supernatant was then centrifuged at $100,000 \times g$ for 1 h to remove any unsolubilized material. All steps were performed at 4 °C. Aliquots (1.3 ml) were incubated overnight with 100 μ l anti-FLAG agarose (0.6 mg/ml, Sigma) or anti-VKORC1 Sepharose (2 mg/ml), followed by centrifugation ($10,000 \times g$, 1 min). The resins were washed using five rounds of incubation (1 min in 1 ml of buffer B (25 mM Tris-HCl, 0.3% CHAPS, 0.2% phosphatidyl choline and 100 mM NaCl, pH 7.4)) and centrifugation ($10,000 \times g$, 1 min). VKORC1 was then eluted in buffer B containing 100 μ g/ml FLAG peptide (Sigma) or VKORC1 peptide (21), followed by centrifugation ($10,000 \times g$, 1 min) and filtration using a micro bio-spin column (Bio-Rad) to collect resin-free material. Samples were then assayed by Western analysis using anti-FLAG or anti-VKORC1 antibodies (both at 0.4 μ g/ml) and goat polyclonal antibody conjugated to IRDye 800 CW (LICOR Biosciences, 0.2 μ g/ml).

VKORC1 Purification—r-VKORC1_{FLAG} was purified from r-VKORC1_{FLAG} 293 cells and SF21 insect cells infected with baculovirus (r-VKORC1_{FLAG}) (multiplicity of infection of 5). In both cases, large scale preparations (10^9 cells) were used to generate microsomes, as before (22). Microsomal pellets were resuspended in buffer A at a final protein concentration of 4 mg/ml, as determined by a BCA assay (Pierce). Initial tests were performed to determine the optimal conditions for solubilizing the microsomes. Varying concentrations of CHAPS (0.5–1.5%) and NaCl were added, followed by nutation (1 h) and then centrifugation ($100,000 \times g$, 1 h), all at 4 °C. The starting material and supernatants were then assayed for KO to K reduction as described (16) to identify the conditions that gave the best recovery of activity. r-VKORC1_{FLAG} was purified from insect cells following solubilization with 1% CHAPS, 0.2 M NaCl, and ultracentrifugation ($100,000 \times g$, 1 h, 4 °C). Supernatant (1 ml) was incubated overnight with anti-FLAG-agarose (100 μ l, 0.6 mg/ml) and the bound material was then washed with buffer B and eluted as described above. The purification from r-VKORC1_{FLAG} 293 cells was similar, except that solubilization used 0.5% CHAPS and no NaCl.

Testing KH₂ Stability in the Standard VKOR Activity Assay—KH₂ was prepared as described (23), and stability was assessed in the standard assay used to monitor VKORC1 reduction of KO. KH₂ (2800 pmol) was incubated for 1 h at 21 °C in 200 μ l of buffer A containing 0.75% CHAPS and 5 mM EDTA, either without added antioxidants or with 1 mM butylhydroxytoluene, γ -tocotrienol, β -hydroxytoluene, octyl gallate, or *tert*-butylhydroquinone (all from Supelco). The samples were extracted with 500 μ l of a 1:1 mixture of ethanol and hexane, then vortexed for 1 min and centrifuged ($1000 \times g$, 5 min). The organic phase was evaporated using oxygen-free nitrogen (Praxair), and vitamin K was resuspended in 60 μ l of ethanol, followed by HPLC analysis using a C18 column (Thermo Scientific, 250 \times

4.6 mm, 5 μ m), 100% methanol as the mobile phase, and absorbance detection at 226 nm.

Establishing a Sealed Vial Assay for Quantitating KH₂—The effect of an oxygen-free environment on KH₂ stability was tested. Buffer A (200 μ l) was added to vials (12 \times 32 mm, National Scientific) sealed with a 9-mm screw cap fitted with a polytetrafluoroethylene/silicone/polytetrafluoroethylene septum (Supelco). The vials were flushed with nitrogen for 10 min, and KH₂ was then added using a syringe (Hamilton). Nitrogen that was oxygen-free was used throughout the procedure, which was performed at 21 °C. The samples were incubated for 1 h, with continuous flushing of the vials with nitrogen. Vitamin K was then extracted by adding ethanol and hexane (250 μ l each) that had been bubbled with nitrogen for 10 min in screw-capped vials (15 \times 45 mm). The vitamin K samples were gently vortexed and then centrifuged ($750 \times g$, 2 min), and the upper organic layer was transferred by syringe to an empty sealed vial that had been preflushed with nitrogen. The samples were evaporated to dryness with nitrogen and then resuspended in 100 μ l of nitrogen-flushed ethanol. The samples were transferred to preflushed empty HPLC vials sealed with 9-mm caps fitted with slit polytetrafluoroethylene-silicone septa (Waters) and then analyzed immediately.

HPLC analysis was performed using a C18 column (Thermo Scientific, 250 \times 4.6 mm, 5 μ m), followed by a column packed with zinc powder (50 \times 3.9 mm, 100 mesh). The mobile phase contained 5 mM acetic acid, 5 mM sodium acetate, and 10 mM ZnCl₂ in 100% methanol and was continuously degassed with helium. The zinc reactor and mobile phase together reduce all vitamin K forms to KH₂ (24), resulting in fluorescence that was detected using an excitation wavelength of 320 nm and an emission wavelength of 418 nm.

This assay was then used to monitor KH₂ production by purified VKORC1. Samples (200 μ l) were flushed with nitrogen for 10 min in sealed vials, followed by the addition of KO (65 μ M, prepared as described (25)) or K (65 μ M, Sigma) and 5 mM DTT. After 1 h, the reactions were quenched by the addition of nitrogen-flushed ethanol and hexane (250- μ l each), followed by vitamin K isolation as described above. HPLC analysis included vitamin K standards (KO, K, KH₂) for quantitation.

Assaying Cellular Reduction of KO—293 cells with or without r-VKORC1 (10^7 cells) were incubated overnight in DMEM/F12 media containing 10% charcoal-treated serum and 1 μ g/ml KO. Cells were harvested and rinsed twice with PBS (10 ml), followed by lysis at 4 °C with 500 μ l of buffer A that contained 0.75% CHAPS. Aliquots (45 μ l) were removed for Western analysis with anti-VKORC1 antibody, and the remainder was extracted with 2 volumes of a 1:1 mixture of hexane and ethanol. The samples were vortexed and centrifuged ($3000 \times g$, 5 min, 20 °C), and the organic phase was then dried under nitrogen. HPLC and absorbance detection were then performed as described above in the section on the standard VKOR assay. KO reduction was also monitored following treatment of the cells with siRNA that targeted the carboxylase. Cells were transfected with siRNA (20 nM, Ambion), and KO (1 μ g/ml) was added to the cells 2 days later. Vitamin K was isolated as above, and Western analysis was performed with anti-carboxylase (26) and anti-VKORC1 antibodies.

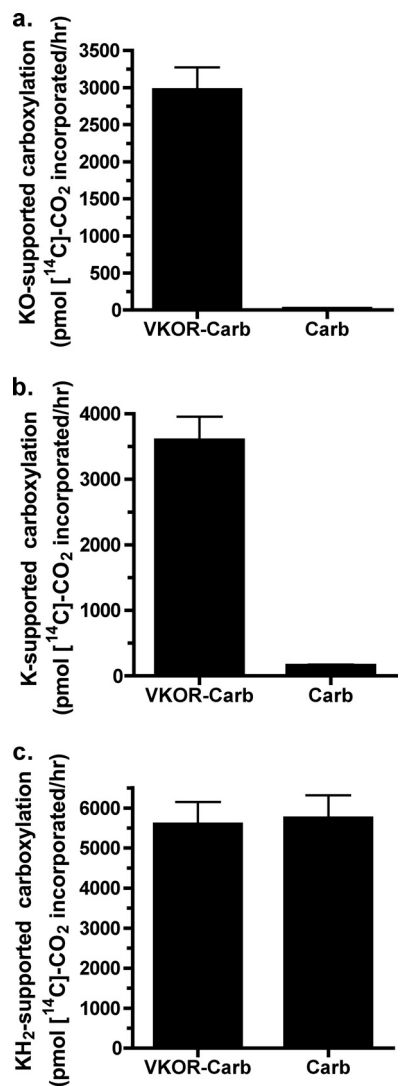


FIGURE 2. VKORC1 reduces vitamin K epoxide to hydroquinone to drive carboxylation. Microsomes were prepared from insect cells coinfecting with baculoviruses containing r-VKORC1_{FLAG} (VKOR) and r-carboxylase (*Carb*) or infected only with baculovirus containing the carboxylase. The microsomes were incubated with KO (a), K (b), or KH₂ (c), and carboxylation was quantitated by measuring [¹⁴C]CO₂ incorporation into the peptide FLEEL.

Monitoring VKORC1-supported Carboxylation in Microsomes—Microsomes containing both VKORC1_{FLAG} and the carboxylase were prepared by coinfecting SF21 insect cells (10⁹ cells) with baculoviruses containing VKORC1_{FLAG} (multiplicity of infection of 2) and the carboxylase (multiplicity of infection of 5). Control microsomes containing only the carboxylase were also prepared. The microsomes underwent a high pH wash that removes peripheral proteins, which was performed as described previously (16) except that the washed microsomes were resuspended in buffer A. Test and control microsomes were washed and assayed on the same day. Carboxylase activity was assessed in mixtures (200 μl) containing washed microsomes (0.8 mg/ml), 0.8 M ammonium sulfate, 50 mM BES pH 6.9, 5 mM DTT, 2.5 mM Phe-Leu-Glu-Glu-Leu (FLEEL, Anaspec), 10 μM factor X propeptide, 1.3 mM [¹⁴C]sodium bicarbonate (PerkinElmer Life Science), 0.16% CHAPS (Sigma), and 0.16% phosphatidyl choline (Sigma). Assays were initiated by the addition of either KO (65 μM) or K (65 μM), and after 90

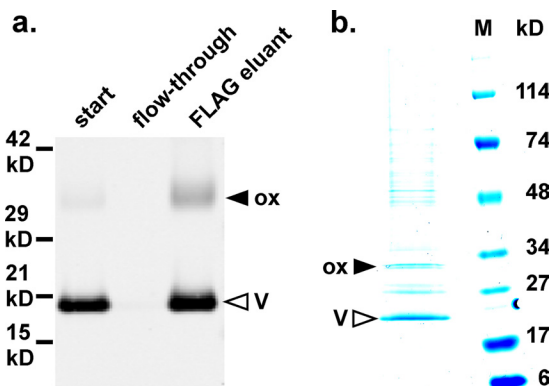


FIGURE 3. Purification of r-VKORC1_{FLAG} from insect cells. a, fractions were monitored in a Western blot using anti-VKORC1 antibody. b, purified VKORC1_{FLAG} was also analyzed by SDS-PAGE and Coomassie staining. VKORC1 (V, empty arrowheads), and a small amount of oxidized VKORC1 (ox, filled arrowheads) that is generated during purification and not resolved in the reducing gel, are indicated.

min, the reactions were quenched by the addition of 10% trichloroacetic acid (1 ml). The samples were centrifuged and then boiled, and the incorporation of [¹⁴C]CO₂ into FLEEL was quantitated by scintillation counting.

Microsomes from 293 cells, wild type r-VKORC1_{FLAG} and r-VKORC1(C132A/C135A)_{FLAG} 293 cells (10⁹ cells each) were also tested for VKORC1-supported carboxylase activity. The reaction was as above, except that 400 μl was assayed and two reductants were tested. The reductants were 5 mM DTT or thioredoxin, thioredoxin reductase, and NADPH, present at final concentrations of 25 μM, 12.5 μg/ml, and 1 mM, respectively. The reactions were initiated by the addition of KO (65 μM), and after a 90-min incubation, the samples were boiled and then subjected to two rounds of chromatography to increase the signal to noise. This approach was required because of the low level of VKORC1 activity in the microsomes, and the details for this method have been described previously (27). Briefly, samples were chromatographed on P-2 columns (Bio-Rad), and carboxylated FLEEL was then isolated by HPLC and quantitated by scintillation counting.

RESULTS

VKORC1 Reduces KO to KH₂ to Drive Carboxylation—VKORC1 function was tested in the SF21 insect cell expression system, which has been valuable because it lacks carboxylation components (28). Recombinant VKORC1 was tagged at the C terminus with the FLAG epitope, and baculovirus containing r-VKORC1_{FLAG} was generated and used to infect insect cells. Microsomes containing r-VKORC1_{FLAG}, which is an integral membrane protein, were prepared, and the specific activity was determined using Western analysis and an assay that measures KO to K reduction (16). A value similar to that observed with endogenous human VKORC1 in 293 cells was obtained (data not shown), indicating the suitability of this expression system for analyzing VKORC1 function.

r-VKORC1_{FLAG} and r-carboxylase were coexpressed in SF21 insect cells and then assayed for carboxylation, which requires the KH₂ cofactor. The reactions were performed using either K or KO, which are reduced to KH₂ in one or two reactions, respectively (Fig. 1). Controls included assaying microsomes

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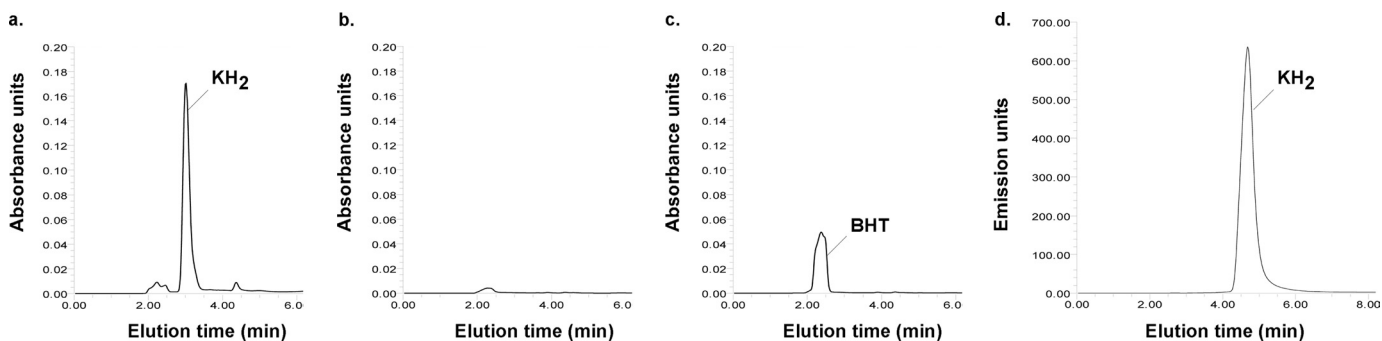


FIGURE 4. **A new assay for quantitating the production of KH_2 .** KH_2 was directly injected onto an HPLC column (a) or was first processed through the organic extraction method used to isolate vitamin K (b). c, extraction in the presence of 3,5-di-tertbutylhydroxytoluene (BHT) was also performed, showing KH_2 oxidation even in the presence of antioxidant. KH_2 is oxidized to K, which elutes at later times (not shown). d, KH_2 extracted under nitrogen in a sealed vial prevented oxidation. This sample was analyzed using a post-column zinc reactor, which accounts for the difference in elution times from those in a–c.

from cells expressing carboxylase but not VKORC1 and assaying both test and control microsomes using KH_2 that is used directly by the carboxylase. The experiment was performed using intact microsomes because solubilization disrupts the functional interaction between VKORC1 and the carboxylase (16). Microsomes were incubated with the peptide FLEEL, [^{14}C]CO $_2$ and vitamin K, and carboxylation was quantitated by [^{14}C]CO $_2$ incorporation into FLEEL. When KO was used as the substrate, carboxylation was observed that was clearly due to VKORC1 because none was detected in the control microsomes expressing only the carboxylase (Fig. 2a). The rate of K-supported carboxylation was only slightly higher in the microsomes from carboxylase-VKORC1 $_{\text{FLAG}}$ -expressing cells (Fig. 2b). Importantly, very little activity was observed in the microsomes lacking VKORC1 $_{\text{FLAG}}$. This result indicates that a quinone reductase other than VKORC1 $_{\text{FLAG}}$ cannot account for the large amount of KO-supported carboxylation observed in the microsomes containing carboxylase and VKORC1 $_{\text{FLAG}}$ (Fig. 2a). The amount of K- and KO-supported carboxylation was within a factor of two of that observed with saturating concentrations of KH_2 (Fig. 2c). The results strongly suggest that VKORC1 performs both reactions to fully reduce KO to KH_2 .

Purified VKORC1 Efficiently Reduces KO to KH_2 —The results with intact microsomes (Fig. 2) are different from what was previously reported with purified VKORC1, where reduction of K to KH_2 was 50-fold lower than reduction of KO to K (20). That study monitored activity using a VKORC1 preparation that was initially inactivated during membrane solubilization and then reconstituted for activity. This method had the potential to disrupt structure and consequent function, and we therefore developed a method in which VKORC1 activity was retained throughout the purification. Microsomes from insect cells expressing r-VKORC1 $_{\text{FLAG}}$ were solubilized using different conditions of detergent and salt, and activity was monitored before and after the ultracentrifugation step that isolates the solubilized material. These tests revealed that the concentration of detergent was critical for retaining activity. Thus, high concentrations of detergent recovered more VKORC1, as indicated by Western analysis, but also resulted in inactivation (data not shown). When optimal concentrations (1%) of the detergent CHAPS were used for solubilization, 73% activity was recovered when compared with the intact microsomes.

To purify VKORC1 $_{\text{FLAG}}$, solubilized material was incubated with anti-FLAG-agarose, and the resin was then washed and incubated with FLAG peptide to elute VKORC1 $_{\text{FLAG}}$. Most of the VKORC1 $_{\text{FLAG}}$ bound to the anti-FLAG resin (Fig. 3a), and the overall activity recovery was 35%. The purified preparation was highly enriched for VKORC1 $_{\text{FLAG}}$, *i.e.* ~1400-fold compared with starting material based on a protein assay, and VKORC1 $_{\text{FLAG}}$ was the most prominent band observed by Coomassie staining of an SDS-PAGE gel (Fig. 3b). The turnover of the purified preparation was 0.07 s $^{-1}$, as determined by combined activity assay and Western analysis. This value was 7-fold higher than that of the starting material, possibly due to the removal of an inhibitor during purification. Importantly, VKORC1 activity was retained throughout the purification.

To test KO to KH_2 conversion by purified VKORC1 $_{\text{FLAG}}$, we developed a new assay. Thus, KH_2 is rapidly oxidized during isolation (Fig. 4, a and b), which has been a major impediment in quantitating its production. Consequently, almost all previous studies have assayed only the first reaction, *i.e.* KO to K (14, 15, 16, 18). Previously, the antioxidant β -hydroxytoluene was used to try to stabilize KH_2 (20); however, tests with multiple antioxidants, including β -hydroxytoluene, were ineffective in blocking KH_2 oxidation (*e.g.* Fig. 4c). We therefore developed an alternative approach in which the reaction and subsequent vitamin K isolation were performed in sealed vials under a nitrogen environment, followed by immediate HPLC analysis. This method, which is described in detail under “Experimental Procedures,” resulted in excellent (85%) recovery of KH_2 (Fig. 4d). A second modification was to use post-column zinc reduction that resulted in fluorescence of all vitamin K forms (24), which improved the sensitivity of the assay.

Purified VKORC1 $_{\text{FLAG}}$ was then analyzed using the sealed vial assay. Incubation with KO resulted in the production of substantial amounts of KH_2 as well as K (Fig. 5a), with a KH_2 to K ratio of 42%. No KH_2 was detected in a control sample purified from mock-infected cells (Fig. 5b), indicating that VKORC1 $_{\text{FLAG}}$ was responsible for the reduction of KO. The preparation isolated from mock-infected insect cells was also monitored for K to KH_2 reduction to test for the presence of a non-VKORC1 vitamin K quinone reductase. Very little K reduction was observed (Fig. 5c), *i.e.* the K to KH_2 ratio was 2%, which was 20-fold lower than that observed with purified

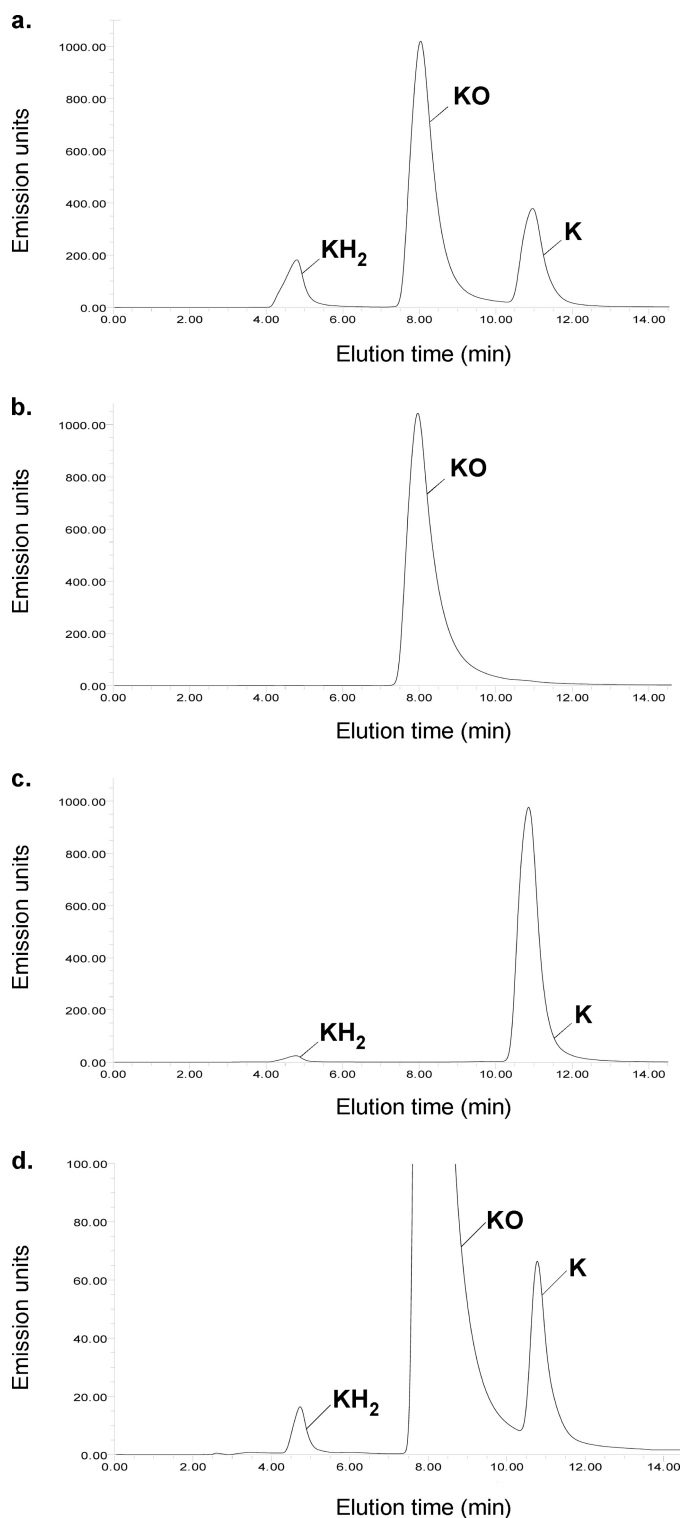


FIGURE 5. Purified VKORC1 efficiently reduces KO to KH_2 . Samples purified from insect cells infected with baculovirus containing VKORC1_{FLAG} (a) or from mock-infected cells (b) were incubated with KO, followed by vitamin K isolation, all under nitrogen in sealed vials. The reactions were staggered so that the vitamin K products (K and KH_2) could be analyzed immediately after isolation. c, purified sample from mock infected cells was also assayed with K to test for the presence of quinone reductases other than VKORC1. d, VKORC1 purified from 293 cells expressing r-wild type VKORC1_{FLAG} was also assayed for KO reduction. Control reactions performed in the absence of reductant showed no reduction of KO (not shown).

VKORC1_{FLAG} (Fig. 5a). A similar result was obtained with catalytically inactive VKORC1 (described below) purified from insect cells, indicating that association of a non-VKORC1 quinone reductase with VKORC1 did not account for K to KH_2 reduction. Thus, purified VKORC1_{FLAG} is efficient for the reduction of KO to KH_2 .

VKORC1 Has a Multimeric Structure—A possible explanation for how VKORC1 performs two reactions to reduce KO to KH_2 was revealed by studies on wild type recombinant VKORC1 expressed in 293 cells. r-VKORC1 was C-terminally tagged with the FLAG epitope to distinguish it from endogenous human VKORC1. r-VKORC1_{FLAG} did not affect endogenous VKORC1 levels (Fig. 6a), and overexpression of protein led to a proportional increase in activity (shown for one clone in Fig. 6b). When immunopurification was performed using an antibody against VKORC1 followed by Western analysis with the same antibody, both endogenous and r-VKORC1_{FLAG} were detected, as expected (Fig. 6c). Interestingly, both forms were also observed when immunopurification was performed using an anti-FLAG antibody (Fig. 6, d and e). The results show that VKORC1 exists as a multimer, which is most likely a dimer as only this form was observed on nonreducing gels (data not shown). For simplicity VKORC1 will be referred to as a dimer. A VKORC1 monomer provides two electrons that can only catalyze one step of the reaction; however, a dimer provides four electrons that could allow both steps. r-VKORC1_{FLAG} purified from 293 cells was able to convert KO to KH_2 (Fig. 5d), consistent with this possibility. As in insect cells, the purification of VKORC1_{FLAG} from 293 cells was strongly impacted by the solubilization step, as high detergent concentrations significantly decreased activity (data not shown).

A Dysfunctional VKORC1 Monomer Has a Dominant Negative Effect on KO Reduction in Cells—To assess the functional consequence of a dimeric structure, we tested whether the presence of a catalytically inactive monomer in the dimer had an effect on function. This test was performed using an assay that monitored KO reduction in cells, and this approach was first validated by determining whether KO reduction reflects cellular levels of VKORC1. 293 cells and r-VKORC1_{FLAG} 293 cells (Fig. 6a) were incubated with KO and then washed, and vitamin K was isolated by organic extraction. HPLC analysis of KO and K, which comprises both K and the KH_2 form that is oxidized during isolation, revealed an increase in KO reduction in the r-VKORC1_{FLAG} 293 cells (Fig. 7, a and b). The cells contain endogenous carboxylase, whose ability to recycle KH_2 to KO was unknown because the carboxylase requires activation by VKD proteins (29, 30), which were present in only trace amounts in these cells (31). Therefore, the experiment was repeated in cells pretreated with carboxylase siRNA prior to the addition of KO. Western analysis indicated efficient elimination of carboxylase with little effect on VKORC1 expression (Fig. 7, c and d). The siRNA pretreatment resulted in a small increase in KO reduction, and r-VKORC1_{FLAG} 293 cells still showed a large increase in KO reduction compared with the 293 cells (data not shown). The results indicated that this assay could be used to test the effect of a dysfunctional VKORC1 mutant on KO reduction in cells.

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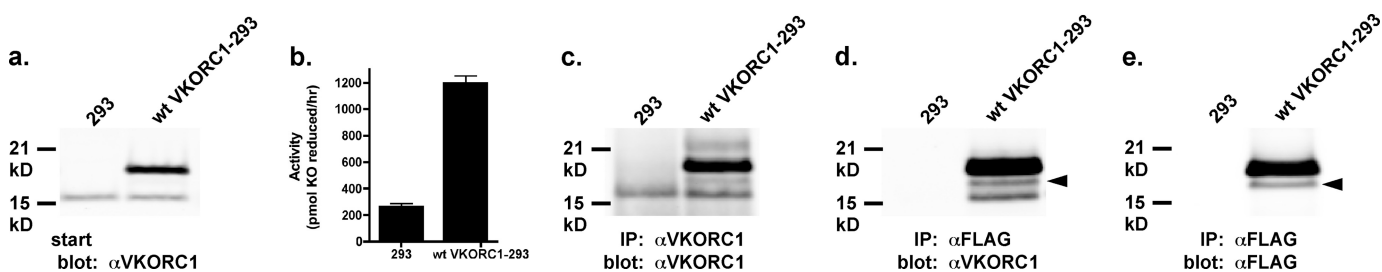


FIGURE 6. Expression of r-wild type VKORC1_{FLAG} in 293 cells reveals association with endogenous VKORC1. Equivalent amounts of lysates (25 μ g) from 293 cells expressing endogenous VKORC1 or also expressing r-wild type VKORC1_{FLAG} were assayed by Western analysis (a) or for the reduction of KO to K (b). Immunoprecipitation (IP) was performed using either anti-VKORC1-Sepharose (c) or anti-FLAG-agarose (d and e), followed by Western analysis with the indicated antibodies. VKORC1 contains reactive thiols (9, 10, 14, 15), and oxidized forms (arrowheads) are not completely reduced during SDS-PAGE.

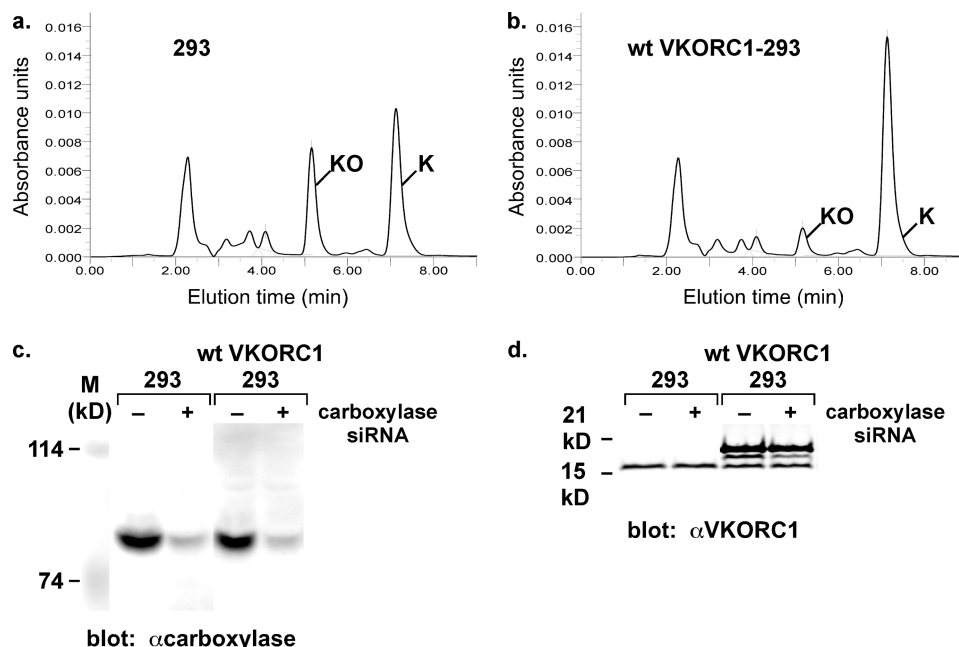


FIGURE 7. An assay to monitor KO reduction in cells. 293 cells (a) and r-wild type (wt) VKORC1_{FLAG} 293 cells (b) were incubated with KO, and vitamin K was subsequently extracted and analyzed by HPLC to monitor KO reduction to K. Similar results were obtained in cells pretreated with siRNA against the carboxylase, which decreased carboxylase (c) but not VKORC1 (d) expression. *M* indicates molecular weight markers.

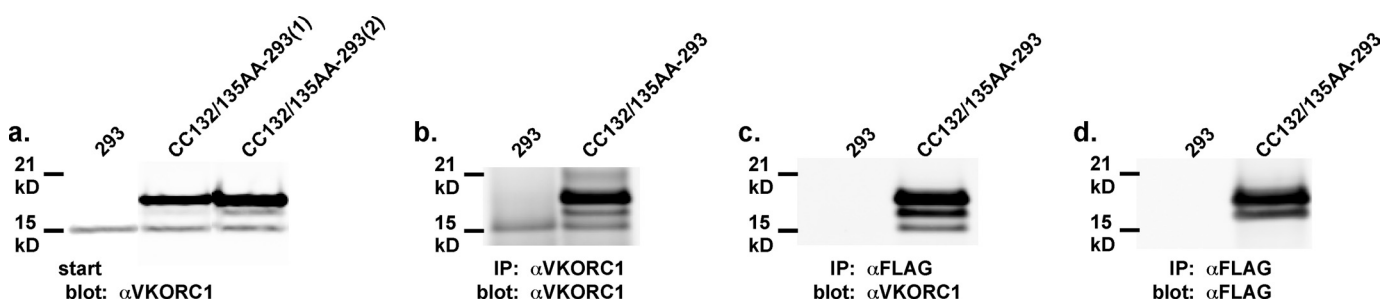


FIGURE 8. Catalytically inactive r-VKORC1(C132A/C135A)_{FLAG} associates with wild type endogenous VKORC1 in 293 cells. a, r-VKORC1(C132A/C135A)_{FLAG} was overexpressed 5- or 7-fold in 293 cells (clones 1 and 2, respectively). b–d, the mutant in clone 1 was tested for association with endogenous VKORC1 by immunoprecipitation (IP) using anti-FLAG-agarose and Western analysis with the indicated antibodies. CC132/135AA, C132A/C135A.

The mutant that was analyzed was r-VKORC1(C132A/C135A)_{FLAG}, which was chosen because substitution of Cys-132 and Cys-135 inactivates VKORC1 (11–13). The mutant was tagged with the FLAG epitope at the C terminus and overexpressed in 293 cells (Fig. 8a), and combined immunoprecipitation and Western analysis indicated association of the mutant with endogenous VKORC1 (shown for one clone in Fig. 8, b–d). The consequence of such association is the repartitioning of

endogenous VKORC1 from wild type homodimers into heterodimers that contain the r-VKORC1(C132A/C135A)_{FLAG} variant. Cells overexpressing the mutant showed a significant decrease in KO reduction, both when carboxylase was present at normal levels (Fig. 9a) or at low levels due to siRNA pretreatment (Fig. 9b). Thus, the mutant showed a dominant negative effect that disrupted VKORC1 reduction of KO. The 293 cells expressing the highest level of r-VKORC1(C132A/C135A)_{FLAG}

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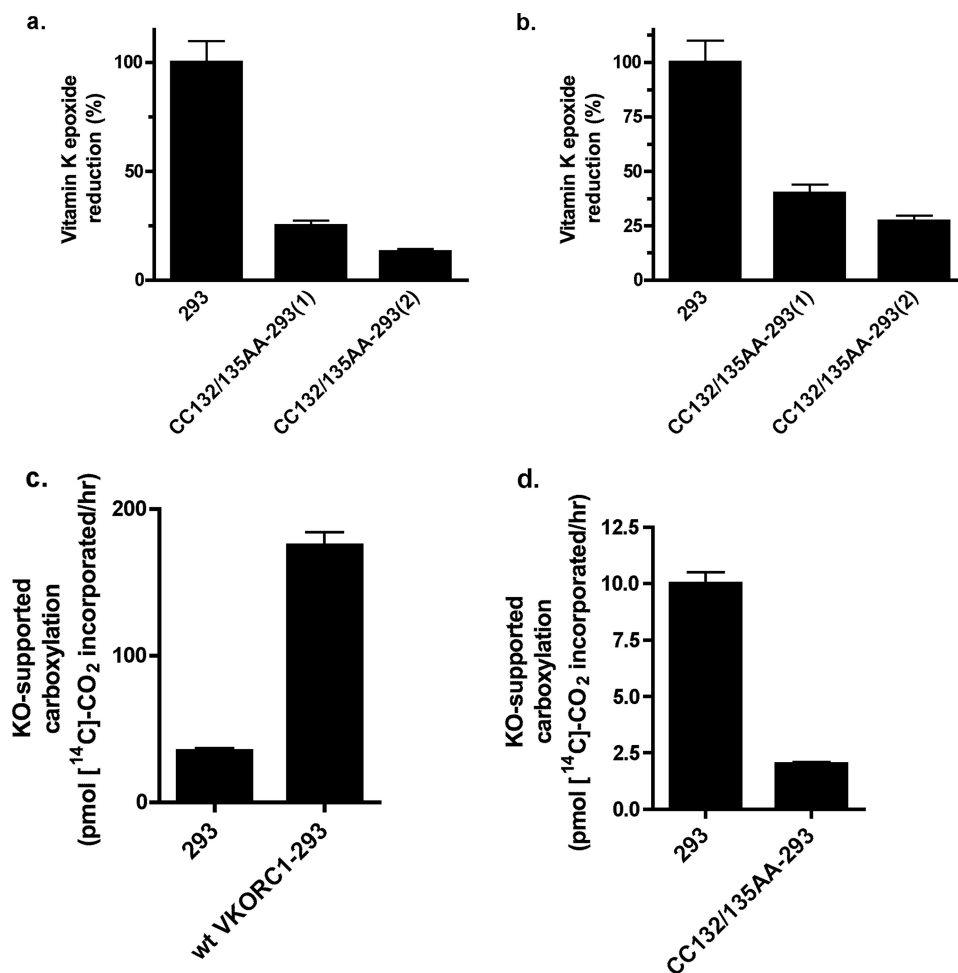


FIGURE 9. Catalytically inactive r-VKORC1(C132A/C135A)_{FLAG} shows a dominant negative effect on KO reduction in cells and carboxylation *in vitro*. 293 cells expressing only endogenous VKORC1 or also expressing r-VKORC1(C132A/C135A)_{FLAG} were assayed for KO reduction as described in the legend to Fig. 7. The cells contained normal levels of carboxylase (a) or decreased amounts (b) due to pretreatment with carboxylase siRNA that altered carboxylase but not VKORC1 protein expression, as described in Fig. 7. The effect of the mutant on carboxylation was also tested. Microsomes prepared from cells expressing only endogenous VKORC1 or also expressing r-wild type VKORC1_{FLAG} (c) or the r-VKORC1(C132A/C135A)_{FLAG} mutant (d) were assayed for FLEEL carboxylation (*i.e.* [¹⁴C]CO₂ incorporation) that reflects KH₂ production.

showed the lowest level of KO reduction (Figs. 8a and 9, a and b), as might be expected because higher levels of mutant expression would result in more heterodimer and less wild type homodimer.

A Dysfunctional VKORC1 Monomer Has a Dominant Negative Effect on Carboxylation—The effect of the r-VKORC1(C132A/C135A)_{FLAG} mutant on carboxylation was also analyzed. This test was possible because VKORC1 is rate-limiting for carboxylation (21, 32) and because of a previous development that improved the sensitivity of the carboxylation assay (27). Endogenous levels of VKORC1 in 293 cells are low, and this assay (described below) therefore enabled analysis of VKORC1 activity. Microsomes were prepared from 293 cells expressing only endogenous VKORC1 or also expressing either r-wild type VKORC1_{FLAG} or the r-VKORC1(C132A/C135A)_{FLAG} mutant and then assayed for VKORC1-supported carboxylation. Activity was monitored using two different reductants to activate VKORC1. Thus, most VKORC1 assays have been performed using the reductant DTT that can directly reduce the Cys-132-Cys-135 disulfide bond. However, we recently showed that VKORC1 is activated through an electron relay pathway (16)

and that a protein redox system (thioredoxin/thioredoxin reductase) acts only through the pathway. As the entire reaction with electron relay had the potential to impact the dominant negative effect, both reductants were tested.

Microsomes were incubated with reductant, the peptide FLEEL, [¹⁴C]CO₂, and KO, and [¹⁴C]CO₂ incorporation into FLEEL was quantitated after two sequential purifications, first using size exclusion chromatography and then HPLC. These isolations substantially increased the signal to noise ratio, consequently allowing sensitive detection of carboxylated FLEEL that quantified KH₂ production. When 293 microsomes were compared with r-wild type VKORC1_{FLAG} microsomes, we observed a 5-fold increase in carboxylation with thioredoxin/thioredoxin reductase (Fig. 9c) and a 3-fold increase with DTT (data not shown). The 5-fold increase with thioredoxin/thioredoxin reductase is similar to the amount of r-wild type VKORC1_{FLAG} overexpression (Fig. 6a), and the results show that carboxylation depends upon the levels of VKORC1 activity. In the case of microsomes containing the r-VKORC1(C132A/C135A)_{FLAG} mutant, a significant decrease in carboxylation was observed when compared with 293 microsomes, *i.e.* 5-fold with

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thioredoxin/thioredoxin reductase (Fig. 9*d*) and 3-fold with DTT (data not shown). The mutant thus had a dominant negative effect on VKORC1-mediated carboxylation.

DISCUSSION

Continuous VKD protein carboxylation requires recycling of KO to KH_2 , which occurs in two reactions, and this study shows that VKORC1 efficiently performs both reactions. Analysis of VKORC1 and the carboxylase coexpressed in insect cells that lack endogenous carboxylation components showed that VKORC1 converts KO to KH_2 to drive carboxylation (Fig. 2). Substantial amounts of KO conversion to KH_2 were also observed with purified VKORC1 (Fig. 5), using a novel assay that we developed that prevents KH_2 oxidation and therefore allows quantitation of this product (Fig. 4). VKORC1 exists as a multimer (Fig. 6), most likely a dimer. Reduction of KO to KH_2 requires four electrons, only two of which are provided by a VKORC1 monomer, and a dimeric structure can therefore explain how VKORC1 accomplishes the full reduction of KO. We found that a catalytically inactive mutant, VKORC1(C132A/C135A), had a dominant negative effect on KO reduction in cells and on carboxylation *in vitro* (Fig. 9). The simplest explanation for these combined data is that a dimeric structure facilitates the ability of VKORC1 to perform two reactions to fully reduce KO to KH_2 .

VKOR exists in mammals to bacteria, and our results indicate that mammalian VKORC1 acquired a new function (reducing KO to K) rather than losing an ancestral function (reducing K to KH_2), as proposed previously (17). That proposal was based on the observation that KH_2 production by purified VKORC1 was inefficient, *i.e.* ~50-fold lower than what we obtained (Fig. 5). One explanation for the difference in results is that our new assay that prevents KH_2 oxidation (Fig. 4) allowed detection of the actual levels of KH_2 . Alternatively, the difference may be due to the enzyme preparation. Previously, VKORC1 was inactivated during detergent solubilization of membrane, and activity was subsequently reconstituted (20), which may have disrupted the dimer to alter the normal course of the reaction. We used an activity assay to optimize membrane solubilization, which retained activity and may have been significant in revealing that VKORC1 efficiently reduces KO to KH_2 .

The observations that VKORC1 exists as a dimer and performs two reactions to reduce KO to KH_2 raise questions about the mechanism. For example, VKORC1 is activated by the transfer of electrons from the extramembrane loop Cys residues to the Cys pair that is membrane-embedded (16), and so a relevant question is whether electron transfer occurs within a single subunit or between two subunits. The nature of the active site that results from a dimeric structure is also of interest. The crystal structure of the bacterial VKOR homolog from *Synechococcus* revealed a cage of four transmembrane domains surrounding ubiquinone, which co-crystallized with the enzyme (8). *Synechococcus* VKOR is a monomeric quinone reductase, and so movement of substrate in and product out of the cage is required for continuous reduction. A similar mechanism for mammalian VKORC1, then, may involve vitamin K movement between the cages present in each monomer.

However, mammalian VKORC1 has been proposed to have a different topology than in bacteria, *i.e.* with three rather than four transmembrane domains (33, 34). A mammalian VKORC1 with a different structure may therefore have a distinct mechanism that cannot be extrapolated from the bacterial structure.

A dimeric structure could increase the efficiency of KO reduction to KH_2 by retaining the K intermediate in the active site and may also be important for accommodating the intermediate. Thus, the thiols that reduce KO to K form a disulfide bond whose accessibility to VKORC1 residues for subsequent reduction could be blocked by the K intermediate; however, K to KH_2 reduction can still occur due to the availability of another pair of thiols in the second subunit of the dimer. These considerations raise the possibility that VKORC1 reduces KO to KH_2 by a processive mechanism. The carboxylase is processive, with VKD protein binding resulting in the conversion of multiple Glu to Gla (35). A processive VKORC1 mechanism that maintains high local concentrations of vitamin K is attractive, as vitamin K levels in the diet and in tissue are low. VKORC1 processivity is consistent with the observation that the amount of carboxylation resulting from VKORC1 reduction of KO was similar to that obtained with saturating concentrations of the KH_2 cofactor (Fig. 2). Processivity is also consistent with the dominant negative effect of the r-VKORC1(C132A/C135A) mutant on intracellular KO reduction (Fig. 9, *a* and *b*). Overexpression of the mutant partitions much of the endogenous wild type VKORC1 into a wild type mutant heterodimer that may be less efficient in KO turnover than the wild type homodimer. Decreased reactivity that stalls the enzyme in a nonproductive complex with K intermediate would decrease the total amount of KO reduction, as observed (Fig. 9, *a* and *b*). Other explanations for the dominant negative effect are also possible, and future studies that address the question of VKORC1 processivity will be important for defining how vitamin K is recycled.

A VKORC1 dimer is of interest with regard to the *in vivo* consequences of naturally occurring VKORC1 mutations. Patients with these mutations will have up to 50% of VKORC1 existing as a heterodimer, depending upon the relative stability of mutant *versus* wild type enzyme. The heterodimer could therefore strongly impact phenotype. VKORC1 mutations have been identified in patients with warfarin resistance (14), which refers to the requirement for higher doses of warfarin to control hemostasis. These higher levels will eliminate wild type homodimer activity, and so mutant homodimer and/or wild type mutant heterodimer will support carboxylation. If the wild type monomer in the heterodimer is resistant to warfarin, then the heterodimer could be fully functional. However, if the wild type monomer in the heterodimer is warfarin-sensitive, then the heterodimer may only be partially functional, making the mutant homodimer the most physiologically important form during warfarin therapy. Alternatively, if the mutations decrease activity, then the heterodimer may be the most important form because the wild type monomer can compensate for the mutant defect. These considerations highlight the significance of understanding the functional properties and warfarin susceptibility of mutant wild type VKORC1 heterodimers to understand the mechanism of warfarin resistance.

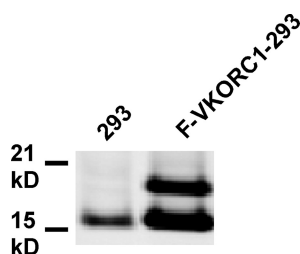


FIGURE 10. Endogenous VKORC1 up-regulation in cells expressing a r-FLAG-VKORC1. Equivalent amounts of lysate (25 μ g) prepared from cells expressing only endogenous VKORC1 or also expressing N-terminally FLAG-tagged r-VKORC1 were assayed by Western analysis using anti-VKORC1 antibody.

A VKORC1 dimer also impacts the interpretation of r-VKORC1 mutant analysis in cells, as what is being studied is a mixed population of VKORC1 dimers. Understanding the functional properties of each type of dimer will therefore be important. Determining the stoichiometry of recombinant and endogenous enzyme will also be important because the relative amounts of heterodimer *versus* mutant homodimer will depend upon the level of r-VKORC1 expression. This stoichiometry can be altered if r-VKORC1 impacts endogenous enzyme levels, which we observed in initial tests to express r-VKORC1 in cells. Although VKORC1 FLAG-tagged at the C terminus had no effect on endogenous expression (Figs. 6 and 8), a FLAG tag at the N terminus resulted in \sim 4-fold up-regulation of endogenous enzyme (Fig. 10). Monitoring only activity and the expression of the FLAG-tagged VKORC1 would have missed the contribution of increased endogenous levels. Thus, determining the levels of both recombinant and endogenous VKORC1 during cellular analysis is essential for obtaining interpretable results.

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