Identification of Key Functional Residues in the Active Site of Vitamin K Epoxide Reductase-like Protein (VKORC1L1)

Abstract

Background/Purpose: Vitamin K is involved in the gamma-carboxylation of the vitamin K dependent proteins. Due to the limited intake of vitamin K, its regeneration is necessary and involves the vitamin K 2,3-epoxide reductase (VKOR) activity. This activity is catalyzed by VKORC1 and/or VKORC1L1 proteins. Warfarin is able to inhibit both enzymes, but VKORC1L1 appears to be 30-fold more resistant to warfarin than VKORC1.

Methods: To predict functional peptide regions or amino acid residues important for VKOR activity or resistance to vitamin K antagonists (VKA) of human VKORC1L1, we conducted a multiple alignment of VKORC1L1 and VKORC1 sequences. The role of conserved amino acid residues between VKORC1 and VKORC1L1, but also the role of conserved amino-acid residues in VKORC1L1 but not in VKORC1 were challenged by systematic engineering of point mutations combined with in vitro functional assays.

Findings: Interestingly, engineering mutants at position 130 allowed us to obtain a VKORC1L1 as susceptible to VKA as wild type VKORC1. Our results also suggested the involvement of Cys-43(+7), Cys-51(+7), Cys-132(+7) and Cys-135(+7), in the transfer of the redox power to vitamin K epoxide.

Conclusion: Altogether, this study provides novel insight into VKORC1L1 active site functional domains. Glu-130 is a key residue governing the natural resistance of VKORC1L1 to VKAs.

Keywords: Vitamin K; Protein; VKOR

Introduction

The vitamin K epoxide reductase (VKOR) activity has been described since the early 70s [1]. This VKOR activity allows the reduction of vitamin K epoxide in order to regenerate vitamin K quinone. This activity is a key step of the vitamin cycle and is the target of vitamin K antagonist (VKA). The VKORC1 gene has only been identified in 2004 [2,3]. This gene encodes for a 163 amino acid protein with, probably, four transmembrane domains and a luminal loop. This protein contains also a C_{12}XXC_{135} redox motif, as previously hypothesized by Silverman [4]. This motif is located in the fourth transmembrane domain.

VKA actively inhibit VKORC1 in the liver and thus limit the gamma-carboxylation of hepatic VKD proteins resulting in an intense anticoagulant effect. VKA are extensively used worldwide for prevention and treatment of thromboembolic disorders. Many missenses mutations have been detected in VKORC1 gene in humans and rats and are sometimes associated to a VKA resistance phenotype [5-7].

Until 2011, VKORC1 was considered as the only protein able to catalyze the VKOR activity. Nevertheless, Westhofen et al. [8] described that a paralogous enzyme of VKORC1, the VKORC1L1, could also reduce vitamin K>0 to vitamin KH when expressed in HEK293T cells. Resulting from a gene duplication of an early
common VKOR ancestor, there is 50% of homology between this protein and VKORC1 [9]. The VKORC1L1 gene encodes for a 176 amino acids protein with almost the same structure than VKORC1. However, this VKOR activity was described with a low enzymatic efficiency and then they propose that VKORC1L1 is responsible for driving the vitamin K mediated intracellular antioxidation pathway critical to cell survival by generating vitamin KH₂ [8].

In another hand, Hammed et al. [10] emphasized two main differences between VKORC1L1 and VKORC1. VKORC1L1-catalyzed VKOR activity is 30-fold more resistant to warfarin than that catalyzed by VKORC1. The tissue distributions of the corresponding mRNA are quite different. Liver expression of Vkorc1L1 mRNA is very low in comparison to Vkorc1. On the contrary, in other tissues such as lung or testis, the expression of these two genes is similar. These data argue for the fact that VKORC1L1 does not interfere with the coagulation process which is supported by the liver. On the contrary, these data argue for the fact that VKORC1L1, being resistant to VKA, could protect extra-hepatic tissues against the effects of VKAs. These results provide an interesting explanation for the low or even the absence of clinical consequences on the gamma carboxylation of vitamin K-dependent proteins (VKDP) produced by extra-hepatic tissues such as matrix Gla protein (MGP) [11], osteocalcin (OC) [12] or Gas 6 [13]. Indeed, the absence of gamma carboxylation of MGP should create ectopic mineralization [14] and the absence of gamma carboxylation of osteocalcin should have consequences on energetic metabolism [15,16].

Unlike VKORC1 proteins, which have been subjected to extensive investigation, our understanding of the mechanism of action of VKORC1L1 is limited. As the contrast between the susceptibility to VKAs of VKORC1 and VKORC1L1 appears to be an important determinant for the biological function of the VKORC1L1 protein, the objective of this paper is to analyze the structural basis of the natural resistance of VKORC1L1 to VKAs in the absence of 3D-structures of VKORC1L1 proteins. To highlight important residues for VKORC1L1 function, sequences alignments were first performed between VKORC1 proteins and VKORC1L1 proteins. Finally, using site directed mutagenesis, we analyzed the functional implications of these highlighted amino acid residues. Our results suggest the central function of Glu-130(+7) amino acid residue in the natural resistance to VKAs of VKORC1L1 compared with VKORC1.

Methods

Plasmid constructions

Human VKORC1 and VKORC1L1 coding sequences fused with a c-myc tag via a flexible (GGG) in its 3’-extremity was optimized for heterologous expression in yeast and synthesized by GenScript (Piscataway, NJ, USA). Synthesized nucleotide sequences included EcoRI and Xbal restriction sites at their 5’- and 3’-extremities, respectively. These nucleotide sequences were subcloned into pPICZ-B (Invitrogen, Cergy Pontoise, France) and sequenced on both strands. Construction of amino acid substituted mutants of VKORC1 or VKORC1L1 was carried out using pPICZ-VKORC1 or pPICZ-VKORC1L1 as template, respectively, with the QuikChange site-directed mutagenesis kit (Stratagene). Mutants were systematically checked by sequencing, and the various mutants were individually expressed in P. pastoris.

Heterologous expression in Pichia pastoris

Heterologous expressions of VKORC1 and VKORC1L1 proteins were performed in Pichia pastoris as described previously [5,17]. pPICZ-VKORC1 or VKORC1L1 vectors were individually transformed into the P. pastoris SMD1168 yeast strain using the P. pastoris Easy Comp Transformation kit (Invitrogen). Transformants were selected on YPD plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) containing 100 µg/ml Zeocin (Invitrogen). The cells were grown in BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base, and 1% (v/v) glycerol). Expression was induced by methanol (1%, v/v) for 48 h at 30°C in a rotary shaker (200 rpm). Yeast cells were collected by centrifugation (3000 g-10 min) and immediately frozen at −20°C.

Subcellular fractionation of recombinant yeast cells

Yeast microsomes were prepared from thawed yeast cells by differential centrifugation. Briefly, yeast cells were resuspended in 50 mM Phosphate Buffer (pH 7.4) containing 1.15% (w/v) of KCl. Yeast cells were broken with Zircon beads using Dispermät® LC30 (VMA-GETZMANN, germany) (15 min-3500 rpm) a continuously at 4°C and further submitted to differential centrifugation. The 100,000 g pellet corresponding to the membrane fraction was resuspended by Potter homogenization in HEPES glycerol buffer (50 mM Hpes, 20% glycerol, pH 7.4). Protein concentrations were evaluated by the method of Bradford using bovin serum albumin as a standard. Microsomes were frozen at −80°C and used for kinetic analysis.

Immunoblot analysis

Expression level quantification of VKORC1 and VKORC1L1 proteins in microsomal fractions were determined by western blotting as described previously [6]. Microsomal proteins were separated on 12% SDS-polyacrylamide gel electrophoresis, transferred onto Immobilon-P membranes and probed with anti-c-myc antibodies (Invitrogen, Cergy Pontoise, France). The resulting immunocomplexes were visualized using alkaline phosphatase-conjugated anti-mouse immunoglobulins as secondary antibodies and a BCIP/NBT solution. Quantification of the stained bands was performed by densitometry using the Scion Image software. The relative intensity (RI) of the signal was correlated to the quantity of microsomal proteins. The relation was linear from 0 to 50 µg for microsomal proteins.

To evaluate the expression level of VKORC1 and VKORC1L1 proteins, the expression of wild type human VKORC1 was designated as the basal expression. For the quantification of wild type or mutant VKORC1 or VKORC1L1, the same unique pool of yeast microsomes containing human VKORC1 was used. Therefore, its expression factor was by definition 1. The expression level of VKORC1 or VKORC1L1 proteins was evaluated by comparison to the expression of human VKORC1. For this purpose, various amounts (from 0 to 20 µg) of microsomal proteins containing human VKORC1 and various amounts (depending on the
expression level) of microsomal proteins containing VKORC1 or VKORC1L1 proteins were analyzed on the same western blot. Two linear relations (RI = a × quantity of microsomes loaded) were obtained, the first one for microsomes containing human VKORC1 (characterized by a specific slope $a_{\text{VKORC1}}$), the second one for microsomes containing VKORC1 or VKORC1L1 proteins (characterized by a slope atarget). Ratio $a_{\text{VKORC1L1}}/a_{\text{VKORC1}}$ allowed us to determine the expression factor characterizing the expression level of VKORC1 or VKORC1L1 proteins in the microsomal fraction compared with the expression level of the human VKORC1.

**Vitamin K epoxide reductase activity (VKOR) assays and kinetics**

Microsomal vitamin K epoxide reductase (VKOR) activity was assayed as described previously [5,17]. Briefly, standard reactions were performed in 200 mM Hepes buffer (pH 7.4) containing 150 mM KCl, 1 mM dithiothreitol, 0.25 to 2 g.L⁻¹ of total proteins containing membrane VKORC1. The reaction was started by the addition of vitamin K₃ solution in 1% Triton X-100 and incubated at 37°C for 30 min. In these conditions, the reaction was linear according to the time of incubation and the quantity of incubated proteins. After incubation at 37°C for 30 min, the reaction was stopped by adding of 4 mL of iced 1:1 isopropanol/hexane solution. After centrifugation at 5000 g for 10 min, the hexane layer was removed and dried under nitrogen. The dry residue was immediately dissolved in 0.2 mL of isopropanol and reaction product was analyzed by liquid chromatography-mass spectrometry.

The LC-APCI/MS/MS used was a 1100 Series LC/MSD ion Trap VL with an Atmospheric Pressure Chemical Ionisation (APCI) interface and a LCMS Chemstation software from Agilent Technologies (Palo Alto, CA, USA). Chromatographic separation was performed using a SunFire reverse phase C8 column (4.6 mm × 150 mm, 5 µm, Waters, Milford, MA, USA) with a mobile phase of methanol, 0.1% acetic acid (95:5) in isocratic conditions. The column temperature was 45°C. The flow rate in the LC column was 1 ml/min. The injection volume was 20µl. The temperature of the autosampler tray was set to 5°C and the samples were protected from the daylight. Detection was by MS/MS with APCI source in positive mode. Nebulizer pressure was set to 60psi, dry gas temperature was set at 350°C, dry gas flow to 5 L/min, and vaporizer temperature to 400°C. Capillary voltage was set to 4000V, corona needle to 4000 nA, and CID to 1V. Collision gas in the trap was helium with a pressure of 0.6×10⁻⁵ mbar. Identification criteria for vitamin K₃ were the retention time (tr= 4.1 min) and the product ion 451 ≥ 187 (m/z(+)=187). Identification criteria for vitamin K₃ ≥ 187 (m/z(+)=307). Linearity and accuracy were tested from 25 to 2000 ng/ml (n=20). The response was linear throughout the concentration range tested with a coefficient of correlation ($r^2$) above 0.99. Accuracy was between 80 and 120% of the theoretical concentrations.

$K_{\text{m}}$, $V_{\text{max}}$, and $K_i$ values were obtained from at least three separate experiments performed on two different batches of protein. The estimation of $K_{\text{m}}$ and $V_{\text{max}}$ values was achieved by the incubation of at least 9 different concentrations of vit K₃ (from 0.003 to 0.2 mM) to the standard reaction. Incubations were performed in duplicate. Data were fitted by nonlinear regression to the Michaelis-Menten model using the R-fit program. In order to evaluate the inhibiting effect of warfarin on VKOR activity, $K_i$ were determined after addition of various concentrations of anticoagulant to the standard reaction in the presence of increasing amounts of vit K₃ (from 0.003 to 0.2 mM) using anticoagulant concentrations from about 0.05 to 20×$K_i$. Data were fitted by non-linear regression to the non-competitive inhibition model $v= (V_{\text{max}}/(1+(L/K_i)))*(S/(K_m+S))$ using the R-fit program.

**Results**

**Comparative inhibition of VKOR activity catalysed by VKORC1 or VKORC1L1**

The susceptibility of wild type VKORC1L1 to various VKAs used either in human medicine or as rodenticide was evaluated comparatively to that of wild type VKORC1. Results are presented in Table 1. While VKORC1 was inhibited by all the VKAs used in this study with Ki lower than 0.5 µM (except for warfarin and phenprocoumon), VKORC1L1 was particularly resistant to some VKAs with Ki systematically higher than 0.5 µM. More exactly, VKORC1L1 was roughly 30-fold more resistant to warfarin, a 4-hydroxycoumarin-derivative and the most VKA used worldwide, than VKORC1 (52 µM vs 1.7 µM). In the same way, fluphenidine, an indanedione derivative largely used in France was completely ineffective to inhibit VKORC1L1 with a Ki 80-fold higher than that obtained for VKORC1 (21.3 µM vs 0.25 µM). Acenocoumarol and phenprocoumon were more effective to inhibit VKORC1L1 than warfarin and fluphenidine, but Ki obtained (i.e., 2.7 and 5.4 µM, respectively) were still largely higher than those obtained for VKORC1 (i.e., 0.3 and 0.7 µM, respectively). VKORC1L1 was also resistant to tecarfarin, a new VKA developed to avoid the CYP-450-dependent metabolism pathway [16]. Ki towards tecarfarin obtained with VKORC1L1 was 30-fold higher than that obtained with VKORC1 (2.4 vs 0.08 µM). The most potent VKAs, such as difenacoum, brodifacoum and difethialone, which possess very low Ki towards VKORC1, were also assayed in this study. These VKAs are used as rodenticide due to their good efficiency even on resistant strains of rats. Even towards these very effective VKAs, VKORC1L1 was approximately 7-fold more resistant than VKORC1, (Difenacoum 0.7 µM vs 0.1 µM, Brodifacoum 0.6 µM vs 0.15 µM, Difethialone 0.9 µM vs 0.2 µM).

**VKORC1 and VKORC1L1 alignment**

To gain insight into potential functional domains or amino acid residues of VKORC1L1 involved in VKOR activity and/or resistance to VKAs in the absence of 3D-structure of VKORC1L1 proteins, we performed a multiple alignment of 20 selected VKORC1L1 sequences plus 26 VKORC1 sequences, taken as an outgroup (Figures 1 and 2). VKORC1L1 proteins contain 176 amino acid residues, while VKORC1 proteins contain 163 amino acid residues. The sizes of the N-terminal and the C-terminal domains are longer in the VKORC1L1 group compared with the 26 VKORC1 proteins. The sequence comparison highlighted i) a very strong identity between VKORC1L1 sequences (between 85 % and 100 % according to the species considered) and ii) a limited degree of
Table 1 $K_i$ values of human VKORC1 and human VKORC1L1 proteins towards various VKA. Inhibition parameters were assessed using anticoagulant concentrations from about $0.05$ to $20 \times K_i$.

| Molecule       | Chemical structure | $K_i$ hVKORC1 (µM) | $K_i$ hVKORC1L1 (µM) | $K_i$ hVKORC1L1 / $K_i$ hVKORC1 |
|----------------|--------------------|--------------------|----------------------|-------------------------------|
| Warfarin       | ![Warfarin Structure](image) | 1.65 ± 0.79        | 52.0 ± 3.0            | 32                            |
| Acenocoumarol  | ![Acenocoumarol Structure](image) | 0.33 ± 0.18        | 2.69 ± 0.8            | 8                             |
| Phenprocoumon  | ![Phenprocoumon Structure](image) | 0.72 ± 0.31        | 5.36 ± 1.6            | 7                             |
| Fluindione     | ![Fluindione Structure](image) | 0.25 ± 0.14        | 21.3 ± 1.3            | 85                            |
| Tecarfarine    | ![Tecarfarine Structure](image) | 0.08 ± 0.01        | 2.40 ± 0.40           | 30                            |
| Difenacoum     | ![Difenacoum Structure](image) | 0.12 ± 0.03        | 0.71 ± 0.09           | 6                             |
| Brodifacoum    | ![Brodifacoum Structure](image) | 0.15 ± 0.02        | 0.57 ± 0.08           | 4                             |
| Difethialone   | ![Difethialone Structure](image) | 0.18 ± 0.03        | 0.87 ± 0.16           | 5                             |

Identity between VKORC1L1 sequences and VKORC1 sequences (~50% of identity) with two remarkable conserved peptide domains with an identity higher than 75%. More precisely, 47 amino acid positions are identical, both in the VKORC1 outgroup and in the VKORC1L1 family. Among these conserved positions, 9 are located in the first conserved motif and 17 are located in the second conserved motif; the others are distributed all along the alignment.

The first conserved domain from amino acid 64 to amino acid 72...
of VKORC1L1 sequences (i.e., the “SRWGRGFL” motif) is strictly conserved between VKORC1L1 and VKORC1 sequences (Figure 1). Nevertheless, none of these residues were described to be of importance in VKORC1. Close to the first domain are found the Cys-43 and Cys-51 strictly conserved in VKORC1 proteins and proposed as important residues in VKORC1 proteins. These Cys are also strictly conserved between VKORC1L1 proteins in position 50 and 58, respectively (Figure 1).

The second conserved domain between VKORC1 and VKORC1L1 proteins contain amino acid 123 to amino acid 146 in VKORC1L1 (i.e., corresponding to the residues 116 to 139 in VKORC1). This domain is highly conserved with 75% of identity between VKORC1L1 sequences and VKORC1 sequences (Figure 2).

The presence of 7 additional amino acids in the first 20 amino acids of VKORC1L1 leads to a gap of the numbering of amino acid residues between VKORC1 and VKORC1L1 sequences. To facilitate the understanding of the results, we decide to number amino acids of VKORC1L1 by the number of the corresponding amino acid in VKORC1 and to add to this number (+7) in index.

Putative warfarin-binding interface I in hVKORC1 (from Leu 22 to Val 29)  
Putative warfarin-binding interface II in hVKORC1 (from Ser 52 to Phe 55)

Analysis of the importance of the “CXXC” motif in the function of VKORC1L1

To address the functional contribution of the “CXXC” motif in the vitamin K epoxide reductase activity catalyzed by VKORC1L1, we conducted a systematic site-directed mutagenesis approach.
Individual cysteines were subjected to non-conservative substitutions to generate 2 mutants VKORC1L1-C132(+7)A and VKORC1L1-C135(+7)A. Wild-type and mutant VKORC1L1 enzymes were expressed at a similar level in Pichia pastoris (data not shown) and tested for in vitro vitamin K epoxide reductase activity using K₁₀₀ as substrate. Activity of the recombinant enzymes determined in the presence of 200 µM of K₁₀₀ was normalized to the amount of expressed protein, and values are presented in Figure 3. The recombinant wild type VKORC1L1 exhibited high activity up to 20 pmol·min⁻¹·mg protein, although it was undetectable in mock-transformed yeasts. On the other hand, replacement of each cysteine of the “CXXC” motif completely abolished the in vitro enzyme activity (i.e. less than 2% of the recombinant VKORC1L1 activity), emphasizing the prevalent function of the “CXXC” motif for the vitamin K epoxide reductase activity of VKORC1L1.

Analysis of the importance of Cys-43(+7) and Cys-51(+7) in the function of VKORC1L1

To further investigate the importance of conserved Cys-43(+7) and Cys-51(+7) in the function of VKORC1L1, we analyzed the catalytic effects of non-conservative mutation of these residues into Ala. Upon expression, VKORC1L1-C43(+7)A and VKORC1L1-C51(+7)A mutants were expressed at similar or higher levels than that of the wild-type enzyme (data not shown). Interestingly, replacement of Cys-43(+7) or Cys-51(+7) of VKORC1L1 by Ala abolished the in vitro VKOR activity (i.e. less than 2% of the recombinant VKORC1L1 activity) in the presence of saturating concentration of K₁₀₀, suggesting the major role of both Cys in the function of VKORC1L1.

Analysis of the importance of the nucleophilic amino acid residues surrounding the “CXXC” motif of VKORC1L1

In the next series of experiments, we explored the specific function of Lys-129(+7), Glu-130(+7) and Tyr-139(+7) amino acid residues surrounding the “CXXC” motif. To address the importance of these invariant amino acids in VKOR activity, a series of mutants exhibiting non-conservative and/or conservative substitutions at each position were engineered to generate 6 mutants VKORC1L1-K129(+7)Y, VKORC1L1-E130(+7)A,-E130(+7)H,-E130(+7)D, VKORC1L1-Y139(+7)A and -Y139(+7)F. The recombinant proteins were produced in yeast P. pastoris. Immunoblot analysis indicated
that these mutants were all produced and their expression factor was comprised between 0.3 to 2 compared with the expression of recombinant wild type VKORC1 protein used as a standard (data not shown).

To evaluate the consequences of the mutations on the enzyme function, we analyzed the activity of mutated VKORC1L1 proteins (Figure 3). VKORC1L1-K129(Y) exhibited up to 200% vitamin K epoxide reductase activity in the presence of 200 µM of K1=0 compared with wild-type VKORC1L1 enzyme; VKORC1L1-E130(A) and VKORC1L1-E130(H) exhibited up to 67 and 86% vitamin K epoxide reductase activity. The non-conservative mutation of Tyr-139(Y) in Ala abolished the VKOR activity suggesting the important role of this residue in VKOR activity (Figure 3). On the contrary, the replacement of Tyr-139(Y) by Phe, another aromatic amino acid residue led to a mutant that exhibited up to 14% vitamin K epoxide reductase activity, indicating that Phe and Tyr residues are to some extent interchangeable at this position. Kinetic parameters and susceptibility to VKA were thus determined for active mutated VKORC1L1 proteins and are presented in Tables 2 and 3, respectively.

**Discussion**

Even if VKORC1L1 was initially proposed to be responsible for driving the vitamin K mediated intracellular antioxidation pathway [8], its crucial role in the gammacarboxylation of extrahepatic VKDP is becoming increasingly obvious. In the absence of VKA treatment, involvement of VKORC1L1 in the gamma-carboxylation of extrahepatic VKDP is at least as important as VKORC1. On the other hand, during long-term anticoagulation therapy with warfarin, VKORC1L1, which is 30-fold more resistant to warfarin than VKORC1 [10], is certainly the only enzyme supporting the VKOR activity in extrahepatic tissues and thus, the gammacarboxylation of extrahepatic VKDPs. Warfarin was the first molecule developed as VKA. Since then, numerous vitamin K-antagonists molecules were developed. While these molecules are all much more potent to inhibit VKORC1 than warfarin, their ability to inhibit VKORC1L1 is still unknown. This question is of major interest if we consider that some of these molecules are also used in human medicine during long-term anticoagulation and that their use could naturally inhibit the coagulation process, but also other extrahaepatic VKDP-dependent physiological processes not inhibited by the use of warfarin.

To address this question, recombinant VKORC1L1 was expressed in *P. pastoris* and VKOR activity was characterized by a DTT-driven VKOR assay, as previously described [5,10]. This experimental approach is a direct approach based on the measurement of the VKORC1 reaction product, the vitamin K. The results obtained by this method, and particularly the resistance factors to VKAs obtained by this method, were successfully confronted to results obtained by *ex vivo* and *in vivo* assays [17,18-20]. This experimental approach was recently challenged by other indirect approaches designed as cell-based VKOR assays [6,7,21] based on the measurement of the gamma-carboxylation of overexpressed clotting factors by the constitutive GGCX in the presence of vitamin K hydroquinone produced by the overexpressed VKORC1. Since the results obtained by the different developed cell-based VKOR assays are conflicting between them, we chose intentionally to use the DTT-driven VKOR approach, as previously used [10] to characterize the VKOR activity of VKORC1L1.

Seven molecules, known to inhibit VKORC1, were tested in this study as inhibitor of VKORC1L1. These molecules are either
Table 2 Apparent kinetic parameters towards K>0 of human VKORC1, VKORC1L1 and mutated VKORC1L1 proteins. V_{max} values determined at saturating concentration of K>0 were evaluated after normalization of the recombinant proteins expression level by immunoquantification. Each data point represents the mean ± SD of three individual determinations.

| Protein          | K_{i} (µM) | V_{max} (pmol min^{-1} mg^{-1}) | V_{max}/K_{i} (nL min^{-1} mg^{-1}) |
|------------------|------------|--------------------------------|------------------------------------|
| hVKORC1          | 21.5 ± 4.2 | 7.1 ± 0.5                      | 330                                |
| hVKORC1L1        | 24.1 ± 3.0 | 20.7 ± 0.9                     | 859                                |
| K129^Y           | 22.2 ± 11.8| 39.2 ± 5.1                     | 1766                               |
| E130^A           | 29.8 ± 8.9 | 13.1 ± 4.4                     | 440                                |
| E130^H           | 31.1 ± 8.3 | 19.0 ± 5.1                     | 611                                |
| E130^D           | 19.1 ± 5.7 | 3.8 ± 0.7                      | 199                                |
| Y139^A           | _           | _                              | _                                  |
| Y139^F           | 25.1 ± 1.2 | 2.7 ± 0.1                      | 108                                |

Table 3 K_{i} values of human VKORC1, VKORC1L1 and mutated VKORC1L1 proteins towards warfarin. Inhibition parameters were assessed using anticoagulant concentrations from about 0.05 to 20×K_{i}.

| Protein          | K_{i} (µM) |
|------------------|------------|
| hVKORC1          | 1.65 ± 0.79|
| hVKORC1L1        | 52.0 ± 0.79|
| K129^Y           | 12.3 ± 1.9 |
| E130^A           | 3.2 ± 1.1  |
| E130^H           | 3.6 ± 1.1  |
| E130^D           | 3.2 ± 0.8  |
| Y139^A           | _          |
| Y139^F           | >300       |

4-hydroxycoumarin or 4-hydroxy-1-thiocoumarin or indanedione derivatives. Whatever the structure of the molecule is, K_{i} obtained for VKORC1L1 systematically increases as compared to human VKORC1, as it was already observed with warfarin [10]. Hydrophobicity and length of lateral chain of the molecules seems to favor the interaction of anticoagulant with VKORC1L1 (Table 1) and (Figure 4), as previously described for VKORC1 [17,19]. Contrary to human VKORC1, 4-hydroxycoumarin structures seems to interact with VKORC1L1 more efficiently than indanedione structures. Among these molecules, four are used (i.e., acenocoumarol, phenprocoumon, fluindione) [22] or developed to be used (i.e., tecarfarin) [18,23] in human medicine and three are used as rodenticides (i.e., difenacoum, brodifacoum, difethialone) [24]. All these molecules are able to inhibit human VKORC1L1 in a non-competitive manner, as observed for human VKORC1 [5]. Nevertheless, all the molecules used in human medicine (i.e., acenocoumarol, phenprocoumon, fluindione, tecarfarin) are very bad inhibitors of VKORC1L1 (with K_{i} higher than 2 µM) while they are able to efficiently inhibit human VKORC1 (i.e., with K_{i}<0.7 µM). It is thus likely that the use of these molecules during long-term anticoagulation therapy allow VKORC1L1 to still support the VKOR activity in extrahepatic tissues, as previously described for warfarin [10]. The other molecules (i.e., difenacoum, brodifacoum, difethialone) with K_{i} less than 1 µM are probably able to inhibit VKORC1L1 in extrahepatic tissues and, thus inhibit or at least decrease the gamma-carboxylation of extrahepatic VKDP. The use of some of these molecules during long-term anticoagulation could lead to serious unwanted side effects due to the undercarboxylation of extrahepatic VKDP such as OC [12], MGP [11], Gas 6 [13]. Fortunately, these molecules are never used in human medicine and are exclusively used for the pest control management.

To pinpoint potential amino acid residues of VKORC1L1 involved in catalysis and/or substrate recognition or responsible for the natural resistance to VKAs of VKORC1L1 in the absence of 3D-structure of VKORC1L1 proteins, we carried out a multiple sequences alignment analysis between VKORC1 and VKORC1L1 proteins. This study allowed us to highlight 1) conserved amino acid residues between VKORC1 and VKORC1L1 potentially involved in catalysis (i.e., Cys-43^(+7), Cys-51^(+7), Asp/Glu-130^(+7)), Cys-132^(+7), Cys-135^(+7), Tyr-139^(+7) and/or substrate binding and 2) conserved amino acid residues in VKORC1L1 (i.e., Lys-129^(+7)), but not in VKORC1 (i.e., Tyr-129) that may cause a different susceptibility to VKAs between enzymes. Our analysis was centered on the area surrounding the presumed VKORC1 catalytic site [25,26], in which mutations leading to the most severe resistances to VKA have been detected [3,17,27]. This area was also proposed as putative-warfarin binding site [7,28]. These highlighted residues were subjected to systematic site-directed mutagenesis and we assessed the functional consequences of these mutations.

The Cys-132^(+7) and Cys-135^(+7) are totally conserved between VKORC1L1 sequences. These cysteines were also previously shown to be evolutionarily conserved in all known VKORC1 sequences [29]. In VKORC1L1, both cysteines are also in a “CXXC” motif (Figure 2). This motif was shown to be implicated in redox reactions in VKORC1 [30,31] but also in other redox proteins [32]. Recently, from two crystal structures of bacterial homologs captured in different reaction states, the second cysteines of the motif was proposed to link covalently to the C1 of the quinone ring of vit K1>0 [33]. In agreement with these results, mutations of cysteines 132 and 135 in alanine in hVKORC1 lead to an inactivation of the enzyme. In hVKORC1L1, the replacement of Cys-132^(+7) and Cys-135^(+7) of the “CXXC” motif by Ala, a non-conservative amino acid residue, totally abolished the VKOR activity. These results suggest the involvement of Cys-132(+7) and Cys-135(+7) in the transfer of the redox power from VKORC1L1 to vitamin K epoxide, as already proposed for VKORC1 [30].

Besides Cys-132 and Cys-135, two other cysteines Cys-43 and Cys-51 are strictly conserved in VKORC1 proteins of vertebrates, Drosophila, plants, bacteria and arachaea. In VKORC1L1 proteins, both cysteines are also strictly conserved. In bacterial VKOR homologues, both cysteines are present in the predicted luminal loop separating α-helices 1 and 2. These loop cysteines were proposed to reduce the CXXC motif [31,33]. For VKORC1 proteins, there is still a debate between Cys-43 and Cys-51 being involved in the internal electron transfer. Indeed substitutions of Cys-43 or/Cys-51by non-conservative residues lead to contradictory results [30,34-36]. To address the functional contribution of both residues in VKORC1L1, Cys-43^(+7) or Cys-51^(+7) were replaced by Ala, a non-conservative residue. These mutations totally abolished activity of recombinant VKORC1L1 enzyme, suggesting a crucial role of these conserved residues in the internal electron transfer from the redox protein partner to the internal catalytic site, as suggested recently by Tie et al. [37].

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A theoretical reaction mechanism for converting vitamin K>0 to K by VKOR enzymes was proposed by Silverman based on a chemical model study [38]. This mechanism was revisited later by a quantum chemical study [39]. The theoretical model implied the CXXC catalytic site and one nearby acidic amino acid residue involved in the protonation of the epoxide oxygen. Nevertheless, this proposed reaction mechanism was never experimentally confirmed even for VKORC1 enzymes. In hVKORC1l, only two acidic amino acid residues Glu-130 (+7) and Tyr-139 (+7) are located close to the CXXC catalytic site. Both residues are totally conserved between VKORC11 proteins. To explore their respective role in the reaction mechanism of VKORC11, non-conservative and conservative mutations of these residues were performed.

The non-conservative substitution of Tyr-139 (+7) by Ala in hVKORC1l totally abolished VKORC11 activity, while the conservative substitution of Tyr-139 (+7) by Phe partially restored VKORC11 activity with a Kᵢ similar to wild type VKORC1. These results highlight the importance of an aromatic residue at this position. The Tyr-139 (+7) may be involved in the substrate binding via stacking interaction. Nevertheless, the replacement of this residue by Phe at position 139, dramatically impaired the Vₘₐₓ and thus the catalytic efficiency. The Tyr-139 (+7) of VKORC11 might be involved in the protonation of the reaction intermediate produced by catalysis, as was proposed for VKORC1 [40]. Finally, even if VKORC11 is naturally 30-fold more resistant to VKAs than VKORC1, the replacement of Tyr-139 (+7) by Phe leads also, as described for VKORC1 [27,41], to an increase of Kᵢ, of VKORC11 towards VKAs. This result suggests a similarity of VKA-binding sites between VKORC1 and VKORC11 and the involvement of Tyr-139 (+7) in the binding of VKA by VKORC11 as proposed for VKORC1 [28].

The non-conservative substitutions of Glu-130 (+7) by Ala or His in hVKORC11 did not modify the VKOR activity. These results are thus not in favor of the involvement of Glu-130 (+7) in the reaction mechanism of hVKORC11 and more precisely in the protonation of the epoxide oxygen. Nevertheless, while the substitution of Glu-130 (+7) by Ala or His does not modify VKOR activity, the replacement of the same Glu-130 (+7) by Asp drastically decreases VKOR activity with an activity lower than 20% compared with that of the wild type enzyme. Therefore the catalytic efficiency of this mutant is extremely decreased while the enzyme-substrate interaction is unchanged (Kᵢ similar to that of the wild type enzyme). This loss of activity is very surprising because in VKORC1 proteins an Asp is present at this corresponding position. The weak activity of VKORC11-E130 (+7)D is potentially due to a modification of the steric hindrance of the CXXC catalytic site, leading to a change in the positioning of the substrate in the catalytic site. In view of these surprising results, we then evaluated the susceptibility of the VKORC11-E130 (+7)A, -E130 (+7)H, -E130 (+7)D to VKAs. Indeed, the domain surrounding the CXXC motif from Phe-131 to Thr-137 was recently proposed as putative warfarin-binding site for VKORC1 [27]. While VKORC11 is naturally very resistant to VKA (30-fold than VKORC1 towards warfarin), the simple substitution of Glu-130 (+7) by Ala, His or Asp (i.e., the corresponding amino acid found in VKORC1) drastically modified susceptibility to VKAs. Their susceptibility to VKAs becomes similar to that of VKORC1. These results suggest that the natural resistance to VKA of VKORC11 is partially due to the steric hindrance of VKA interaction site by the lateral chain of Glu-130 (+7). Nevertheless, the replacement of Asp-130 by Glu in VKORC1 does not modify the susceptibility of VKORC1 to VKA (data not shown), suggesting structural properties different between VKORC1 and VKORC11.

**Conclusion**

In conclusion, this structure-function study allowed us, 1) to demonstrate the importance of Cys-132 (+7) and Cys-135 (+7) but also of Cys-43 (+7) and Cys-51 (+7) in the transfer of the redox power from VKORC11 to vitamin K epoxide, 2) to compare the susceptibility to all VKAs of VKORC1 and VKORC11, 3) to demonstrate the implication of Tyr-139 (+7) in the interaction of VKORC11 with warfarin, 4) to associate the natural resistance of VKORC11 to VKA with the steric hindrance of VKA interaction site by the lateral chain of Glu-130 (+7). Interestingly, a single mutation is able to modify drastically the susceptibility of VKORC11 to VKAs.

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