Inhibition of bacterial disulfide bond formation by the anticoagulant warfarin

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Blood coagulation in humans requires the activity of vitamin K epoxide reductase (VKOR), the target of the anticoagulant warfarin (Coumadin). Bacterial homologs of VKOR were recently found to participate in a pathway leading to disulfide bond formation in secreted proteins of many bacteria. Here we show that the VKOR homolog from the bacterium *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, is inhibited by warfarin and that warfarin-resistant mutations of mycobacterial VKOR appear in similar locations to mutations found in human patients who require higher doses of warfarin. Deletion of VKOR results in a severe growth defect in mycobacteria, and the growth of *M. tuberculosis* is inhibited by warfarin. The bacterial VKOR homolog may represent a target for antibiotics and a model for genetic studies of human VKOR. We present a simple assay in *Escherichia coli*, based on a disulfide-sensitive β-galactosidase, which can be used to screen for stronger inhibitors of the *M. tuberculosis* VKOR homolog.

vitamin K epoxide reductase | DsbB | DsbA | *Mycobacterium tuberculosis*

The stability of many secreted proteins depends on the presence of structural disulfide bonds. Disulfide bond formation in *Escherichia coli* requires two cell-envelope proteins, DsbA and DsbB (1). The periplasmic protein DsbA, a thioredoxin family member, is the direct catalyst of disulfide bond formation. The cytoplasmic membrane protein DsbB maintains DsbA in the oxidized, active state by transferring electrons from DsbA to membrane-bound quinones. Unlike *E. coli*, many other bacteria do not have a DsbB protein encoded in their genomes, but instead have an alternative pathway for disulfide bond formation (2, 3). In place of DsbB, these bacteria use a homolog of an enzyme required for blood coagulation in humans. This enzyme is vitamin K epoxide reductase (VKOR), the target of the most widely used oral anticoagulant, warfarin (Coumadin).

We previously showed that the VKOR homolog from *Mycobacterium tuberculosis* was capable of replacing DsbB in *E. coli* and thereby restoring disulfide bond formation to an *E. coli ΔdsbB* strain (2). Similar results were obtained with a VKOR homolog from a cyanobacterium (3). Although bacterial VKOR homologs do not show sequence similarity to DsbB, these results suggest that they may be carrying out similar reactions to those of DsbB: the oxidation of DsbA-like proteins followed by the reduction of quinones.

Although the cellular processes in which the bacterial VKOR (and DsbB) and human VKOR are involved (disulfide bond formation and blood coagulation) are quite different, the enzymatic reactions that they can carry out are analogous. In both cases, the enzymes mediate the transfer of electrons from a thioredoxin-like protein to a quinone. Human VKOR can transfer electrons from protein disulfide isomerase, also a thioredoxin family member and the primary catalyst for disulfide bond formation in eukaryotic secreted proteins, to vitamin K, a quinone, in the endoplasmic reticulum membrane (4–8). This reaction produces reduced vitamin K, which is required as a cofactor for the enzyme γ-carboxylase, allowing this enzyme to make post-translational modifications critical to the activity of several clotting factors (9). The reduction of vitamin K by VKOR is the reaction inhibited by the anticoagulant drug warfarin (Coumadin) (10).

In this article, we show that the VKOR homolog from *M. tuberculosis* is also sensitive to warfarin. In addition, we have found that mutations conferring warfarin resistance on this protein are located at sites similar or identical to such mutations found among humans who require higher doses of warfarin as a blood thinner. Further, we find that warfarin inhibits the growth of *M. tuberculosis* and that deletion of the *vkor* gene from *Mycobacterium smegmatis* results in a severe growth defect. Finally, we show that the activity of the *M. tuberculosis* VKOR can be assayed in *E. coli* using a disulfide-sensitive β-galactosidase fusion protein, which provides a cell-based, positive screen for stronger inhibitors of the enzyme.

Results

***M. tuberculosis* VKOR Can Efficiently Replace *E. coli* DsbB.** We have previously reported that when the *M. tuberculosis* (*Mtb*) VKOR homolog is expressed in *E. coli* from a weakened *trc* promoter [plasmid pDSW206 (11)], it is able to restore motility, although only partially, to a *dsbB* mutant (2). The motility phenotype is frequently used to assess the effectiveness of the *E. coli* disulfide bond formation pathway because the flagellar P-ring protein FlgI requires disulfide bonds for its function (12). To study the properties of *Mtb*VKOR more effectively, we switched to using two plasmids from which the VKOR would be produced at higher levels. These new plasmids use the more potent Ptrc promoter [in pTrc99a (13)] to express either the *Mtb*VKOR ORF alone (pRD31, untagged *Mtb*VKOR, see Table S1 for strains and plasmids used in this study) or the open reading frame (ORF) with a 6xHis tag at its amino terminus (pRD33, his-*Mtb*VKOR). When fully induced, both the untagged and his-*Mtb*VKOR show significantly improved motility, near wild-type levels, which suggests that the expression of *Mtb*VKOR was limiting its ability to complement the *dsbB* mutant (Fig. S1). The his-*Mtb*VKOR shows higher activity than the untagged version, possibly because the amino-terminal tag improves expression of the mycobacterial protein in *E. coli.* These two plasmids give us the ability to test different ranges of *Mtb*VKOR expression on various phenotypes, as described below. We point out that, importantly, all of the experiments with *E. coli* reported in this article can be done with growing cells because the elimination of the disulfide bond formation pathways does not interfere significantly with growth.

We asked directly whether the presence of *Mtb*VKOR in an *E. coli* lacking DsbB would restore oxidation of DsbA and FlgI. Although in a *dsbB* mutant DsbA is overwhelmingly in the reduced form (Fig. 1*A*, lane 5), the efficiently complementing his-*Mtb*VKOR plasmid restores full oxidation of DsbA to the strain

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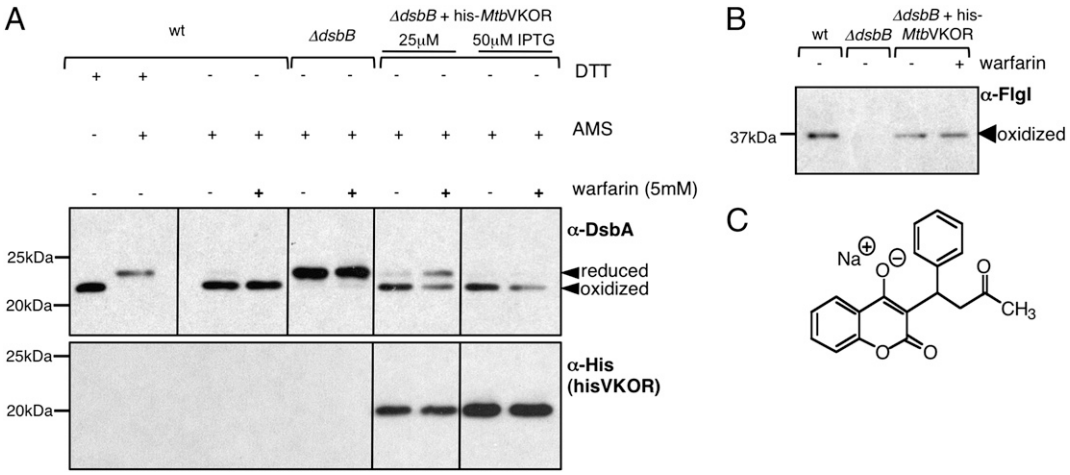

Fig. 1. Oxidation of *E. coli* DsbA by *M. tuberculosis* VKOR. (*A*) We performed in vivo cysteine alkylation to observe the oxidation of DsbA promoted by VKOR. Samples from wild-type *E. coli* that were DTT-treated (lane 1); DTT-treated followed by alkylation with AMS (0.5 kDa), which will react with free cysteines in the reduced protein but with not oxidized cysteines (DsbA has two cysteines, resulting in a shift of 1 kDa upon alkylation) (lane 2); and alkylated with AMS (without prior reduction) (lane 3). Lanes 3–10 represent the redox state in cultures grown either without or with 5 mM warfarin added to the growth media. Oxidation of DsbA in wild-type cells (lane 3) is not affected in the presence of warfarin (lane 4). DsbA is in the reduced state in a *dsbB* mutant (lane 5) and oxidized when the *dsbB* mutant is complemented by expression of his-*Mtb*VKOR from a plasmid (lane 7). Warfarin addition (5 mM) causes approximately one-half of the DsbA to shift to the reduced state (lane 8). Increasing the expression of his-MtbVKOR (using 50 μM IPTG) decreases the effect of warfarin (lanes 9 and 10). We have seen that, in repetitions of this experiment, that the DsbA levels are lower in the strains expressing *Mtb*VKOR. We cannot explain this finding, although it is known that DsbA is subject to regulatory effects (28). (*B*) The redox state of FlgI, which is unstable and thus degraded in the absence of disulfide bond formation (in the *ΔdsbB* mutant), was analyzed, and the expression of *Mtb* VKOR was shown to restore oxidation to substrates of DsbA. (*C*) The chemical structure of warfarin (sodium salt form).

(Fig. 1*A*, lane 7). Similarly, in the *dsbB* mutant, the FlgI protein cannot be detected on gels due to its reduced state and subsequent degradation, whereas the *Mtb*VKOR restores oxidized FlgI (Fig. 1*B*).

***M. tuberculosis* VKOR Homolog Is Warfarin-Sensitive.** Because human VKOR is the clinical target of warfarin, we wanted to ask whether *Mtb*VKOR might also be warfarin-sensitive. Although inhibitory effects on bacterial VKOR activity might be assayed in vivo in *E. coli* lacking DsbB by assessing the oxidation state of either DsbA or FlgI, we sought a more sensitive assay for defects in disulfide bond formation. To this end, we employed a protein fusion that makes the enzyme β-galactosidase sensitive to disulfide bond formation (14). This fusion protein has a portion of the membrane protein MalF attached to the amino terminus of β-galactosidase (MalF–βgal), such that β-galactosidase protrudes into the periplasm. In this location, the normally cytoplasmic enzyme is inactivated by disulfide bond formation.

Wild-type strains, in which disulfide bond formation occurs, show low β-galactosidase activity of the fusion protein. However, when disulfide bond formation is impaired in *dsbA* or *dsbB* mutant backgrounds, the strains exhibit high β-galactosidase activities up to as much as three orders of magnitude higher than that seen in the wild-type background. Thus, the presence and the degree of functioning of the disulfide bond formation pathway can be monitored by a colorimetric assay of β-galactosidase in growing cells. This assay is particularly sensitive, giving measurable differences in β-galactosidase activity when there are only slight defects in disulfide bond formation (14–16).

As seen above with the detection of oxidized DsbA and FlgI, expression of *Mtb*VKOR (untagged) in an *E. coli* strain lacking DsbB restores efficient disulfide bond formation, resulting in low β-galactosidase activity of the MalF–βgal fusion protein (Fig. 2*A*). However, when warfarin is added to the growth media of this strain, β-galactosidase activity increases, indicating that disulfide bond formation is inhibited (Fig. 2*A*). In contrast, warfarin added to wild-type *E. coli*, in which DsbB is the oxidant of DsbA, or to the *dsbB* mutant causes no change in β-galactosidase activity. Although these results indicate that the drug targets *Mtb*VKOR-promoted disulfide bond formation and not that promoted by DsbB, we cannot rule out the possibility that warfarin could inhibit DsbB under some other conditions.

Millimolar concentrations of warfarin (1–10 mM) are required for inhibition of *Mtb*VKOR expressed in *E. coli*, whereas, for human VKOR, low micromolar concentrations are effective (17). This difference may result either from the lower affinity of warfarin for *Mtb*VKOR due to sequence divergence (*Mtb*VKOR is 18% identical and 32% similar to human VKOR) or from the restricted accessibility of the protein to warfarin in bacteria. The anticoagulant phenindione also inhibits *Mtb*VKOR, but at a concentration an order of magnitude lower than warfarin.

The inhibition of *Mtb*VKOR was further confirmed by analyzing the redox state of DsbA in the presence of warfarin. Whereas DsbA is fully oxidized when his-*Mtb*VKOR is expressed in a *dsbB* mutant (Fig. 1*A*, lane 7), the addition of 5 mM warfarin to the growth media results in ≈50% of the DsbA being found in the reduced state (Fig. 1*A*, lane 8). Increasing the expression level of his-*Mtb*VKOR with additional isopropyl–thiogalactosidase (IPTG) leads to a decrease in the ability of warfarin to inhibit disulfide bond formation (Fig. 1*A*, lanes 9–10).

Warfarin-Resistance Mutations in the *M. tuberculosis* VKOR Homolog Align to Sites of Warfarin-Resistance Alleles in Human VKORC1. To show that warfarin directly inhibits this bacterial VKOR homolog, we sought warfarin-resistant mutants of *Mtb*VKOR. In these experiments, we mutagenized plasmid pRD31 (medium-complementing) containing *Mtb*VKOR and then selected for plasmids that restored motility to an *E. coli ΔdsbB* strain in the presence of warfarin. The high-complementing pRD33 plasmid was not used for these experiments because there is not sufficient inhibition by warfarin of *Mtb*VKOR in this complemented strain to lower the amount of FlgI and, thus, affect motility (see Fig. 1*B*). We obtained and characterized four warfarin-resistant mutations of *Mtb*VKOR. DNA sequencing of

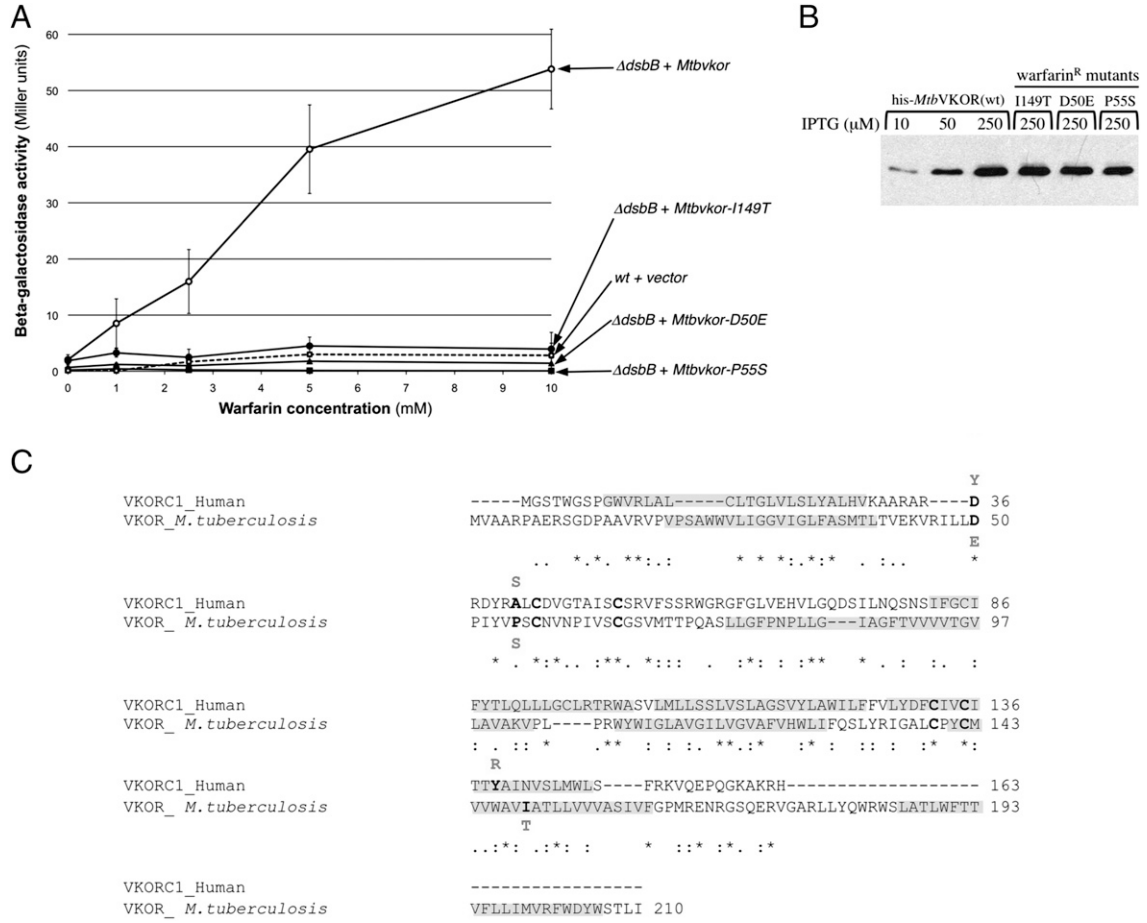

Fig. 2. *M. tuberculosis* VKOR is warfarin-sensitive and mutations conferring warfarin resistance mimic naturally occurring warfarin-resistance alleles in human and rat populations. (*A*) The MalF–βgal fusion exports β-galactosidase to the periplasm and makes it sensitive to disulfide bond formation (14). Disulfide bonds inactivate β-galactosidase, resulting in low activity in the wild-type *E. coli* background (0.1 ± 0.1 Miller units), high activity in the *dsbB* mutant (216.9 ± 18.5 Miller units), and low activity when the *dsbB* mutant is complemented by *Mtb*VKOR (2.0 ± 0.8 Miller units). Addition of warfarin does not affect the activity of wild type or *dsbB* mutant *E. coli* carrying the fusion, but results in an increase in β-galactosidase activity in the VKOR-complemented strain. The maximal activity observed (at 10 mM warfarin, 53.8 ± 7.0 Miller units) is 26% of the activity of the uncomplemented strain (*ΔdsbB*, not shown). Each of the warfarin-resistant mutants (I149T, D50E, and P55S) complement for disulfide bond formation; however, they do not respond to warfarin. Error bars represent the standard deviation based on three separate experiments. (*B*) 6xHis-tagged versions of the warfarin-resistant mutants were constructed to check expression levels of these proteins. Wild-type his-*Mtb*VKOR was expressed with increasing concentrations of IPTG (10, 50, 250 μM). (*C*) VKOR homologs from *M. tuberculosis* (Rv2968c; gi:15610105) and humans (VKORC1, gi:46241834) were aligned with ClustalW2.0 (29). The mutations identified in this study that confer warfarin resistance to the mycobacterial VKOR are below the sequence. Previously characterized mutations in similar sites associated with warfarin-resistance phenotypes in the human VKOR are above the sequence. Predicted transmembrane domains [made using Scampi (30)] are shaded.

the mutations revealed that they mapped to locations similar to those of the alleles of VKOR detected in humans who require higher warfarin doses (Fig. 2*C*).

One of the warfarin-resistant *Mtb*VKOR mutations results in an Asp50-to-Glu change. A comparison of the sequences of *Mtb*VKOR with that of human VKORC1 reveals an alignment in which Asp50 corresponds to Asp36 in humans. In Ashkenazi Jewish and Ethiopian populations, an Asp36-to-Tyr change is associated with warfarin resistance (18, 19). Although the Asp-to-Glu mutation represents a rather conservative change, both this and the Tyr replacement found in the human enzyme result in bulkier residues, which may interfere with the binding of warfarin. Another mutation results in a Pro55-to-Ser change. This site aligns to Ala41 in humans. One report indicates that patients with a VKORC1 Ala41-to-Ser mutation (heterozygous) require the highest overall doses of warfarin (20). Another mutation in *Mtb*VKOR results in an Ile149-to-Ser change. This alteration lies two amino acids downstream of Tyr139 in human VKOR. Several alterations at Tyr139 give warfarin resistance in rats and mice (21), and the Thr–Tyr(139)–Ala motif has been proposed as the binding site for warfarin (22). Another warfarin-resistant mutation does not map to the ORF, but instead to the –1 position of the gene and may influence VKOR expression. We have observed that increasing expression of *Mtb*VKOR in *E. coli*, by varying IPTG levels or the strength of the promoter, leads to an increase in the concentration of warfarin required for inhibition. In humans, mutations in the VKOR promoter region can confer increased dose requirements for warfarin (20, 23).

These mutants all retained the ability to catalyze disulfide bond formation (this was a condition of the original selection), thereby inactivating the MalF–βgal fusion (Fig. 2*A*, at 0 mM warfarin), and no longer showed sensitivity to warfarin (Fig. 2*A*, 0–10 mM warfarin). The three warfarin-resistant mutations that affect the coding sequence do not alter the amounts of protein, because, when these mutations are introduced into the plasmid with the amino-terminal 6His-tag, Western blots show that they are expressed in the same amounts as wild-type VKOR (Fig. 2*B*).

Deletion of the VKOR Homolog Causes a Severe Growth Defect in Mycobacteria. A high-throughput transposon mutagenesis study of *M. tuberculosis* indicated that VKOR is essential for growth of this pathogen (24) and thus could represent a target for antituberculosis drugs. To further assess the essentiality of VKOR in mycobacteria, we constructed a clean deletion of the *vkor* gene (Msmeg_2411) in *M. smegmatis*, a fast-growing relative of *M. tuberculosis*. The strain deleted for *vkor* has a severe growth defect, forming tiny colonies on rich medium (NZ) and not growing at all on minimal medium (7H10) (Fig. 3 and Fig. S2). These growth defects are eliminated by expression of the VKOR homolog from *M. tuberculosis* or by expression of DsbB from *E. coli* (Fig. 3). Complementation of the *vkor* mutation by *E. coli* DsbB provides further support for the role of VKOR in disulfide bond formation in mycobacteria. Addition of cysteine (10% final concentration) to the growth media (either NZ or 7H10) also improves growth, possibly by providing a source of redox-active thiol groups for direct oxidation of substrate proteins.

Mycobacteria Are Warfarin-Sensitive. We next asked whether warfarin could inhibit the growth of *M. tuberculosis*. Growth was inhibited at minimal inhibitory concentrations (MIC) in the low millimolar range (1.6 and 4.5 mM for *M. tuberculosis* and *M. smegmatis*, respectively). This is similar to the concentration required to inhibit the enzyme when expressed in *E. coli.* We also tested an anticoagulant, phenindione, which inhibited growth at lower concentrations (250 μM for *M. smegmatis*, no data for *M. tuberculosis*). However, when the *Mtb*VKOR warfarin-resistant mutants obtained in *E. coli* are introduced into the *M. smegmatis Δvkor* mutant, these strains, and the DsbB-complemented strain, remained sensitive to warfarin, showing the same MIC as wild type. Although our data show (1) that the *M. tuberculosis* VKOR homolog is inhibited by warfarin, (2) that loss of VKOR results in severe growth defects of mycobacteria, and (3) that growth of *M. tuberculosis* is inhibited by warfarin, these last results suggest that warfarin may have additional essential targets in mycobacteria, or perhaps less likely, that VKOR is not accessible to warfarin in mycobacteria. Identifying an additional target of warfarin in *M. smegmatis* may be achieved by isolating warfarin-resistant mutants in *M. smegmatis* directly or in strains that already carry one of the VKOR warfarin-resistant mutants isolated in *E. coli.*

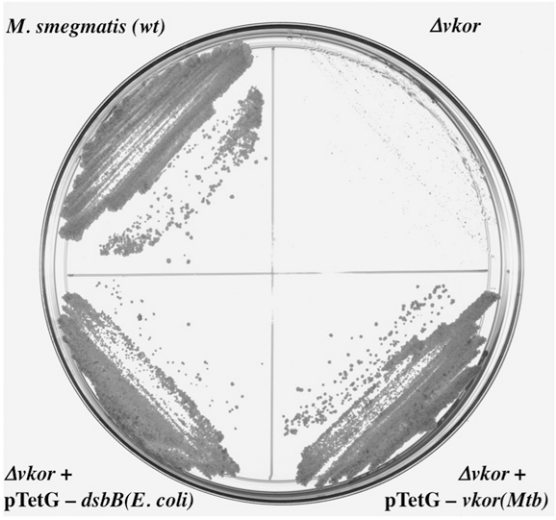

Fig. 3. Deletion of the VKOR homolog (Msmeg_2411) from *M. smegmatis* results in a severe growth defect. This strain produces tiny colonies on rich media (NZ, shown) and fails to grow on minimal media (7H10, Fig. S2). The growth defect is complemented by expression [from an episomal plasmid, pTetG (31)] of the VKOR homolog from *M. tuberculosis* or by expression of DsbB from *E. coli.*

Discussion

We have previously shown that *M. tuberculosis* VKOR can replace DsbB in *E. coli*, allowing disulfide bond formation to take place in a DsbA-dependent manner (2). Here, we increased expression levels of *Mtb*VKOR in *E. coli* and confirmed directly that expression of *Mtb*VKOR promotes in vivo oxidation of DsbA, thus allowing disulfide bond formation in substrate proteins.

Although *Mtb*VKOR is similar in function to DsbB, it also shares common features with human VKOR. In addition to the homology, the two proteins carry out electron transfer processes using very similar substrates (5, 8). We show here that disulfide bond formation promoted by *Mtb*VKOR in *E. coli* is inhibited by the drug warfarin, the most widely used oral anticoagulant, which targets VKOR of humans and other vertebrates. The inhibition by warfarin seen in *E. coli* appears to be specific to *Mtb*VKOR because disulfide bond formation promoted by DsbB is not affected by warfarin. The finding of warfarin-resistant mutations that alter the *Mtb*VKOR gene further supports our proposal that warfarin is, in fact, targeting this enzyme. Three of the four warfarin-resistant mutations that we isolated are located in the *Mtb*VKOR-coding region. Consistent with our suggestion that bacterial and human VKOR share common features is the finding that these three mutations alter amino acid residues that correspond to the same or similar sites as those found among humans who require higher doses of warfarin (7, 18–23). These mutations do not alter expression of the protein and therefore may be affecting the binding of warfarin to the protein or altering the redox activity of the protein to overcome the inhibition.

Although the finding that warfarin-resistance mutations map to similar sites in both human and bacterial VKOR suggests that these two homologs may interact similarly with warfarin, it remains to be seen whether other aspects of the functioning of human VKOR, such as reduction of the epoxide form of vitamin K, are conserved across the entire protein family (17). However, our results raise the possibility that bacterial VKOR homologs may provide a model for the human enzyme. In addition, applying genetic approaches in *E. coli* to the human enzyme could also be very informative. Therefore, we are currently attempting to express human VKORC1 in *E. coli* and assess its potential to participate in the disulfide bond-forming pathway.

Bacterial VKOR homologs, in addition to serving as models for the VKOR protein family, could also represent previously undescribed targets for the development of antibiotics. Because bacterial virulence, in many cases, depends on secreted proteins, many of which contain disulfide bonds, inhibiting a pathway that ensures the stability of secreted proteins could prove effective in interfering with pathogenesis (25). Our results demonstrating that *Mtb*VKOR is capable of being inhibited by small molecules by using high concentrations of warfarin, that the growth of *M. tuberculosis* is sensitive to high concentrations of warfarin, and that deletion of *vkor* from *M. smegmatis* results in a severe growth defect and previous work suggesting that *vkor* is essential for the growth of *M. tuberculosis* (24) indicate that stronger inhibitors of the *Mtb*VKOR homolog could represent a unique class of antituberculosis drugs. If stronger inhibitors of the bacterial enzyme are found, they may be useful in that they could inhibit the growth of *M. tuberculosis* and cause defects in disulfide bond formation in secreted proteins, which may include virulence factors.

We describe a sensitive colorimetric assay for *Mtb*VKOR activity in *E. coli* that will allow a screen for stronger inhibitors of that enzyme. Inhibitors of disulfide bond formation promoted by *Mtb*VKOR provoke a significant increase in the β-galactosidase activity of the MalF–βgal fusion. This activity can be monitored in liquid or agar-based media by various colorimetric and fluorescent

substrates of β-galactosidase, long used in biology. These assays should allow high-throughput screening for inhibitors of *Mtb*VKOR via a cell-based, positive screen. On the basis of previous work from this laboratory, we anticipate that the compounds that will give a positive signal with the MalF–βgal fusion in this strain will be limited mainly to those inhibiting the *Mtb*VKOR-dependent disulfide-bond-forming pathway (14–16). The screening may be particularly advantageous because the assay for inhibition is carried out under in vivo conditions where bacterial growth (of *E.coli*) is not inhibited. This approach is not currently possible in *M. tuberculosis* itself, first, because no assay for disulfide bond formation is available for the organism and, second, because the organism exhibits severely retarded growth in the presence of the inhibitors of *Mtb*VKOR. Given the findings with warfarin, such a screen may yield compounds that also inhibit human VKOR. Such potential alternative anticoagulants to warfarin could be tested by in vitro assays for inhibition of the human enzyme.

Methods

To generate warfarin-resistant mutants, a plasmid carrying the *Mtb*VKOR (pRD31: pTrc99a-*Mtbvkor*) was mutagenized in the *E. coli* mutator strain XL1-red (Stratagene). The mutagenized plasmid library was then transformed into a *ΔdsbB* strain of *E. coli*, and warfarin-resistant mutants were selected by plating transformants on motility plates with 10 mM warfarin (Sigma). FlgI, a flagellar protein required for motility, requires a disulfide bond for stability. Plasmids from bacteria that showed motility in the presence of warfarin were isolated and sequenced, and the VKOR gene was subcloned and then retested for warfarin resistance. β-Galactosidase assays were performed as described previously (16) with the cells grown at 30°C in M63 minimal medium (27) with maltose added (0.2%). The 6xHis tagged versions of MtbVKOR (pRD33 and the warfarin-resistant mutants to check expression levels) were constructed by cloning the ORFs into the plasmid pET14b (Invitrogen), which places a 6xHis tag on the amino terminus of the protein. These constructs were then subcloned into the expression plasmid pTrc99a (13).

A clean deletion of the VKOR homolog of *M. smegmatis* was made using previously described methods (26). Alkylations were performed as described previously using 4-acetimido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) (15). The minimal inhibitory concentration of warfarin and phenindione (Sigma) against mycobacteria was determined using an alamar blue assay (27). *M. tuberculosis H37Rv* was grown in 7H10 (Difco) with oleic acid-dextrose-catalase (OADC) enrichment (Becton Dickinson), *M. smegmatis mc²155* was grown in 7H10 with AD enrichment or NZ rich medium. All experiments with *E. coli* were performed in M63 minimal medium (27) with either glucose or maltose added as a carbon source. Antibiotics were added when appropriate (ampicillin, 200 μg/mL; hygromycin, 50 μg/mL; kanamycin 50 μg/mL). IPTG was used to induce expression of *Mtb*VKOR, and anhydrotetracyline (100 ng/mL) was used to induce expression of complementing plasmids in *M. smegmatis*.

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