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From Protein Folding to Blood Coagulation: Menaquinone as a Metabolic Link between Bacteria and Mammals

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Abstract

Menaquinones have long played a central role in bacterial metabolism due to their solubility in membranes and their ability to mediate electron transfer reactions between a large variety of enzymes. In addition to acting as important nodes in fermentation and respiration, menaquinones are critical to the formation of disulphide bonds in the periplasm. Their utility as molecular wires has also led to their incorporation into redox reactions in higher-order organisms, where they participate in numerous physiological processes, including blood coagulation. Through studying the menaquinone-dependent pathways in organisms across the phylogenetic spectrum, researchers have begun to uncover intriguing metabolic links and have identified novel compounds for modulating these vital pathways.

Keywords: menaquinone, vitamin K, vitamin K epoxide reductase, DsbB, disulphide bond formation

1. Rise of the quinones

As life began to emerge in the seas of primordial Earth, one of the first orders of business was the construction of a plasma membrane to protect and concentrate biomolecules in the cytoplasm of what would become the first cell. This served to increase the efficiency of the biochemical reactions necessary for growth and propagation. The constant influx of salt water across the plasma membrane, however, would have led to extremely high internal osmotic pressure in what were essentially bags of chemicals, necessitating the invention of efflux pumps to drive

ions back out into the surrounding milieu. These early efflux pumps most likely exported protons at the expense of ATP, which the cells were forced to make through substrate-level phosphorylation, an inefficient process. However, leakage of protons back into the cell could drive the ATPase in the reverse direction, thus linking the extrusion of protons to the creation of cellular energy.

Aside from the issue of osmotic pressure, the plasma membrane also created a conundrum in that the organic molecules necessary to drive metabolism were prevented entry. Thus, cells developed membrane-associated transporters capable of importing such nutrients. Catabolism of these organic molecules provided the cells with necessary building blocks and resulted in the liberation of electrons, which could then be collected on redox-active molecules like NAD⁺ or FAD⁺, reducing them to NADH and FADH₂.

To regenerate the pools of electron carriers, these ancient microbes resorted to fermentation, the process by which electrons are dropped onto self-derived organic molecules. This freed up NAD⁺ and FAD⁺ to participate in more rounds of catabolism of carbon sources, thus driving metabolism. However, fermentation is an inefficient process and thus limited the growth rate and abundance of these microbes. Only when the enzymes involved in fermentation (i.e., nitrate reductase, fumarate reductase) evolved to associate with the plasma membrane did these ancient microbes begin to tap into the power they needed to flourish. Now, the process of passing electrons onto terminal acceptors could be coupled to the extrusion of protons into the extracellular space. With greater numbers of protons pumped out of the cell, their leakage back across the membrane could greatly increase the amount of ATP generated [1]. In essence, the cells could target these molecular machines to the membrane to produce the chemical energy necessary to fuel metabolism. The effect could be further amplified by linking these redox reactions together, but that required cofactors capable of accepting and donating electrons to act as molecular wires. The earliest such cofactors were likely iron-sulfur clusters and flavins, but these were not readily inserted into the highly lipophilic environment of the plasma membrane. Thus arose the quinones, which are fat-soluble redox molecules capable of associating with membrane-embedded enzymes. By linking together several modular redox complexes into an electron transport chain capable of extruding protons, quinones potentiated a huge leap forward in bioenergetics and greatly increased the capacity for complexity in biological systems.

To maximize the utility of having quinones available in their membranes, these ancient microbes needed to find a way to tap into more plentiful electron donors and acceptors. One potential source of electrons present in abundance on primordial Earth was water. However, the vast amounts of energy necessary to pull electrons from water presented a formidable obstacle to its utilization. Only when high-energy solar radiation came to be employed in the process known as photosynthesis were "cyanobacteria" successful in linking the fixation of CO₂ to hydrolysis [2]. Quinones were key to the evolution of photosystem I, yet another example of their power and adaptability in biological systems.

While the increased access to electrons represented a potential windfall to energy-starved cells, the development of photosynthesis was nonetheless catastrophic to life on Earth. Concomitant with the liberation of electrons from water by photosynthesis was the production of a new and

toxic gas–oxygen. Among the many harmful effects of an oxygenated atmosphere were the generation of reactive oxygen species, which poisoned the metabolic pathways of early microbial cells by destroying important cofactors and enzymes [3]. In this noxious environment lay opportunity, however, as oxygen could serve as an extremely effective electron acceptor if cells could evolve mechanisms for reducing it as part of their electron transport chains. Once again quinones mediated a metabolic breakthrough, linking the reduction of oxygen to membrane components via cytochrome oxidases. This process of aerobic respiration led to an estimated 16-fold increase in the capacity to generate ATP [4] and may have opened the door to the development of complex eukaryotic life [5].

The metabolic flexibility of quinones means that their use is not limited simply to the respiratory chains of microbes or the photosynthetic centers of plants. Many higher-order organisms not only incorporated quinones into their respiratory chains, but have utilized these highly effective molecular wires in many different redox-dependent reactions. While the quinones are ancient, they remain very important to life on this planet.

2. Biosynthesis of menaquinone and phyloquinone

The major quinones found in nature are ubiquinone (UQ), menaquinone (MK), and phyloquinone (K1) (**Figure 1**). They differ not only in structure but also in their redox potentials, so the incorporation of one or the other as a cofactor allows for fine-tuning of electron transfer reactions. The distribution in nature of genes involved in menaquinone biosynthesis suggests that it was most likely the original quinone; this is supported by the observation that menaquinone is readily oxidized in aerobic environments, suggesting that it existed long before the appearance of oxygen [6]. There are many different species of menaquinone, though they differ only in the length of their isoprenyl side chains. These differences are reflected in the nomenclature of menaquinones, wherein the number of isoprene units is indicated (i.e., MK-4). Phyloquinone, usually considered distinct from the menaquinones, is merely MK-4 with a more heavily saturated lipophilic tail.

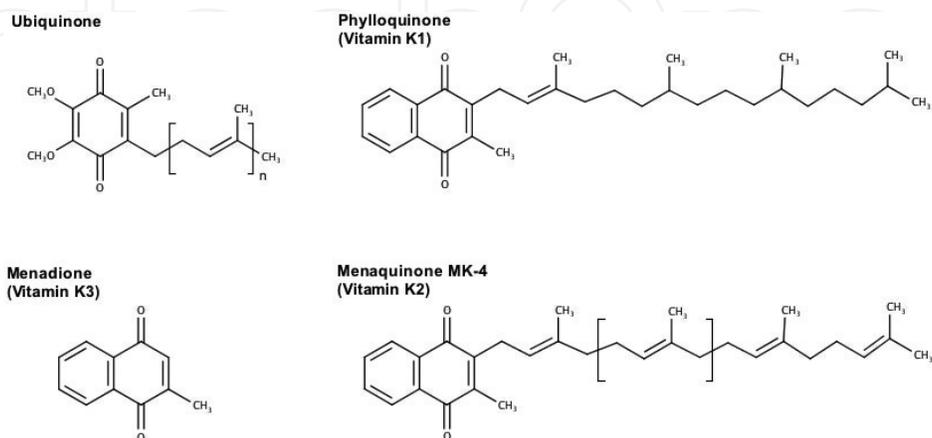


Figure 1. Structures of common quinones.

The canonical pathway for menaquinone biosynthesis in bacteria is well-established and has been reviewed in great detail elsewhere [7]. Of particular interest to this review, however, is the prenylation reaction mediated by MenA in which the lipophilic tail is attached to 1,4-dihydroxy-2-naphthoic acid (**Figure 2**). In essence, this is the critical step that links the redox-active quinone to the membrane. The lipophilic substrate of MenA is made up of repeating isoprenal subunits, the exact number of which is determined by the octaprenyl pyrophosphate (OPP) synthase encoded by the particular microbe. The ultimate chain length of these products is determined by a molecular ruler mechanism wherein bulky amino acid residues at the bottom of each of OPP's active sites block chain elongation [8], and it is this step that controls the identity of the primary MK produced by an organism. There is some evidence to suggest, however, that growth temperature also plays a role in the length and degree of saturation of the aliphatic side chain [9]. Phylloquinone biosynthesis in cyanobacteria is predicted to proceed via a pathway very similar to that of MK biosynthesis. However, the cyanobacterial MenA incorporates a mostly saturated phytyl tail at position C-3 rather than the partially unsaturated isoprenyl side chain associated with MK [10]. Recently, an alternative pathway

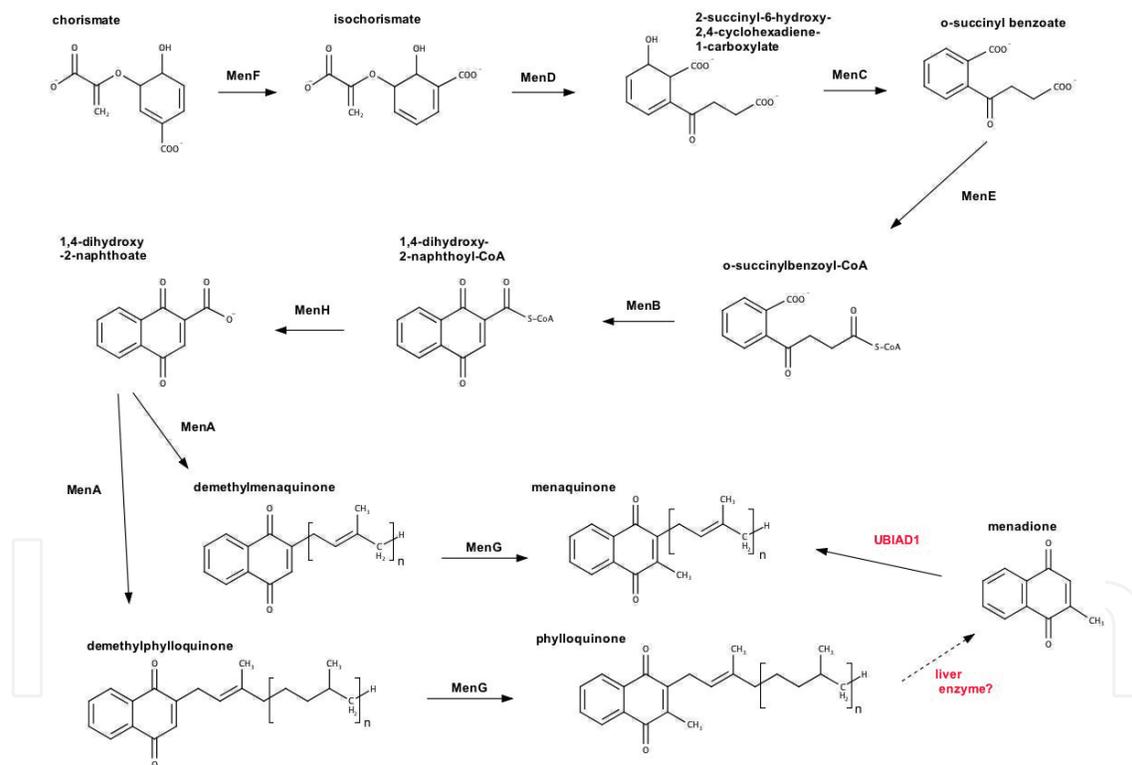


Figure 2. Biosynthesis of menaquinone and phylloquinone. The highly conserved sequence of reactions required for conversion of chorismate to menaquinone is shown. The pathway for phylloquinone biosynthesis is thought to proceed via the same steps, with the exception of the prenylation step mediated by MenA. The production of demethylphylloquinone versus demethylmenaquinone is determined by which substrate is provided to MenA by octaprenyl pyrophosphate synthase (OPP). A phytyl chain results in the production of demethylphylloquinone, whereas a more highly unsaturated isoprenyl chain results in demethylmenaquinone. In mice, metabolism of phylloquinone in the liver has been shown to release menadione, which in turn can be prenylated by the MenA homolog UBIAD1 to produce menaquinone.

for menaquinone biosynthesis has been described in several Achaea and Gram-negative bacteria, including *Helicobacter pylori*, Chlamydia species, and spirochetes [11]. While both pathways start with chorismate, the formation of the quinone proceeds via completely different reactions. The diversity of pathways for biosynthesis of MKs serves to underscore the importance of its role in metabolism.

Phylloquinone biosynthesis in plants is not as well understood as in cyanobacteria, though the pathway likely mirrors that of menaquinone. One striking difference is that the first four reactions proceeding from chorismate, mediated by the products of the *menF*, *D*, *C*, and *H* genes in bacteria, are accomplished in plants by one fusion protein known as PHYLLLO [12]. Later steps are compartmentalized between the chloroplast and peroxisome, adding an additional level of complexity to production in plants [13].

Unlike the organisms mentioned above, higher-order organisms are incapable of *de novo* synthesis of MKs. It is therefore imperative that MKs be supplied through diet, thus classifying MKs as a “vitamin” for mammals. The identification of MKs as essential vitamins arose from the Nobel Prize-winning work of Edward Adelbert Doisy and Henrik Dam in 1943. Their recognition that a fat-soluble compound played a key role in blood coagulation led them to name it “vitamin K” (“K” for the German word “Koagulationsvitamin”). Purification and characterization revealed two forms of vitamin K—phylloquinone thus became known as vitamin K1 and menaquinone as vitamin K2 with regards to their requirement in mammals.

Though humans cannot synthesize vitamin K *de novo*, several homologs of menaquinone biosynthetic enzymes can be found in the human genome, including one for the prenylating enzyme MenA (UBIAD1). This enzyme was expressed in an insect cell line and was shown to be capable of converting menadione (K3) and K1 into MK-4 [14]. Such a result suggested that while humans cannot make their own MKs, they may be capable of converting biosynthetic intermediates into the final product. Indeed, recent results show that the phytyl tail of K1 is cleaved in the intestine of mice to make K3 and then prenylated by UBIAD1 in the cerebrum [15, 16]. Furthermore, human subjects fed K1 exhibited increased levels of K3, indicating that dietary K1 may play a major role in overall MK levels [16], and gnotobiotic rats fed K1 saw increased levels of MK4 in tissues, indicating that the conversion is not dependent on gut bacteria [17]. UBIAD1 has also been demonstrated to be essential for the embryonic development of mice [18], so vitamin K's route through the body is not completely understood and the complexities begin with the vitamin's source.

3. Sources of vitamin K

While the reactions requiring vitamin K in human metabolism are becoming clearer, the source of the vitamin K is still not completely understood. The presence of large numbers of bacteria in the human colon capable of synthesizing K2 would perhaps suggest that absorption of this bacterial byproduct might fulfill the human requirement. In fact, MK-6 is made by *Eubacterium lentum*, MK-7 by *Veillonella*, MK-8 by *Enterobacteria*, and MK-10 and MK-11 are made by *Bacteroides* [19, 20]. The bacterial contribution to vitamin K pools in humans is supported by

studies done with gnotobiotic rats fed vitamin K-free diets. The rapidly developing hemorrhagic conditions in these rats could be reversed by supplying bacteria from conventionally raised rats, suggesting that absorption from the bowel provided sufficient quantities of K2 [21]. Concordant with this is the observation that taking broad-spectrum antibiotics can reduce vitamin K production by more than 70% [22]. However, K2 is embedded within the bacterial inner membrane, and as such would appear to be inaccessible to passive absorption. MKs have been shown to be secreted by some organisms [23], and it is also possible that water-soluble precursors of MK biosynthesis might be more readily available [7]. However, this scenario is further complicated by the fact that there is very little evidence that the large intestine is capable of absorption of MKs. Uptake has been shown to be poor in rats [24] and infants [25]. Even the finding that antibiotic treatment lowered vitamin K production does not conclusively identify bacteria as a major source of human vitamin K2 pools, as some antibiotics have been shown to inhibit the human enzymes necessary for recycling vitamin K2 [26]. The role of the microbiome in the production of K2 is therefore questionable and would suggest that perhaps vitamin K stores in humans might be the result of dietary intake.

Low concentrations of vitamin K2 can be found in dairy, meat, and fermented foods like natto [27], but makes up only 10% of total dietary vitamin K intake. While K1, found in a variety of green leafy plants and vegetable oils, is present in much higher amounts, it is not readily absorbed in the intestines as it is strongly bound to vegetable fiber [28]. Vitamin K is not transported by specific plasma carrier proteins like other fat-soluble vitamins, but is instead shuttled by lipoproteins. The small fraction of K1 that is absorbed is almost exclusively incorporated into the triacylglycerol-rich lipoprotein (TGRLP) fraction, while dietary K2 is associated with low-density lipoprotein (LDL) fraction [29]. These divergent pathways would deliver large amounts of K1 to the liver, but efficient delivery to extrahepatic tissues would only occur for K2. Measurements of the concentrations of vitamins K in various tissues mostly back this up, showing that K1 levels are low in the brain, kidneys, and lungs but high in the liver, heart, and pancreas; K2 (in the form of MK-4) was found to be in high concentration in the brain, kidneys, and pancreas but in low concentration in the liver, heart, and lungs. As for longer chain K2s, MK6-11 were found in the liver and trace amounts of MK6-9 were found in the heart and pancreas [30]. MK10 and MK11 may be major contributors to the hepatic pool of K2 [26], and the presence of these long-chain MKs again raise the possibility that the commensal population of colonic bacteria may somehow contribute to overall vitamin K levels in the host, as analysis of tissue samples has only shown the ability to synthesize MK-4 from K1. However, the presence of potential homologs for other prenyl diphosphate synthases in the genome further suggests that humans may be capable of producing longer chain MKs as well. Overall the data clearly indicate that dietary K1 is a major contributor to vitamin K levels in the body, but a full accounting of its sources has yet to emerge.

4. Uses of vitamin K

While the side chains of K1 and the various MKs differ, the redox-active portion of the molecules (the naphthoquinone) remains unchanged. The reactivity of these various species

should therefore be very similar, a fact underlined by the nearly identical mid-point redox potentials as determined by voltammetry [31, 32] (**Figure 3**). The degree of lipophilicity in the tails most likely dictates mobility of the quinones in the membrane, with the partially saturated isoprenyl tail of MK allowing for greater freedom of movement compared to the mostly unsaturated chain of K1. Additionally, longer chain MKs are likely stiffer and more viscous in the membrane due to the greater surface areas available for van der Waals interactions. For these reasons, the preferential incorporation of one MK over another into a redox-active enzyme is most likely due to availability within the membrane as well as the ability of the enzyme to accommodate different length side chains. In microsomal fractions, MK2 and MK3 were shown to have much higher activities than K1 [33], while a partially purified enzymatic system showed similar activities for MK2-6 compared to K1. MKs with seven or more isoprenoid units were not as active [34].



Figure 3. Resonance structures of naphthoquinone species. Two electrons (e^-) can be accepted or donated in step-wise transfers from partner proteins.

Vitamin K2 has been found to play a role in protection against oxidative stress and inflammation in mammals [35], and improved locomotion defects in mutant fruit flies [36], suggesting that it might benefit human patients suffering mitochondrial pathologies. Mounting evidence suggests that MK-4 is an important component of sphingolipid biosynthesis and can inhibit the proliferation of several cancer cell lines [37]. The exact role of vitamin K2 in these processes is unknown however—its most thoroughly understood use is in protein modification.

Numerous proteins in vertebrates are modified post-translationally as a means of regulating and enhancing their activity. One such modification is the carboxylation of glutamate residues within Gla domains, which is mediated by the enzyme gamma-glutamyl carboxylase (GGCX). This modification allows for the high-affinity binding of calcium ions, which in turn mediates a conformational change necessary for proper folding of the protein. Gla-containing proteins play important roles in the venom of snakes and the toxins of cone snails [38], and they have numerous functions in humans including bone development, calcification, and sphingolipid metabolism [35, 39]. The cell-signaling activities of the vitamin K-dependent proteins Gas6 and protein S may also be crucial to cognitive processes [35]. Among the Gla-containing proteins, however, those involved in blood coagulation have received the most attention. Carboxylation of several of these factors activates them and thereby sets off a cascade leading to clotting. The

GGCX glycosylation reaction is coupled to the oxidation of vitamin K hydroquinone to vitamin K 2,3-epoxide, and it is this step that shows sensitivity to anticoagulants like warfarin. When this vitamin K cycle is disrupted or insufficient quantities of vitamin K are present in the diet, excessive bleeding can and does occur, as was the case in the initial discovery of vitamin K's role in nutrition.

While the flexibility of K2s is crucial to all of these redox-driven processes, short circuits occur wherein reduced menaquinones donate their electrons to “inappropriate” acceptors like oxygen. Such reactions result in the production of reactive oxygen species and can lead to massive damage to proteins and DNA [40, 41], underlining the importance of properly regulating the expression and distribution of MKs.

5. VKORs

Clearly, MKs play a critical role in mediating the activity of numerous proteins in mammals, yet the levels of this important cofactor in tissues is relatively low. After passing electrons onto the appropriate acceptors, MK is oxidized to its inactive, oxidized form. In bacteria, MK is quickly reduced again by the flow of electrons from the electron transport chain or to a lesser extent by the delivery of electrons from the disulfide bond pathway. To recharge and replenish their redox-active pool of MKs, mammals have evolved enzymes capable of reducing of vitamin K 2,3-epoxide (KO) to vitamin K and vitamin K hydroquinone (KH₂). These two steps occur via a warfarin-sensitive pathway as well as a warfarin-insensitive pathway, suggesting that two or more enzymes may be required to efficiently complete the reaction. While the enzymatic activity of vitamin K epoxide reductase (VKOR) had first been assayed in 1974 and VKOR had long been known to be the target of the anticoagulant warfarin, identification of the enzyme responsible for the regeneration of vitamin K did not come until 2004 [42, 43]. While this discovery set the stage for in-depth analysis of the kinetics of blood coagulation, one of the most surprising early findings was that VKOR homologs could be found not only in a large family of vertebrates, but also in insects, plants, bacteria, and archaea [44]. What role could VKOR possibly play in organisms that do not contain blood? The discovery of vitamin K-dependent proteins in sea squirts [45] suggests that this modification arose much earlier than the blood coagulation cascade and that vertebrates simply repurposed Gla-modified proteins.

To fully understand the function of a membrane-bound protein, it is important to determine the topology of the enzyme within the membrane. This allows for greater insights into the catalytic site as well as to possible interactions with partner proteins. The topology of VKOR in the endoplasmic reticulum (ER) membrane, however, has been fraught with controversy. Initial reports suggested an enzyme with 4 transmembrane domains (TM) [44], though there is also mounting evidence that VKOR may adopt a 3-TM structure (**Figure 4**). Of particular importance to this debate is the potential positioning of critical cysteine residues. VKOR contains a total of four conserved cysteines, two of which are present in a C-X-X-C motif characteristic of redox-active thioredoxins. These two cysteines (C132 and C135) have been

shown to be essential for the reduction of vitamin K₀ to vitamin K and vitamin K_{H2} using purified VKOR [46]. The second set of conserved cysteines (C43 and C51) lie within a loop region between TMs. The 3-TM model for VKOR places the N-terminus of the protein and the active site cysteines on the ER side of the membrane, with the loop cysteines and C-terminus in the cytoplasm. On the other hand, the 4-TM model places both termini in the cytoplasm, while the active site and loop cysteines both face the ER lumen. This 4-TM topology would immediately suggest an enzymatic mechanism wherein the loop cysteines receive electrons from interactions with redox partners in the lumen, and then pass them on to the active site C-X-X-C. Because the 3-TM model predicts that the two sets of cysteines are on opposite sides of the ER membrane, it is difficult to imagine how they might interact, and it suggests a distinctly different mechanism for reduction. The fact that several mutations encoding

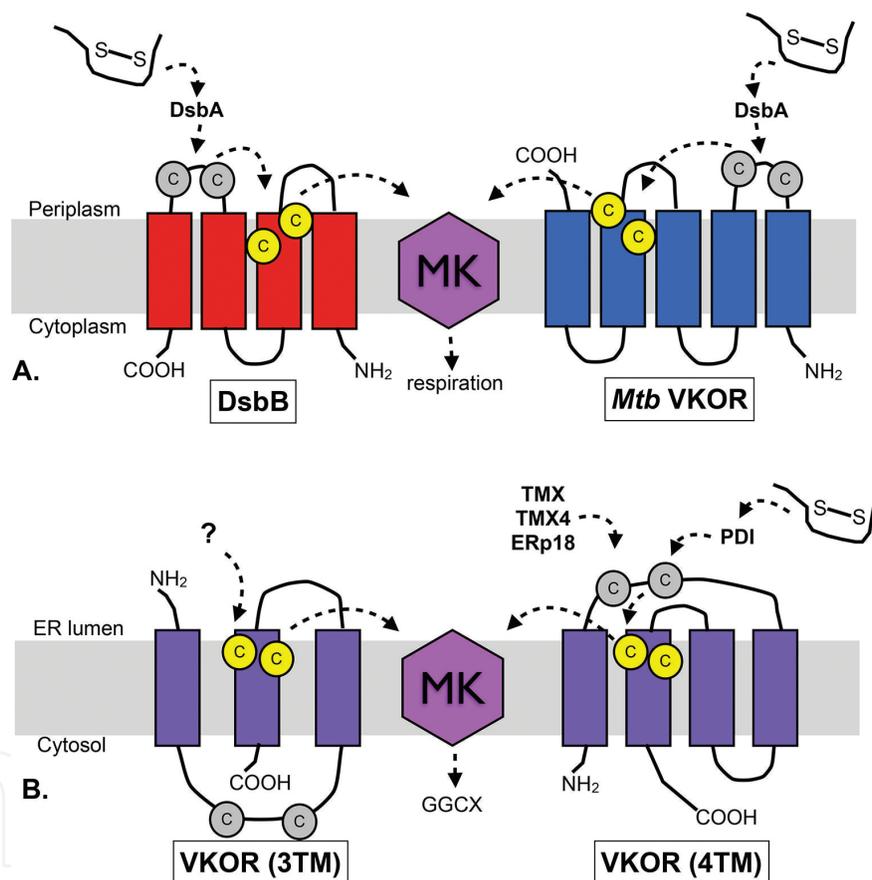


Figure 4. Representative topologies of membrane redox proteins. (A) Topologies of DsbB and *Mtb* VKOR in the bacterial plasma membrane. Critical cysteine residues required for transfer of electrons to menaquinone are represented by yellow circles, while loop cysteines required for accepting electrons from the periplasmic protein DsbA are represented as gray circles. Note that the order of these residues is reversed in the two enzymes with respect to the amino acid sequences. (B) Proposed topologies of the mammalian VKOR in the membrane of the endoplasmic reticulum. In the 3-TM structure, the loop cysteines and those required for transfer of electrons to menaquinone (vitamin K₂) are located in different cellular compartments, while in the 4-TM model, all four are found near the interface of the membrane with the ER lumen. While several luminal proteins have been found to be capable of transferring electrons to the catalytic site of VKOR in the 4-TM model, it is not yet clear which proteins would perform this role in the 3-TM model. MK = menaquinone. GGCSX = γ -glutamate carboxylase.

resistance to warfarin map to the loop region containing Cys43 and Cys51 further suggests that these loop cysteines may play a key role in VKOR activity.

The loop cysteines were not required for the enzymatic activity of VKOR with the purified enzyme, though C51 was found to be important along with C132 and C135 for activity in cell extracts [47]. Expression of Cys 43 and Cys 51 mutants in reporter cells in which endogenous VKOR and VKORL1 were knocked out show that these mutant alleles retain ~90% activity [48]. However, challenges to such results have emerged. Results with purified VKOR showing the non-essentiality of the loop cysteines were obtained using dithiothreitol (DTT), a non-physiological reductant. Because DTT is membrane permeable, it is possible that Cys43 and Cys51 are important for shuttling electrons to the active site cysteines under physiological conditions, but DTT bypasses this necessity. To this end, experiments utilizing the membrane impermeable system of NADPH, thioredoxin, and thioredoxin to drive reduction gave results showing that the loop cysteines were actually required for VKOR activity [49].

The membrane topology of VKOR has been directly tested through a number of biochemical approaches. The Stafford lab fused green fluorescent protein (gfp) to either the N- or C-terminus and tested protease susceptibility. These studies showed that only the C-terminus was proteolytically cleaved, which suggested that while the N-terminus faced the ER lumen, the C-terminus must face the cytoplasm. This architecture placed the two confirmed active site cysteines (Cys-132 and Cys-135) on the luminal side of the membrane, while the two conserved loop cysteines (Cys-43 and Cys-51) were on the opposite side [50]. An important caveat to this work is that the protease sensitivity assay was performed after permeabilization with digitonin, a process not thought to affect the topology of membrane proteins. However, a very similar approach using live (i.e., non-permeabilized) cells and a redox-active gfp clearly demonstrated that both the N- and C-termini are located within the cytoplasm [51]. Further experiments showed that the loop region containing Cys43 and Cys51 could be glycosylated by machinery within the ER lumen and that the loop cysteines could form mixed disulfides with luminal proteins, thereby placing this loop region firmly in the ER in accordance with a 4-TM topology.

The overall architecture of VKOR becomes most germane when attempting to identify its redox partners. If VKOR had three membrane-spanning domains, the loop cysteines would not be accessible to soluble redox partners, yet the active site cysteines would need to be directly accessible to a partner. To achieve this, the partner must also be membrane bound, as has been suggested for GGCX [52], or must have a hydrophobic domain capable of inserting into the membrane during electron transfer. A potential membrane complex of VKOR and GGCX would explain how a transfer reaction between these two enzymes could be facilitated during blood coagulation, but it does not offer any insights into how electrons might be supplied to VKOR in the first place. Despite the fact that molecular dynamic simulations indicate that the 3-TM model of human VKOR has a structural advantage in terms of protein stability over a VKOR with 4 TM [53], questions regarding this model still remain.

In a 4 TM structure of VKOR, the active site cysteines face the ER lumen. It has therefore been hypothesized that VKOR's redox partner must be a luminal protein that most likely bears at least some homology to thioredoxin-like proteins, which encode cysteines in a C-X-X-C motif. The proposed reaction scheme posits that Cys-43 of VKOR forms a mixed disulfide with its

redox partner, which subsequently attacks Cys-51 to form an intramolecular disulfide bond in VKOR and releases the redox partner. By mutating the resolving Cys-51, the mixed disulfide can be trapped, thus allowing identification of the redox partner. Such an approach identified several intriguing candidates including soluble proteins and the membrane-bound TMX, TMX4, and ERp18 as forming mixed disulfides with VKOR, although it is not clear what the downstream effects of such interactions might be [54].

Early studies with microsomal fractions indicated that protein disulfide isomerase (PDI) might be an important source of electrons for VKOR [55], which is consistent with PDI's localization to the ER lumen. PDI can act as an electron acceptor by interacting with proteins containing multiple cysteine residues. By accepting electrons from such proteins, disulfide bonds form between these cysteines, which can serve to stabilize or activate these substrates. Following this reaction, the reduced form of PDI is free to donate its electrons to other partner proteins. Studies confirmed that PDI could stimulate VKOR's reductive activity and went on to suggest that VKOR and PDI may even form a complex in the ER membrane [56]. In other words, the reduction of vitamin KO may be driven by the formation of disulfide bonds in the ER lumen. Such a mechanism appears to contribute to the overall redox homeostasis within the ER [57] and has also been suggested to operate in plants as well [58, 59].

While VKORs can be found in numerous classes of organisms, paralogs of VKOR are also quite prevalent. Known as "VKORLs" ("VKOR-like"), the exact role of these enzymes is still unclear. The human VKOR and VKORL1 share 42% identity and 60% similarity. Like VKOR itself, VKORL1 can reduce KO to vitamin K, which may explain why patients treated with anticoagulants do not exhibit significant side effects that would be expected from the inability to turn over vitamin K, like arterial calcifications [60]. Indeed, rat VKORL1 was shown to be up to 50-fold more resistant to warfarin as compared to VKORC1 in one study [61], although such a finding has been contested [62]. However, the rate at which VKORL1 reduces KO may be significantly slower than VKOR [60], and mice missing VKOR (but expressing VKORL1) bled to death shortly after birth [63], suggesting a different function for VKORL1. It has also been suggested that VKORL1 may play a role in the vitamin K cycle by reducing vitamin K to KH_2 [64], although such a suggestion may be premature, as comparisons of VKOR and VKORL1 activity can be problematic [62]. To gain further insight into the differential functions of VKORL1 versus VKOR, these authors looked at expression levels of the two genes in different tissues. They found clear evidence that VKOR and VKORL1 are differentially regulated in rats and mice, with VKOR showing higher expression in rat liver, lung, and kidney, VKORL1 showing higher expression in the brain, and similar expression profiles in the testis. Overall, the levels of VKORL1 were relatively constant across organs, while VKOR showed extremely high levels in the liver but much lower levels in the remaining tissues [61]. Such findings have led some to hypothesize that VKOR may have evolved to provide cofactor to the vitamin K-dependent proteins required for maintaining the high-flux environment of the circulatory system and the homeostasis of a calcified skeleton, while the ability of VKORL1 to reduce vitamin K may be ancillary to its role in antioxidant functions and disulfide bond formation [39]. The differential activity of VKORL1 compared to VKOR is supported by studies conclu-

sively showing that the loop cysteines of VKORL1 are required for activity, in potential contrast to VKOR [62].

The quest to define a role for bacterial VKORs began with an observation that arose from studies of a well-defined, quinone-dependent pathway in bacteria responsible for catalyzing the formation of disulfide bonds in some periplasmic proteins. As covalent bonds between cysteine residues, disulfide bonds can stabilize otherwise energetically unfavorable conformations of certain proteins, thus promoting functionality, similar to binding of calcium ions in the Gla-dependent proteins of eukaryotes. While most disulfide bonds in the bacterial cytoplasm exist transiently as part of an enzyme catalytic cycle, disulfide bonds in the periplasm are much more stable. This is due to the activity of *disulfide-bonding protein A* (DsbA), which utilizes two cysteine residues to accept electrons from substrate proteins [65, 66]. Such an electron-transfer reaction leads to the oxidation of the substrate protein (disulfide-bonded), while leaving the cysteine residues of DsbA in the reduced form (-SH). To regenerate active DsbA, electrons need to be transferred to another protein, DsbB [67, 68]. DsbB is localized to the plasma membrane via four transmembrane-spanning domains and utilizes two pairs of active-site cysteines to accept electrons from DsbA and pass them onto ubiquinone or menaquinone [69–71]. These quinone carriers deposit the electrons onto a final electron acceptor like oxygen or nitrate through the process of respiration, thus completing the disulfide bond generation cycle. While homologs of DsbA and DsbB have been identified in many bacteria, some do not encode these enzymes but contain disulfide-bonded proteins [72]. To identify the enzymes responsible for generating disulfide bonds in these organisms lacking DsbB, bioinformatics analysis was performed. The results of this analysis demonstrated that some bacteria encoded a DsbA-like protein fused to a homolog of eukaryotic VKOR [44, 72]. Because VKOR and DsbB both utilize catalytic cysteine residues in redox-dependent transfer reactions, have multiple membrane-spanning domains, and are known to reduce quinones, it suggested that the bacterial VKOR might function in a manner analogous to DsbB. Concordant with this, VKOR homologs were found only in bacteria- and archaea-lacking DsbB [72]. In fact, the VKOR encoded by *Mycobacterium tuberculosis* (*Mtb*) can restore disulfide bond activity to an *E. coli* strain missing *dsbB* [72], and a VKOR homologue has been shown to catalyze disulfide bond formation in cyanobacteria [73]. Therefore, despite the fact that DsbB and VKOR show no significant homology at the amino acid level, they perform analogous reactions. Perhaps even more striking, the VKOR-dependent disulfide bond activity in this *E. coli* strain can be inhibited by high concentrations of the anti-coagulant warfarin, providing another link between the bacterial and eukaryotic enzymes [74]. Such results are of course strikingly analogous to the potential role of VKORL1 in the formation of disulfide bonds in the ER lumen. The crystal structure of the fused DsbA-VKOR from *Synechococcus* suggests that the reactions performed by bacterial and eukaryotic VKORs may proceed via similar mechanisms and may provide insights into the inhibition by warfarin and other anti-coagulants [75, 76]. The *Synechococcus* VKOR (synVKOR) is, however, slightly different than the mammalian enzyme in that it utilizes ubiquinone as a cofactor rather than MK. The epoxide form of ubiquinone has not been found in *Synechococcus*, as with other bacteria, and synVKOR cannot reduce vitamin KO to the hydroquinone form [75]. While DsbB has been shown to encode four transmem-

brane domains, studies using fusion proteins show that the bacterial VKOR spans the plasma membrane five times, with the fifth-TM segment usually allowing for the fusion to the periplasmic DsbA. Despite this difference in topology, all four active site cysteines face the periplasm [77]. Like DsbB, the cysteines in bacterial VKOR are essential for the formation of a mixed disulfide with DsbA, but the pairs of cysteines are reversed in regards to their order within the amino acid sequence [77]. Phylogenetic analysis of VKOR and DsbB suggests that these two enzymes are in fact related evolutionarily, having diverged from a single lipidic quinone–disulfide oxidoreductase superfamily [78]. The diversion would appear to have led to differential function as well, for while eukaryotic VKORs reduce the epoxide form of K₂ to the quinone and the hydroquinone form in order to provide substrate for the gamma-glutamate carboxylase reaction, there is no evidence for gamma-glutamate carboxylases in bacteria.

Despite the fact that there is little overall homology in the amino acid sequences of human VKOR and *Mtb* VKOR, the enzymes appear to catalyze very similar reactions, at least from a redox perspective. It is therefore interesting to test whether they are interchangeable. Expression of *Mtb* VKOR in mammalian cell lines confirmed that the bacterial VKOR is capable of reducing both vitamin K and vitamin KO to KH₂ [79]. While the loop cysteines of *Mtb* VKOR (Cys-57 and Cys-65) are essential for disulfide bond activity when expressed in *E. coli*, they may not be required for reduction of vitamin K epoxide in mammalian cells [79].

Studies of the functionality of mammalian VKORs in bacteria have been more problematic. Expression of hVKOR in *E. coli* results in the formation of inclusion bodies, thus preventing any attempts to assess function *in vivo*. Additionally, reconstitution of recombinant hVKOR activity from insoluble fractions was strictly dependent on the nature of the membrane composition [80]. Attempts to restore disulfide bond formation to a $\Delta dsbB$ strain of *E. coli* by expressing either the rat or human VKOR have so far been unsuccessful at least in part due to the lack of stable expression [81]. However, selection for a rat VKOR functional in substituting for DsbB in this system yielded a collection of mutants in the rat *vkor* gene that encoded amino acid changes in the protein. Notably, many of these gain of function mutations resulted in changes in the charge of what is predicted to be the first periplasmic loop of rat VKOR when it is expressed in *E. coli*. The charge distribution of amino acids is known to play an important role in establishing the proper topology of membrane proteins [82, 83]. Further, even higher levels of protein are detected when the mutant proteins are expressed in *E. coli* strains carrying mutations that alter the YidC insertase, a protein necessary for membrane localization of some proteins or mutations eliminating the cytoplasmic protease HslV [81]. Mutant strains harboring both *yidC* and *hslV* mutations showed significantly more VKOR activity and protein levels. These results suggest that while the bacterial VKOR can be properly inserted into the ER membrane for the reduction of vitamin KO, the mammalian VKOR may not be able to be properly inserted in the bacterial plasma membrane without initial changes in the protein itself. Nevertheless, the mutant versions of rat VKOR that are expressed in *E. coli* are sensitive to anticoagulants, suggesting that the functional expression even of the mutant enzyme may provide a powerful tool for its study.

6. Vitamin K2 as a target for inhibition

MKs are clearly critical components of many aspects of the growth and proliferation of bacterial and human cells, but most of the enzymes necessary for their biosynthesis are only bacterially encoded and are missing from humans. MK biosynthesis would appear to be an ideal target for the development of small molecule inhibitors as potent antibiotics. Among pathogenic bacteria, *Mtb* poses one of the most significant threats, as it accounts for nearly two million deaths annually. While combinatorial antibiotic therapies have been developed against *Mtb*, serious complications have arisen that compromise the efficacy of these treatments. In addition to excessive length of treatment, the side effects of these drugs can be debilitating, and antibiotic resistance has arisen at a startling rate. In addition, *Mtb* can remain for long periods of time in a dormant state in which traditional antibiotics are not effective. However, even in this quiescent state, *Mtb* requires an active electron transport chain to maintain adequate levels of ATP, and MKs therefore play a key role [84]. To this end, researchers have developed screens specifically targeting the MK biosynthetic pathway of *Mtb*. Early results show that compounds-targeting MenE show some promise [85], and the prenylating enzyme MenA is also being developed as a target [86, 87]. Most strikingly, one MenA inhibitor (allylaminomethanone-A) was shown to be up to 320-times more effective in killing non-replicating *Mtb* than first line drugs currently prescribed for infection [84], and MenA inhibitors have been shown to inhibit growth of *Mtb* resistant to commonly used antitubercular drugs [86]. Caution must be exercised in advancing such therapies, however, as the full scope of vitamin K metabolism in the body has not been elucidated. If gut bacteria do contribute significantly to vitamin K stores in the body, then inhibitors targeting MK biosynthesis may have significant effects on blood coagulation and bone calcification, for example. MenA inhibitors are particularly noteworthy, since off-target effects on the human homolog UBIAD1 could potentially disrupt a number of cellular processes that are only beginning to be understood.

As an inhibitor of vitamin K-dependent reactions, warfarin has long been used as an anticoagulant that at least in part targets human VKOR. While the mycobacterial VKOR has been shown to be sensitive to warfarin, the amount necessary to inhibit the bacterial enzyme is orders of magnitudes higher than the amount needed to prevent blood coagulation [74]. This would suggest that while the human and bacterial VKORs can perform similar functions and do so by similar mechanisms, the divergence in the amino acid sequence of the two is significant enough that treatment of mycobacterial infection with anticoagulants would not be an effective therapeutic strategy. However, ferulenol, an anticoagulant, shown to be approximately 20-fold more potent against human VKOR than warfarin, showed similar potency against the VKOR from *Synechococcus* [75]. It is therefore possible that drug discovery efforts to identify novel anticoagulants may impact the search for inhibitors of bacterial VKOR and vice versa.

Disulfide bond formation appears to be dispensable for *in vitro* aerobic growth of *E. coli* and other bacteria, although many virulence factors absolutely require disulfide bonds for proper assembly and function. Bacteria disrupted in the DSB pathway are rendered less virulent, and *E. coli* cannot grow anaerobically, suggesting that small molecule inhibitors of DsbB- or VKOR-

dependent pathways may be potent anti-virulents and may prevent anaerobic growth of some pathogens. *Mtb* is especially vulnerable to such compounds, as VKOR is essential for growth of this organism, even in aerobic environments [88]. Bacterial DsbBs and VKORs therefore make attractive targets for antibiotic therapies.

The fact that DsbB and *Mtb*VKOR perform complementary functions but lack amino acid homology allowed our laboratory to develop a screen to identify potential small molecule inhibitors that specifically target DsbB or *Mtb*VKOR [89]. β -galactosidase (LacZ) is a cytoplasmic enzyme capable of cleaving the disaccharide lactose to yield galactose and glucose. The activity of this enzyme can be readily monitored in *E. coli* by using the lactose analog Isopropyl 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal)—successful cleavage of X-gal yields an insoluble blue dye that can readily be distinguished by eye. When *lacZ* is fused to the gene encoding the membrane protein MalF, however, the enzyme is exported into the periplasm and is inactivated by the formation of inappropriate disulfide bonds. Strains expressing this fusion construct appear white on X-gal, as the substrate cannot be cleaved. However, strains lacking *dsbB* appear blue on X-gal when expressing this construct, as these cells lack the ability to catalyze the formation of the inappropriate disulfide bonds in LacZ. In such a case, LacZ is active and capable of cleaving X-gal. When the $\Delta dsbB$ strain is complemented with a construct expressing lowered-levels of *dsbB* or with *Mtb vkor*, the strains appear white again, as disulfide bond formation is restored. When libraries of small molecules are applied to *E. coli* strains differentially expressing *E. coli dsbB* (or the *dsbB* from another Gram-negative bacterium) or *Mtb vkor* along with the MalF-LacZ fusion in a high-throughput format, specific inhibitors of DsbB or VKOR can easily be identified by the appearance of a blue color. The differences in the primary structure of DsbB and VKOR would suggest that any compound that inhibits one should not inhibit the other. For this reason, each strain acts as a strong counter screen for the other. We have successfully employed this screen to identify several strong, specific inhibitors of the DsbB from *E. coli* as well as several other important pathogens, and we continue to use it to screen for potential inhibitors of the *Mtb vkor*. Further efforts to express functional mammalian VKOR and VKORL1s in the *E. coli* screening strain would not only provide a means by which to test potential side effects of compounds targeting the bacterial enzymes, but may offer a high-throughput approach to identifying new compounds capable of inhibiting VKOR-dependent processes in mammals. Additionally, because the screening system provides an easily monitored readout for VKOR activity, it might be used to study hVKOR variants shown to be resistant to anticoagulant therapies. Such studies could lead to more precisely targeted and potent blood thinners.

7. Conclusions

Since their incorporation into the electron transfer pathways of ancient microbes, menaquinones have become a cornerstone of redox-dependent reactions in almost every domain of life. Their ability to interact with a large variety of proteins, to readily accept and donate electrons, and to easily move within biological membranes have combined to make MKs flexible and efficient molecular wires. As such, organisms have evolved to integrate MKs into many

metabolic processes, thus plugging into previously untapped sources of power. While researchers have seemed to only scratch the surface of the myriad uses for MKs to this point, further investigation will yield not only fascinating insights into the biochemical pathways critical to life, but may be a crucial starting point for the development of therapies designed to protect and enhance those pathways.

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