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Intramembrane thiol oxidoreductases: evolutionary convergence and structural controversy

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Abstract

During oxidative protein folding, the disulfide-bond formation is catalyzed by thioloxydoreductases. Through dedicated relay pathways, the disulfide is generated in donor enzymes, passed to carrier enzymes, and subsequently delivered to target proteins. The eukaryotic disulfide donors are flavoenzymes, Ero1 in endoplasmic reticulum and Erv1 in mitochondria. In prokaryotes, disulfide generation is coupled to quinone reduction, catalyzed by intramembrane donor enzymes, DsbB and VKOR. To catalyze *de novo* disulfide formation, these different disulfide donors show striking structural convergences in several levels. They share a four-helix-bundle core structure at their active site, which contains a CXXC motif at a helical end. They have also evolved a flexible loop with shuttle cysteines to transfer electrons to the active site and relay the disulfide bond to the carrier enzymes. Studies of the prokaryotic VKOR, however, have stirred debate of whether the human homolog adopts the same topology with four transmembrane helices and uses the same electron-transfer mechanism. The controversies have recently been resolved by investigating the human VKOR structure and catalytic process in living cells with a mass spectrometry based approach. Structural convergence is found between human VKOR and the disulfide donors to underlie the cofactor reduction, disulfide generation, and electron transfer.

Graphical abstract

Thiol oxidoreductases are a large group of enzymes that use cysteines to catalyze redox reactions. Many of these oxidoreductases promote the disulfide-bond formation of proteins

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in the secretory pathway. Forming the correct disulfides is essential to the folding of these proteins into their native conformation and to the stabilization of their folded structure. During this oxidative folding, disulfide formation is a rate-limiting step¹, which requires the catalysis by the oxidoreductases to ensure timely folding of the target proteins, given that spontaneous oxidation into correct disulfides is an inefficient process².

Thiol oxidoreductases in the disulfide-relay pathways

Oxidative protein folding is carried out by dedicated disulfide-relay pathways in both prokaryotes and eukaryotes. These pathways usually comprise a carrier enzyme, that delivers the disulfide bond to target proteins, and a donor enzyme, that generates the disulfide bond *de novo*. Disulfide bond formation protein A (DsbA) or DsbA-like oxidoreductases are the primary disulfide-bond carrier in prokaryotes, located in the periplasmic space of gram-negative bacteria³ (Figure 1A) or at the cell wall of gram-positive bacteria⁴. DsbA is one of the most oxidizing enzymes⁵ with a broad substrate spectrum: ~300 periplasmic proteins in *Escherichia coli* are predicted to be oxidized by DsbA⁶. DsbA receives disulfide bond from DsbB^{7,8} in many bacteria phyla. In disulfide-making bacteria missing a DsbB homolog, homologs of vitamin K epoxide reductase (VKOR)⁶ are usually found, which donate the disulfide to a DsbA- or thioredoxin-like protein⁶. Both DsbB and VKOR are intramembrane enzymes. To generate disulfide bonds, these enzymes couple the cysteine oxidation with the reduction of ubiquinone or other quinones, which are electron carriers that can be subsequently oxidized in the electron-transport chains.

In eukaryotic cells, oxidative protein folding takes place in several subcellular compartments, including the endoplasmic reticulum (ER), the mitochondria, and the chloroplast in plants. The primary disulfide carrier in the ER lumen is protein disulfide isomerase (PDI), a soluble protein of high abundance (Figure 1B). PDI is the first characterized catalyst of protein folding, identified half century ago^{9,10}. Oxidative folding of many proteins probably requires PDI, given the essentiality of this gene¹. There are, however, about 20 PDI-like homologs in humans, some of which may oxidize a specific subset of proteins¹¹. PDI receives disulfide from ER oxidoreductin 1 (Ero1), the primary disulfide donor in the ER. Ero1 is a membrane-associated flavoenzyme that generates disulfide through reducing flavin adenine dinucleotide (FAD), a cofactor different from the quinones used by DsbB and VKOR. DsbB is not found in eukaryotic cells, but VKOR is conserved from bacteria to vertebrates¹² and contributes to the disulfide formation in the ER¹³ (Figure 1B). However, disulfide generation is no longer the primary function of vertebrate VKOR, which instead supports the blood coagulation through reducing vitamin K epoxide, a reaction coupled with disulfide formation¹⁴. In contrast, VKOR found in *Arabidopsis* is fused with a DsbA to promote disulfide formation in the oxidative thylakoid lumen of chloroplast¹⁵. Oxidative folding also occurs in the intermembrane space of mitochondria¹⁶. The mitochondrial Mia40 is a unique disulfide carrier that, unlike PDI and DsbA, does not belong to the thioredoxin superfamily. The disulfide donor in human mitochondria is ALR/Erv1, flavoenzymes that share no sequence similarity with Ero1.

Disulfide-bond formation and isomerization

Most of these soluble and membrane-associated thiol oxidoreductases contain a CXXC motif at their active site, except a CPC catalytic motif in Mia40. Oxidization of the two cysteines in the CXXC motif (or CPC motif) forms a disulfide bond, a process associated with the loss of two hydrogen atoms and hence two electrons. The quinone or FAD cofactor of the donor enzyme accepts these two electrons, resulting in the formation of the disulfide bond between the CXXC. This disulfide is passed to a second pair of cysteines contained in the donor enzyme, which then deliver the disulfide to the CXXC motif of the carrier enzyme, and subsequently to its substrate proteins. This disulfide-relay pathway is accompanied by the electron transfer in the reversed direction. An intermediate state during the electron transfer is a mixed disulfide bond formed between two pairs of cysteines, which can be resolved by the nucleophilic attack of a thiol group from one of the flanking cysteines (Figure 1C). Through this mechanism, the disulfide is exchanged within one thiol oxidoreductase, between a disulfide donor and a carrier, or between the carrier and its substrate proteins.

A similar disulfide-exchange mechanism underlies the disulfide shuffling, an important pathway to complement the disulfide formation. During oxidative folding, incorrect disulfide bonds can be formed in proteins containing more than one pair of cysteines, and these scrambled disulfides need to be isomerized before a native protein conformation can be reached. To alternate the bonding pattern, the eukaryotic PDI catalyzes a disulfide-exchange process not requiring a full reduction and oxidation cycle (Figure 1C). PDI can function both as the protein disulfide isomerase and as the oxidase to promote disulfide formation (Figure 1B). In bacteria, these two activities are separated, with DsbC being the disulfide isomerase and DsbA being the oxidase (Figure 1A). DsbC is maintained in a reduced state by DsbD, which contains a transmembrane domain carrying a pair of catalytic cysteines. These transmembrane cysteines receive electrons from a cytoplasmic protein, thioredoxin, to reduce the periplasmic DsbC. Thus, this pathway is capable of directly shuttling electrons across the cytoplasmic membrane.

Structural convergence between thiol oxidoreductases

At the first glance, structures of the disulfide carrier and donor proteins fall into several different groups, including thioredoxin-like proteins, flavoenzymes, and transmembrane proteins. PDI¹⁷, DsbA¹⁸, and DsbC¹⁹ contains the thioredoxin fold, the most common structure found in thiol oxidoreductases²⁰. PDI is made of four thioredoxin-fold domains, and DsbA contains one thioredoxin fold with an additional helical domain. In contrast, the flavoenzymes, Ero1²¹ and Erv1²², do not contain a thioredoxin fold and their structures are also different from each other. The bacterial DsbB²³ and VKOR²⁴ are integral membrane proteins made of four or five transmembrane helices (TM). These TMs are, however, connected by different topologies in VKOR and DsbB. The transmembrane DsbD is yet different; structure of its archaeal homolog contains a V-shaped buried helix that presumably undergoes large conformational changes to transport electrons²⁵.

Despite of these apparent structural differences, Kaiser and coworkers proposed that a four-helix-bundle core structure is shared by all the disulfide donors²⁶, a prediction validated by the later determined structures of DsbB from *E. coli*²³ and VKOR from a cyanobacteria²⁴. The active sites of these intramembrane enzymes are surrounded by a bundle of four transmembrane helices (TM), similar to the four-helix bundle forming the active site of the flavoenzymes, Ero1 and Erv1 (Figure 2A). The conserved active-site architecture in these soluble and integral membrane proteins, which share no sequence similarity, gives an impressive example of evolutionary convergence.

The CXXC motif is always located at the N-terminus of one of the four helices. The first cysteine is more exposed and the second cysteine interacts with the FAD or quinone cofactor. Both cofactors have a planar ring structure and are always bound a single turn below the helical end (Figure 2B), forming a charge-transfer complex with the second cysteine^{30,31}. In fact, the helical-end location of CXXC is observed for almost all the thiol oxidoreductases containing this active-site motif, many of which are not involved in oxidative folding. These include all proteins with a thioredoxin fold, such as PDI, DsbA, DsbC, and glutathione reductase, and proteins without a thioredoxin fold, such as thioredoxin reductase or AhpD²⁰. The spatial arrangement of CXXC is probably to ensure the accessibility of the first cysteine, which needs to react with other cysteines to accomplish redox processes. In addition, the reactive thiolate of this first cysteine can be stabilized by the positive dipole at the N-terminus of a α -helix, and by a network of hydrogen bonds that are formed between the thiolate and neighboring residues presented by the helix-turn structure³² (example of DsbA in Figure 2C).

The second pair of conserved cysteines is located in a loop region in all the donor enzymes (Figure 2A). This flexible structure allows the shuttle cysteines to transfer electrons back and forth between the active sites of disulfide donors and carriers, which are located on a rigid helix-bundle region in these proteins. Mediation by shuttle cysteines avoids the direct contact between the active sites of the donors and carriers, thereby preventing steric clashing. Taken together, structural convergence is observed at several levels in these thiol oxidoreductases, suggesting that the disulfide generation and relay require certain structural features that are well recognized by evolution.

The controversies of the human VKOR topology

Despite the striking structural convergence observed in the thiol oxidoreductases, the folding topology of the human VKOR (hVKOR) had been controversial. Crystal structure of the bacterial VKOR²⁴, with 25% sequence identity to hVKOR, indicates that the human version almost certainly has a four-TM-bundle structure. However, biochemical analyses of the hVKOR topology generate conflicting conclusions of the three-TM^{33–36} and four-TM^{37,38} models. Although these models agree that the C-terminus of hVKOR is in the cytosol and the CXXC motif on TM4 is at the luminal side^{14,39,40}, the major difference is that TM2 in the four-TM model is part of a long cytosolic loop in the three-TM model (Figure 3A). The orientation of TM1 is reversed in these two models, and consequently, the N-terminus of hVKOR is either in ER lumen or cytosol in the three-or four-TM model, respectively.

The large discrepancy between a structural biology prediction and the conflicting biochemical data deserves critical evaluation in technical details. The earliest report of three-TM topology was conducted in *in vitro* microsomal systems³³. The full-length and TM-truncated hVKOR protein was translated and co-translocated into microsomes, and its topology was mapped by introduced glycosylation sites. The assumptions in this experiment are that 1) the glycosylation only occurs inside the microsomes, which mimic the ER lumen; 2) the hVKOR protein is properly folded *in vitro*; and 3) the truncated protein constructs have the same topology as the full-length protein. In a separate experiment, insect cells expressing full-length hVKOR was disrupted into membrane fragments, which recircle to form the microsomes, presumably in the same orientation. The topology was determined by protease digestion of a short tag attached to the N- and C-terminus of hVKOR; this tag should be protected only if it is enclosed in microsome.

Because these *in vitro* microsomal systems have a potential problem of not preserving the native topology or orientation of hVKOR, later analyses of hVKOR topology switched to cellular systems. In a subsequent work also supporting the three-TM model³⁵, the full-length hVKOR was compared with a short splicing variant only containing TM1. A glycosylation site was introduced after TM1 in both proteins, and was found to be glycosylated only in the short variant, but not in the full-length protein, suggesting that they adopt different topologies. This experiment, however, cannot rule out the possibility that the introduced site is buried from glycosylation in the full-length structure, whereas in the short hVKOR variant this region is unstructured and exposed for glycosylation. Indeed, a recent paper showed that, when the glycosylation site was introduced at a different amino acid position, the same region (after TM1) in full-length hVKOR was exposed for glycosylation³⁸. Thus, the C-terminus of TM1 is in the ER lumen, as predicted by the four-TM model.

Besides glycosylation mapping, GFP was introduced as a reporter protein at the N- or C-terminus of full-length or truncated hVKOR, and their topology was distinguished by protease digestion of the GFP tag, which can be visualized by cell imaging³⁴. For the protease to reach the hVKOR-GFP on the ER membrane, the plasma membrane was selectively permeated, a process requiring delicate control of the experimental conditions. Consequently, the protease digestion was monitored within tens of seconds, and the rate difference of digesting N- and C-GFP hVKOR suggests the three-TM model. Contradictory to this finding, a recent paper using GFP reporter supported the four-TM model³⁸. To avoid potential artifacts generated from cell permeabilization³⁸, a redox-sensitive GFP (roGFP) was introduced to directly detect the hVKOR topology in living cells. The rho-GFP tags attached to N- and C-terminus of the hVKOR was both found to be in reduced form, consistent with their cytosolic location and hence the four-TM topology.

The last contradictory results of hVKOR topology were from cysteine mapping experiments conducted with the selective permeation of plasma membrane, which only allows chemical modification of cysteines at the cytosolic side. One study³⁷ aimed to distinguish the location of N- or C-terminus of hVKOR. A cysteine was introduced at either of the termini, in addition to the seven cysteines contained in hVKOR, including the conserved CXXC motif and the shuttle cysteines. The introduced cysteines at these termini could be labeled, suggesting that both termini are in the cytosol, as in the four-TM model. In contrast, another

study mutated the shuttle cysteines and conclude that they are in the cytosol³⁴, consistent with the three-TM model. In both studies, the time of selective permeation needs to be precisely controlled between samples, as in the GFP experiment described above. Importantly, both experiments could not distinguish which cysteines were modified and could not quantify their modification extent, and the result is confusing because the same gel band represents modifications on multiple cysteines.

Conflicting results from these different assay systems suggest that the native conformation of hVKOR is sensitive to the perturbations introduced in different studies and the experimental conditions being used. Topology of membrane proteins follows a positive-inside rule that their intracellular side tend to contain more positive-charged residues (Arg and Lys) than the extracellular or ER luminal side⁴²; proteins with no positive-charge difference (or small biases) across the membrane sometimes adopt dual topologies⁴³. Notably, a four-TM hVKOR would contain the same number of Lys and Arg residues at the cytosolic and ER luminal side, generating an uncertainty in hVKOR topology (Figure 3B). In addition, the TM2 in hVKOR has a relatively low hydrophobicity (Figure 3B, C), and a lower efficiency of membrane insertion may result in topological diversity⁴³. A further complication is that the native conformation of hVKOR can be altered by different experimental approaches^{44,45}. Conventional biochemistry experiments often need to introduce protein fusions (e.g., GFP) as the topology reporter^{34,38}, and truncate the TM segments³³ to determine the orientation of each TM. These approaches are informative in general, but caution needs to be taken for proteins sensitive to perturbations⁴⁴, fusions with a large protein^{34,38}, especially at the N-terminus, and TM truncations³³ should be avoided.

Intact human VKOR adopts a four-TM topology

To introduce minimal perturbation, topology of native hVKOR was recently determined by probing the location of the seven intrinsic cysteines in this protein⁴⁶; oxidized and reduced cysteines are located at the ER luminal and cytosolic side, respectively, because the ER is much more oxidized than the cytosol (stable disulfide bonds do not form in cytosol)⁴⁷. Live-cell cysteine labeling, combined with quantitative mass spectrometry (MS) analysis, showed that a major fraction of active site CXXC (Cys132 and Cys135) and the shuttle cysteines (Cys43 and Cys51) are oxidized in the cellular environment, as predicted by the 4-TM topology (Figure 3A). This oxidation pattern is inconsistent with the 3-TM model, in which Cys43 and Cys51 are located at the cytosolic side and should remain reduced. The live-cell MS approach is advantageous over the biochemical cysteine mapping^{34,37} in that modifications on each cysteine are identified and quantified by MS, and the modification process does not require selective membrane permeation. Importantly, the powerful MS approach clearly identified a Cys51-Cys132 disulfide, thereby placing Cys51 in the ER lumen, given that the active site Cys132 is known to locate at the luminal surface^{33,48}. Cys51 and Cys132 are within disulfide-bonding distance, a scenario only occurs in the four-TM conformation. This distance restriction essentially eliminates the three-TM topology and other compromising models; for example, Cys51 has been postulated to be in the membrane-buried region facing the cytosolic side (Figure 3A).

Electron transfer of human VKOR in a cellular environment

Only in the four-TM model can the shuttle cysteines (Cys43 and Cys51) in hVKOR mediate the electron transfer to its active site (Cys132 and Cys135), a common mechanism used by thiol oxidoreductases (Figure 1A, B). The electron-transfer mechanism in hVKOR was initially proposed²⁴ because structure of the bacterial VKOR homolog shares similarities with other disulfide donors (Figure 2A, B). For different bacterial VKORs and a human VKOR-like protein, the shuttle cysteines are clearly required for their *in vitro* and *in vivo* activities^{15,24,36,49}. Consistently, it was later showed for hVKOR that, with the shuttle cysteines mutated, its reductase activity cannot be maintained *in vitro*⁵⁰. However, in a cell-based assay, hVKOR with the Cys51Ala mutation, the Cys43/Cys51 double mutation, or a deletion from Cys43 to Cys51 retains most of the activity, although Cys43Ala alone is nearly inactive⁵¹. Because the shuttle cysteines appear dispensable for hVKOR activity, this observation argues against the electron-transfer pathway, and also places doubt on the four-TM topology of hVKOR that supports the electron transfer⁵¹. Thus, the electron-transfer process in hVKOR remain controversial. In addition, the biochemical proofs of electron transfer all rely on whether the cysteine mutants lose activity. However, direct evidence of an active electron-transfer process, especially in a cellular environment, is missing for hVKOR.

The MS-based method enabled the tracking of electron-transfer process through detecting the redox-state changes for each of the cysteines in hVKOR. With substrates added to cells, the Cys132/Cys135 in the CXXC motif and the shuttle cysteines Cys43/Cys51 were found progressively oxidized. This oxidation pattern is expected for active VKOR catalysis and electron transfer in cells, because substrate reduction is coupled to cysteine oxidation to form the Cys132-Cys135 disulfide at the active site, followed by a subsequent electron transfer to generate the Cys43-Cys51 disulfide. Conversely, mutations of these cysteines were found to block the oxidation along the electron-transfer pathway and change the redox pattern owing to a redistribution of disulfide bonds. As an intermediate state during this electron transfer, a mixed disulfide, Cys51-Cys132, is found to be the major cellular state of hVKOR. Taken together, electron transfer indeed occurs between the shuttle cysteines and the catalytic cysteines in hVKOR, a mechanism consistent with the four-TM model.

Structural mechanism of the electron transfer

To transfer electrons at the membrane interface, both the bacterial VKOR and DsbB have evolved an amphipathic helix as a membrane anchor to increase the accessibility of the shuttle cysteines to the CXXC motif in the membrane (Figure 4A). The amphipathic helix, with its hydrophobic surface attached to the membrane, brings the shuttle cysteines close to the active site. In VKOR, the amphipathic helix caps over the four-helix bundle and forms part of the active site. Electron transfer appears to involve conformational changes in the amphipathic helix, whose N-terminal part can unwind into a loop or rewind into a helix in the bacterial VKOR structures captured in two electron-transfer states²⁹. Comparison of these static structures suggests a motion that brings one of the shuttle cysteines (Cys56 in Figure 4A), located at the N-terminus of this region, back and forth to transfer electrons between the other shuttle cysteine (Cys50) and an active site cysteine (Cys130)²⁹. In contrast, the amphipathic helix in DsbB is not part of its active site, but located aside by the

four-helix bundle and more buried in the membrane (Figure 4B). The amphipathic helix does not carry shuttle cysteines. Instead, it seems to function by placing the two flanking loop regions together, each of which contains a shuttle cysteine. The shuttle and active-site cysteines are all lined up in DsbB, allowing unimpeded electron transfer that only requires conformational changes in the flanking loops. Despite these differences, the bacterial VKOR and DsbB use a common strategy of employing the amphipathic helix to exchange disulfide at the membrane interface, thereby suggesting another level of structural convergence.

A structure biology view of the controversies about hVKOR

Proteins only have a limited number of structural folds to perform various functions. Compared to hundreds of millions of proteins sequences available, only ~ 1,000 different protein folds have been identified to date⁵³. Among these, the four-helix bundle is a common fold with a highly stable structure. It provides a rigid scaffold for all the disulfide donor enzymes to form a binding pocket for the quinone or FAD cofactors and to place the CXXC motif at a helical end for the coupled redox reaction (Figure 2B), although these proteins share no sequence similarity. In contrast, VKOR homologs share high sequence similarity¹² and most homologs adopt the four-TM topology^{15,24,49,54}. For example, hVKOR shares 74% sequence similarity to a paralogous protein, human VKOR-like⁵⁵, which is four-TM and has the same activity as hVKOR³⁶. A model of three-TM hVKOR (or a dimer of three TMs), however, would require the use of rearranged residues in this new fold to generate the same enzymatic activity, which is a highly unlikely scenario. Although multiple topologies remain a possibility for hVKOR, the more important question is perhaps not about whether these topologies co-exist, but about which topology is the catalytic form of hVKOR. If nature has only found certain ways of doing certain things, it would be safe to predict that the active form of hVKOR structure should use a four-helix bundle for catalysis, a flexible loop containing the shuttle cysteines to transfer electrons, and an amphipathic helix to anchor the shuttle cysteines. Convergence of these structural components underlies quinone reduction, and disulfide generation and relay.

Remaining questions in the structural mechanism of intramembrane thiol oxidoreductases

A crystal structure of the hVKOR is long awaited to put an end to these controversies, and it will be interesting to see whether the prediction of structure convergence stays correct. The hVKOR structure will also bring new insights into its catalytic mechanism. Compared to the bacterial VKOR, which only catalyzes quinone reduction, hVKOR can reduce both the quinone and epoxide forms of vitamin K. Structure differences must exist to enable the hVKOR to catalyze an addition reaction. Such structural changes may also explain why warfarin, a commonly used drug that targets hVKOR, is not an effective inhibitor of the bacterial VKOR.

Although the electron transfer does occur in hVKOR, it remains unclear why mutants involving one of the shuttle cysteines (Cys51) remain active in the cellular environment, although this is not surprising because thiol oxidoreductases lacking active site cysteines often can retain partial activity^{56,57}. We postulate that mutation or deletion removing the

Cys51-Cys132 linkage, which normally stabilizes the hVKOR structure⁴⁶, would make the HL1-2 region highly flexible. The hVKOR active site may become more accessible by reducing partner proteins or small reducing molecules. This accessibility may be restricted in wild-type hVKOR, but when Cys51 is mutated, a second pathway to reduce hVKOR may be invoked. It remains unclear, however, how much this second pathway contributes to maintain the wild-type hVKOR activity, compared to the Cys43/Cys51 mediated electron transfer.

Apart from the VKOR and DsbB, how DsbD transfers electron across the membrane is highly interesting. The structural mechanism of DsbD-mediated electron transfer remains unclear and likely requires dramatic structural rearrangement. Only the structure of a DsbD homolog, CcdA, has been determined at a ground state²⁵. The two catalytic cysteines in CcdA, Cys16 and Cys118, are ~ 20 Å apart from each other (Figure 4C). Because these cysteines need to physically interact with each other to mediate the disulfide change, a cytosolic loop containing Cys16 has to interact with Cys118, which is on a V-shaped horizontal helix buried in the middle of the membrane. To continue the electron transfer, both Cys16 and Cys118 need to move close to the periplasmic surface to interact with DsbC. These movements require extremely dramatic conformational changes, with the surface loop moving across the membrane and the V-helix moving out to the aqueous interface. To match with the changing hydrophobicity, these local structures probably need to refold. Understanding such dramatic conformational changes would require DsbD structures to be captured in different states and with its partner proteins, thioredoxin and DsbC.

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Figure 1. Oxidative protein folding pathways

A, Disulfide formation and isomerization pathways in *E. coli*. **B**, Pathways in human ER. **C**, Mechanism of disulfide exchange during disulfide formation (top) and isomerization (bottom). The disulfide donor enzymes are colored in yellow, carrier enzymes in blue, protein substrates in green, and all electron donor and carriers in the DsbC-DsbD pathway in blue.

Figure 2. Structural convergence of the active sites in soluble and intramembrane thioredoxoreductases

A-D, Left, the flavoenzymes and quinone reductases share a four-helix-bundle core structure (shown in blue; other part of the structures is dimmed for clarity) surrounding the active site. They also contain a flexible loop (pink) carrying the shuttle cysteines. **Right**, same structural location of the CXXC motif and the cofactors. The PDB models used are 1RQ1²¹ for Ero1; 1JR8²⁷ for Erv2, a yeast homolog of Erv1; 2ZUQ²⁸ for DsbB; and 4NV2²⁹ for VKOR. **E**, The helical-turn motif provides a positive dipole (δ^+) and surrounding residues to stabilize the thiolate form of the first cysteine. DsbA (PDB: 1A2I) is shown as an example.

Figure 3. Topology of human VKOR

A, The controversial topology models. Cysteines located in oxidative ER lumen are shown in red, and those in reductive cytosol in green. **B**, human VKOR contains the same number of positive charges across the membrane. A charged residue, Gln91, is in the middle of TM2 and lowers its hydrophobicity. **C**, TMHMM prediction⁴¹ of the hVKOR topology. The existence of TM2 is unclear owing to the presence of Gln91 and the relative short length of TM2.

Figure 4. Structural mechanisms of electron transfer

A, Electron transfer in the bacterial VKOR requires unwinding of the N-terminal half of the amphipathic helix. The four-helix bundle is shown in blue, and the amphipathic helix and surrounding loops in pink. Rest of the VKOR structure is omitted for clarity. The PDB models used are 4NV2²⁹ (left) and 3KP9²⁴ (right). **B**, Electron transfer in DsbB only requires loop motion. The PDB models used are 2ZUQ²⁸ (left) and 2K74⁵² (right). **C**, Structure of a DsbD homolog, CcdA (PDB code 2N4X²⁵). The arrows indicate the large movement required for the disulfide exchange.