Supplementary Note

Explanation for apparent oxidized fraction of cysteines

Cysteines show an apparent oxidation fraction (**Fig. 2b**) because they either form disulfide bonds or are inefficiently labeled by NEM. At the first step of MS sample preparation (**Fig. 2a**), NEM is added to cells to label free cysteines in membrane-bound hVKOR. However, free cysteines that are buried in the native protein or membrane may react less efficiently with NEM at this step. Subsequently, the protein is fully denatured and reduced; disulfide-bonded cysteines become free after reduction and buried cysteines become exposed after denaturation. Both kinds of cysteines react with the NEM-d_5 isotope, which is taken as the indicator for oxidation (**Fig. 2a**). Although the buried cysteines do not form disulfide bonds, they appear oxidized with this NEM and NEM-d_5 labeling method. To resolve this complication, we pre-treat cells with DTT before the labeling: DTT does not reduce buried free cysteines, but reduces cysteines within disulfide bonds (**Fig. 2b**). Thus, cysteines that are oxidized in disulfide bonds can be distinguished from free cysteines that are more exposed after denaturation.

Explanation and control experiments for NEM labeling

NEM alkylation captures the intracellular redox state of hVKOR and quenches the hVKOR catalyzed reaction. The use of Triton X-100 accelerates NEM diffusion into cells; without Triton X-100, membrane-buried cysteines (C16, C85) are inefficiently labeled by NEM and appear unrealistically highly oxidized (see attached figure; same mechanism reported in Ref 1). Triton X-100 does not change the oxidation states of all catalytic cysteines; with or without triton X-100, C43, C51, C132, and C135, are at almost identical oxidation levels (attached figure). Capturing the evolving redox state of hVKOR also depends on the efficient quenching by this method. To

confirm this, we show that NEM labeling (with triton-100) carried for different times gives similar results (attached figure). Taken together, the NEM labeling method, facilitated by triton X-100, reports the near realistic redox states of hVKOR in cells.

Distribution of alternative disulfide bonds in wild-type hVKOR in absence of substrate

The electron transfer in hVKOR is associated with alternative formation of disulfide bonds. If the transfer occurs sequentially, as proposed from the structure of the bacterial homolog[2,3], three alternative disulfide bonds, Cys43-Cys51, Cys51-Cys132, and Cys132-Cys135 are formed between these four conserved cysteines (**Supplementary Fig. 2**). All of these disulfide bonds have been identified by MS (**Supplementary Data Set 1**), whereas the other potential disulfides (Cys43-Cys132, Cys43-Cys135, or Cys51-Cys135) were not evident from our MS analysis.

Interactions between Cys132 and Cys135, the CXXC motif, is restricted by TM4. Cys132 is at the N-terminal interface of this helix, and Cys135 is separated by about a helix turn and located more towards the inside of the protein (**Supplementary Fig. 2b**). Therefore, Cys135 primarily forms disulfide bond with Cys132, and only Cys132 interact with other cysteines for electron transfer. A similar mechanism was observed for other enzymes catalyzing *de novo* disulfide formation, including soluble proteins, Ero1 and Erv2, and a membrane protein, DsbB[4]. Interestingly, the CXXC motif is always at the N-terminal end of a TM or a helix in soluble proteins. The second cysteine of CXXC is more buried in the structure and only interacts with the first cysteine, which is on the boundary between the helix and a loop region. This first cysteine in turn interacts with other cysteines for electron transfer.

From this knowledge, the relative distribution of the alternative disulfides can be deduced from **Fig. 2b**.

Cys43: ~40% forms Cys43-Cys51 and 60% reduced.

Cys51: ~40% forms Cys43-Cys51, 50% forms Cys51-Cys132, and 10% reduced.

Cys132: ~50% forms Cys51-Cys132, 40% forms Cys132-Cys135, and 10% reduced.

Cys135: ~40% forms Cys51-Cys132 and 60% reduced.

Redox-state changes induced by cysteine mutations are consistent with a redistribution mechanism

Cysteines forming alternative disulfides in wild-type hVKOR are in equilibrium between disulfide-bonded and reduced states (**Supplementary Fig. 2a**). The key to understanding redox changes after a mutation that disrupts a particular disulfide(s) is that its un-mutated partner cysteine is redistributed into other reduced and oxidized fractions. This redistribution generates redox state changes, which can be predicted as follows.

1. C43A mutation breaks the Cys43-Cys51 disulfide. The portion of Cys51 that used to form Cys43-Cys51 redistributes into two fractions by either forming a new disulfide bond with Cys132 or becoming reduced. Therefore, the fraction of reduced Cys51 will increase (compared to Cys51 in the wild type), while more Cys132 will become oxidized because more Cys51-Cys132 is formed. Because Cys132 forms more disulfide bonds with Cys51, more Cys135 will become reduced.

2. C51A mutation breaks the Cys43-Cys51 and Cys51-Cys132 disulfides. Cys43 that used to form Cys43-Cys51 will become reduced. Cys132 that used to form Cys51-Cys132 will either form a disulfide bond with Cys135 or become reduced. Thus, more Cys132 will become reduced and more Cys135 will become oxidized (under the same principle as explained in 1).

3. C132A mutation breaks the Cys51-Cys132 and Cys132-Cys135 disulfides. Cys135 that used to form Cys132-Cys135 will become reduced. Cys51 that used to form Cys51-Cys132 will either

form a disulfide bond with Cys43 or become reduced. Thus, more Cys51 will become reduced and more Cys43 will become oxidized.

4. C135A mutation breaks the Cys132-Cys135 disulfide. Cys132 that used to form Cys132-Cys135 will either form a disulfide bond with Cys51 or become reduced. Thus, more Cys132 will become reduced and more Cys51 will become oxidized. Consequently, more Cys43 will become reduced because Cys51 will shift to form a disulfide bond with Cys132.

5. Because C16A, C85A and C96A do not affect the electron transfer pathway, they should not change the redox states of any cysteines.

These predictions fit well with most of the redox-state changes after cysteine mutations (see *t* test scores in **Supplementary Fig. 3b**). As expected, C16A, C85A and C96A do not affect any other cysteines (**Supplementary Fig. 3a**). However, there are exceptions to our predictions: only a very weak change is observed in Cys51 with the C132A or C135A mutation. In addition, long-range effects are not observed: C43A does not change Cys135, and C135A does not change Cys43.

Warfarin changes the labeling efficiency of cysteines

As discussed above, the apparent oxidation of cysteines observed in our MS experiments is a result of either disulfide-bond formation or limited NEM accessibility. Unlike the KO substrate of hVKOR, warfarin is chemically non-oxidative. Therefore, cysteines are not expected to be oxidized by warfarin, although this drug preferably binds to the oxidized Cys51-Cys132 form of hVKOR. The following evidence further suggests that warfarin increases the apparent oxidation levels of Cys16, Cys43, Cys85, and Cys135 (**Supplementary Fig. 5a**) by lowering their accessibility to NEM, instead of by inducing disulfide-bond formation.

Cys16 and Cys85 do not form disulfide bonds in wild-type hVKOR, because both cysteines show low oxidation levels (**Fig. 2b**). Moreover, mutating Cys16 or Cys85 does not affect the redox state of other cysteines in untreated samples (**Supplementary Fig. 3a**) or warfarin-treated samples (**Supplementary Fig. 5b**), indicating that, in both cases, the mutations do not disrupt any disulfide bond. Therefore, the apparent oxidation of Cys16 and Cys85 after warfarin treatment is due to decreased NEM labeling.

Warfarin binding also changes the apparent oxidation state of the catalytic cysteines Cys43 and Cys135, which are capable of forming Cys43-Cys51 and Cys132-Cys135 disulfide bonds, respectively (**Fig. 3**). However, formation of these alternative disulfides will disrupt the Cys51-Cys132 disulfide bond and is thus incompatible with warfarin binding. Therefore, warfarin increases the apparent oxidation level of Cys43 and Cys135, not by inducing disulfide bonds, but by lowering NEM labeling.

The change in the NEM labeling efficiency of these cysteines indicates a decrease in their accessibility, suggesting that the overall structure of hVKOR may become less flexible after warfarin binding. In addition, warfarin may induce the formation of a tighter pocket and/or physically block access to the affected cysteines.

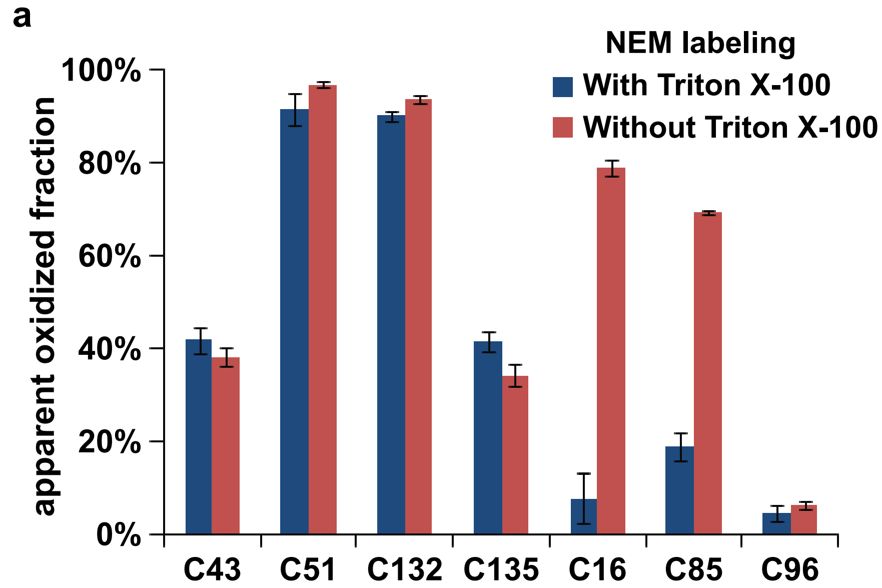
Supplementary References

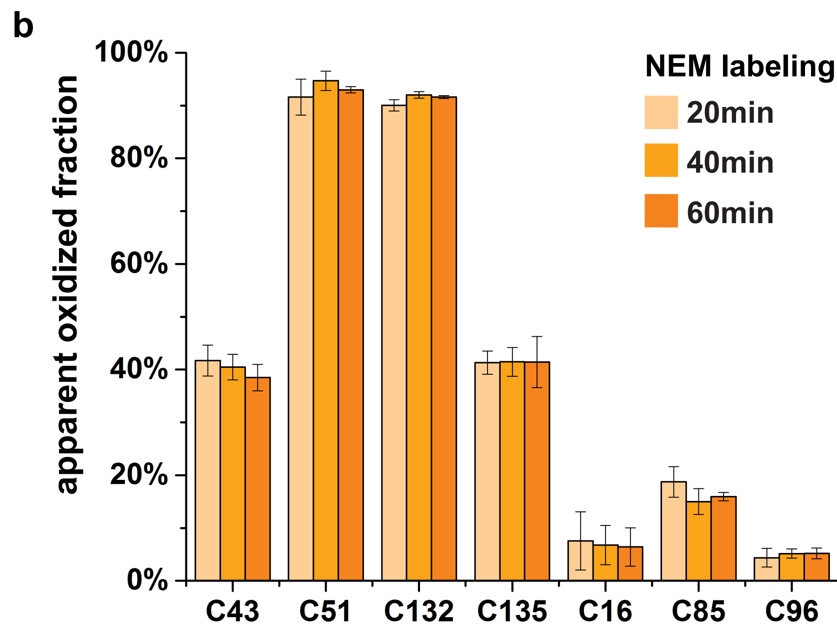
1. Wang, Y., Toei, M. & Forgac, M. Analysis of the membrane topology of transmembrane segments in the C-terminal hydrophobic domain of the yeast vacuolar ATPase subunit a (Vph1p) by chemical modification. *J. Biol. Chem.* **283,** 20696–20702 (2008).

2. Li, W. *et al.* Structure of a bacterial homologue of vitamin K epoxide reductase. *Nature* **463,** 507–12 (2010).

3. Liu, S., Cheng, W., Fowle Grider, R., Shen, G. & Li, W. Structures of an intramembrane vitamin K epoxide reductase homolog reveal control mechanisms for electron transfer. *Nat. Commun.* **5,** 3110 (2014).

4. Sevier, C. S. *et al.* The prokaryotic enzyme DsbB may share key structural features with eukaryotic disulfide bond forming oxidoreductases. *Protein Sci.* **14,** 1630–1642 (2005).





Control experiments to validate the NEM labeling method. (a) NEM labeling of wild-type hVKOR with and without triton-X100. The oxidation level of catalytic cysteines (Cys43, Cys51, Cys132, and Cys135) are almost identical. However, the membrane buried cysteines (Cys16 and Cys85) show large difference. **(b)** Different NEM labeling time in triton X-100 does not change the oxidation level detected by the NEM labeling method. Therefore, the hVKOR catalysis is effectively quenched by this method.