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Crystal structure of an assembly intermediate of respiratory Complex II

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Flavin is covalently attached to the protein scaffold in ~10% of flavoenzymes. However, the mechanism of covalent modification is unclear, due in part to challenges in stabilizing assembly intermediates. Here, we capture the structure of an assembly intermediate of the *Escherichia coli* Complex II (quinol:fumarate reductase (FrdABCD)). The structure contains the *E. coli* FrdA subunit bound to covalent FAD and crosslinked with its assembly factor, SdhE. The structure contains two global conformational changes as compared to prior structures of the mature protein: the rotation of a domain within the FrdA subunit, and the destabilization of two large loops of the FrdA subunit, which may create a tunnel to the active site. We infer a mechanism for covalent flavinylation. As supported by spectroscopic and kinetic analyses, we suggest that SdhE shifts the conformational equilibrium of the FrdA active site to disfavor succinate/fumarate interconversion and enhance covalent flavinylation.
ofactor-assisted enzymes catalyze numerous biochemical transformations, especially the oxidation–reduction reactions important for metabolism and detoxification. The conjugated isoalloxazine ring of flavins allows these versatile cofactors to support both electron transfer and group transfer reactions. Importantly, flavins can be either covalently attached to their protein scaffold or non-covalently associated. While non-covalent enzyme-associated flavins have access to a full range of chemistry, the addition of a covalent linkage has several effects, including increasing the redox potential. This important modification may therefore allow flavoenzymes to act upon substrates with higher redox couples.

Covalent flavin was discovered in Complex II of the mitochondrial respiratory chain where it is attached via an 8a-N(3)-histidil linkage. Integral-membrane Complex II enzymes can act in either aerobic respiration (termed succinate—quinone oxidoreductase (SdhABC) or during anaerobic respiration with fumarate as the terminal electron acceptor (termed quinol: fumarate reductase (FrdABC)). In their mature and fully assembled forms, both SdhABC and FrdABC can catalyze the interconversion of fumarate and succinate at the FrdA subunit (FrdABCD)). In their mature and fully assembled forms, both SdhABC and FrdABC can catalyze the interconversion of fumarate and succinate at the FrdA subunit (FrdABCD). Importantly, the covalent linkage to FAD increases the potential from −145 to −55 mV, a change that is required to catalyze succinate oxidation. This potential remains constant and cannot be oxidized further. The covalent oxidation of the flavin adenine dinucleotide (FAD) covalently attached to the flavin adenine dinucleotide (FAD) covalently attached to the flavoprotein subunit (FrdA or SdhA). Importantly, the covalent linkage to FAD raises the $E_{m7}$ ~100 mV. In the case of FrdABC, this increases the potential from −145 to −55 mV, a change that is required to catalyze succinate oxidation. This potential remains constant and cannot be oxidized further.

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binding mode. However, it is also possible that this peptide mass was a false positive.

In the structure of the FrdA-SdhE assembly intermediate, the electron density for SdhE is weaker than for the FrdA subunit. Regions distal to the FrdA-SdhE binding interface were difficult to model, and there was not interpretable electron density for many side chains, including the side chain of the unnatural amino acid crosslinker, SdhER8BzF. It is possible that there is increased mobility of SdhE as suggested by elevated crystallographic temperature factors (Supplementary Figure 1d). This is consistent with the proposed transient nature of the assembly intermediate, but may also reflect an imperfectly stabilized complex.

SdhE-associated domain rotation within the FrdA subunit. FrdA/SdhA subunits comprise a two-domain architecture consisting of a flavin-binding domain and a capping domain. The bifunctional surface of FrdA/SdhA that interacts with either SdhE or the Fe:S subunit bridges these domains (Fig. 1a–c). We performed structural comparisons between the FrdA-SdhE assembly intermediate and FrdA in the context of the FrdABCD complex (PDB entry 3P4P). In these comparisons, some of the conformational differences may be due to the loss of contacts between FrdA and FrdB rather than the addition of new contacts between FrdA and SdhE. Pair-wise comparisons of the isolated FrdA domains in the FrdA-SdhE assembly intermediate with those in the mature FrdABCD are consistent with similar folds of each component; however, domain motion analysis identifies that changes in interdomain angle accompany the replacement of bound FrdB with SdhE (Fig. 1d). Interestingly, there are two copies of the FrdA-SdhE assembly intermediate in each crystallographic asymmetric unit; one in complex with the dicarboxylate malonate (50 mM in the crystallization conditions) and the other in complex with the carboxylate acetate (100 mM in the crystallization conditions). Both the malonate-bound (Supplementary Figure 3a) and the acetate-bound FrdA-SdhE assembly intermediate (Supplementary Figure 3b) induce a rotation of similar magnitude (10.6°–10.8°) (Fig. 1d, Supplementary Figure 3c, d), yet the rotation angles of the capping domain are subtly different, such that the capping domain rotation differs by a 12.1° rotation between these (Supplementary Figure 3c, e). This suggests some plasticity of the capping domain position in the FrdA-SdhE assembly intermediate.

Prior crystallographic snapshots of FrdA homologs also identified that the flavin-binding and capping domains can adopt different interdomain angles, which may be controlled by the identity of the ligand bound to the active site. This observation...
motivated proposals that interdomain rotation contributes to catalysis by allowing substrate entry to the active site, contributing to transition state stabilization, and protecting the transition state from solvent. However, both the historical and present analysis of capping domain rotation is complicated by crystal packing interactions. In both this FrdA-SdhE assembly intermediate and in mature FrdABCD, the capping domain is involved in crystal packing interactions (Supplementary Figure 4a, b). In order to investigate the alternative explanation that the domain rotation is an artifact of crystal packing, we compared the structure of the FrdA-SdhE assembly intermediate with the mature FrdABCD complex. This overlay identifies that the capping domain would be in steric clash with SdhE in the absence of a rotation. We additionally analyzed whether any previously reported flavinylation-deficient missense mutations would be anticipated to affect domain orientation. Based upon the locations of the residues affected, we propose that several reported flavinylation-deficient mutations of E. coli FrdA (FrdAD288, FrdA E245, and FrdA R287) and yeast Sdh1 (equivalent to SdhA; Sdh1 R382 and Sdh1 C630/Sdh1 R638) likely disrupt domain alignment (Supplementary Figure 4c). Loss of covalent flavinylation in mutants that are expected to affect interdomain interactions is suggestive of a model where interdomain orientation contributes to flavinylation.

Allosteric destabilization of loops near the active site. A related global structural change involves two loops of the FrdA subunit (residues 50-58 and 103-129). When the FrdA subunit is assembled into the FrdABCD complex, the folded loops shield the active site from solvent (Fig. 2a, b). The same region in the context of the FrdA-SdhE assembly intermediate lacks interpretable electron density. If this region is unfolded in the assembly intermediate (Fig. 2c, d) it may form a tunnel into the active site. These loops are conserved in FrdA/SdhA homologs of available structures, including those that contain non-covalent FAD, equivalent loops are unfolded in the FAD-free enzyme but are folded in the FAD-bound enzyme. Surprisingly, this only results in minor perturbations to the FAD environment (Supplementary Figure 5a). In the FrdABCD complex, the positions of these loops are stabilized by interactions with the flavin-binding domain, the capping domain, and the Fe:S subunit (Supplementary Figure 5b). The physical basis for the increase of mobility of these loops within the context of the FrdA-SdhE assembly intermediate may be a combination of the lack of the contacts to the Fe:S subunit, and the rotation of the capping domain.

To test whether destabilization of this region is important for covalent FAD attachment, we developed a variant of FrdA (FrdA F116C/G392C) predicted to tether the larger loop of this unfolded region to the flavin-binding domain via a disulfide bond (Fig. 2e, Supplementary Table 1). Neither substitution is near the SdhE binding site, and should not directly impact SdhE association with the FrdA subunit. Using wild-type FrdA as a positive control and FrdA H44S, which lacks the histidyl ligand to FAD, as a negative control, we measured covalent flavinylation under all conditions tested (Fig. 2f, Supplementary Figure 6).

Although there are caveats to the interpretation of changes in function in disulfide-trapped mutants, the substitution of these residues with cysteines inhibits the covalent flavinylation process. Notably, if this region was fully unfolded during assembly, a tunnel would form (Fig. 2d). While a role for this tunnel cannot be proposed at this time, the tunnel would be large enough to accommodate water molecules, dicarboxylates, or even another protein.

SdhE inhibits succinate/fumarate interconversion. Intriguingly, these conformational differences between the FrdA subunit in the FrdA-SdhE assembly intermediate and the mature FrdABCD

Fig. 2 Active site tunnel in the FrdA-SdhE assembly intermediate. a The FrdA subunit within the context of the assembled FrdABCD complex (PDB entry 3P4P21). Residues 50-58 and 103-129 are highlighted in red. b Surface representation of the FrdA subunit within the context of assembled FrdABCD. The surface contributed by the loops (50-58 and 103-129) is shown in red. c The FrdA-SdhE assembly intermediate lacks interpretable electron density for residues 50-58 and 103-129. d Surface representation of the FrdA-SdhE assembly intermediate lacking these loops. e Design of a variant to tether the loop region to the flavin-binding domain using a disulfide (FrdA F116C/G392C, yellow). f Assessment of covalent FAD in wild type, the FrdA F116C/G392C disulfide-trapped variant, and the FrdA H44S negative control, as monitored by measuring FAD fluorescence of equivalent amounts of protein separated by SDS-PAGE. Covalent FAD is anticipated to co-migrate with the polypeptide, while non-covalent FAD fluorescence would not. Evaluation of the fluorescence of the FrdA subunit therefore reports on covalent flavinylation. Wild-type FrdA subunits have a 1:1 stoichiometry of covalent flavinylation. The FrdA F116C/G392C exhibited reduced covalent flavinylation levels as compared to wild type. Gel is a representative of 12 independent experiments. M molecular weight marker.
It is proposed that the transition state is achieved when FrdAT244 of the capping domain hydrogen-bonds to fumarate and twists this molecule, allowing orients the active site side chains of FrdAH355 and FrdAR390, promoting proton abstraction and resonance stabilization. However, malonate does not hydrogen-bonding interaction to SdhEG16, enabling nucleophilic attack.

3P4P21, gray), malonate (magenta) bound to avian SdhABCD (PDB entry 2H8934, tan) and malonate (yellow) bound to FrdA-SdhE (cyan). The view is of the active site changes catalytic activity bene.

Complex II enzymes is well understood23,32,33 (Fig. 3a). Subsequent experimental evidence shows that fumarate reduction involves: (1) hydride transfer from the N5 of FAD to the C2=Cs double bond of fumarate; and (2) protonation of the intermediate from a catalytic proton donor. When bound to substrate (Fig. 3c), the enzyme promotes these steps through: (1) alignment of the fumarate C2=Cs double bond along FAD, as stabilized by three interactions to side chains of the flavin-binding domain (E. coli FrdAH232, FrdAH355, FrdAR390) and two interactions to side chains of the capping domain (E. coli FrdAT244 and FrdAR287)21; (2) twisting the fumarate molecule to activate the double bond, as promoted by capping domain rotation and a substrate binding threonine (E. coli FrdAT244)23; and (3) transferring a proton from an appropriately positioned catalytic proton donor (E. coli FrdAR287)32. This reaction proceeds on the re-face of the FAD and all of the residues required for this reaction are similarly located on the re-face; succinate oxidation likely proceeds by the reverse of this mechanism.

Associated with SdhE binding to the FrdA subunit are several structural changes that may reduce the rate of succinate/fumarate interconversion. For example, the position of the capping domain (Fig. 1c) alters the position of the catalytically important FrdAT244 so that it is not poised to stabilize the transition state (Fig. 3c, d). This is associated with a 54° rotation of the three-carbon malonate as compared to the position of this ligand when

**Fig. 3** Mechanism of fumarate reduction and covalent flavinylation supported by the FrdA active site. a Chemical mechanism of fumarate reduction requires oriented binding of fumarate so that the C2=Cs double bond is aligned along FAD. The proposed chemical steps involve a transfer of a hydride from the flavin and a proton from FrdAR287. b Probable mechanism of covalent flavinylation requires a new position for dicarboxylate and three steps. The first two steps (top panel) are proton abstraction from C6r and resonance rearrangement to delocalize the resultant negative charge between N1 and C2. The third step (bottom reaction) is the attack by a histidyl ligand. c The active site of the fumarate-bound FrdA subunit (PDB entry 3P4P21) from the assembled FrdABCD complex optimizes dicarboxylate orientation along the FAD with hydrogen-bonding interactions to FrdAH355, FrdAR390, and FrdAT244. It is proposed that the transition state is achieved when FrdAT244 of the capping domain hydrogen-bonds to fumarate and twists this molecule, allowing fumarate to accept hydride from the N5 of FAD and a proton from FrdAR287. d In the FrdA-SdhE assembly intermediate, malonate interacts with and orients the active site side chains of FrdAH355 and FrdAR390, promoting proton abstraction and resonance stabilization. However, malonate does not interact with FrdAH355 or FrdAT244, likely preventing catalysis on the dicarboxylate. The imidazole ring of the FrdAH44 histidyl ligand is oriented by a hydrogen-bonding interaction to SdhE231, enabling nucleophilic attack. e An overlay of the active sites of fumarate (black) bound to FrdABCD (PDB entry 3P4P21, gray), malonate (magenta) bound to avian SdhABCD (PDB entry 2H8924, tan) and malonate (yellow) bound to FrdA-SdhE (cyan). The view is rotated by 90° with respect to c, d, highlighting the 54° rotation of malonate complex affect the active site. For example, several catalytic residues are positioned on the capping domain, and their positions must change with domain rotation. Further, the SdhE-associated tunnel into the active site involves residues adjacent to FAD. These active site changes are anticipated to affect both the oxidoreduction of succinate and fumarate (Fig. 3a) and covalent flavinylation (Fig. 3b). Proposing how SdhE-dependent alteration of the active site changes catalytic activity benefits from careful evaluation of the requirements for each reaction within the context of these conformational changes.

The mechanism of succinate/fumarate interconversion by Complex II enzymes is well understood23,32,33 (Fig. 3a). Substantial experimental evidence shows that fumarate reduction involves: (1) hydride transfer from the N5 of FAD to the C2=Cs double bond of fumarate; and (2) protonation of the intermediate from a catalytic proton donor. When bound to substrate (Fig. 3c), the enzyme promotes these steps through: (1) alignment of the fumarate C2=Cs double bond along FAD, as stabilized by three
bound to avian SdhABCD34 (Fig. 3e). When the four-carbon fumarate is modeled in this rotated position (Supplementary Figure 6a), the C2–C3 bond is no longer poised to accept hydride from the N5 of FAD.

To test whether these changes are associated with reduced succinate/fumarate interconversion, we investigated the impact of SdhE on succinate oxidation. To guide these experiments, we measured \( K_{D} \) values for FrdA/SdhA, which are 0.7 ± 0.1 and 1.5 ± 0.2 \( \mu \)M, respectively (Supplementary Figure 6b). We next assessed whether four-carbon dicarboxylates would misalign in the active site, as predicted by the rotation of the three-carbon malonate. To interrogate dicarboxylate orientation in solution, we focused on oxaloacetate. When oxaloacetate is correctly oriented in the active site, it induces a characteristic spectrum (charge transfer band) attributed to \( \pi-\pi \) interactions between the oxygen of oxaloacetate and the isoalloxazine ring of FAD. Oxaloacetate results in the appearance of the same charge transfer band when added to isolated FrdA or SdhA subunits, indicating that these unassembled subunits also correctly align dicarboxylates for catalysis. However, addition of SdhE to either FrdA or SdhA subunits reduces the charge transfer band, indicating changes in the interaction between oxaloacetate and FAD in the flavoprotein–SdhE assembly intermediate (Fig. 4a).

We predict that the rotation of substrate (Fig. 4a, Supplementary Figure 7a) will reduce the efficiency of succinate/fumarate interconversion. Therefore, we next measured succinate oxidation kinetics of E. coli FrdA subunits (Fig. 4b) in the presence of increasing concentrations of SdhE. SdhE inhibits succinate oxidation in a dose-dependent fashion with the half-maximal inhibitory concentration (IC50) of 1.2 \( \mu \)M (Fig. 4c, Table 2), a value consistent with the \( K_{D} \) measured by optical spectroscopy (Supplementary Figure 7b). We similarly assessed the impact of SdhE on succinate oxidation kinetics of isolated E. coli SdhA and found that the addition of SdhE inhibited catalysis with an IC50 of 1.5 ± 0.2 \( \mu \)M (Table 2).

Taken in aggregate, the structural and biochemical data suggest that two major impacts of SdhE on FrdA and SdhA subunits are: (1) the repositioning of FrdA H244A, which normally stabilizes the transition state, and (2) the misalignment of substrate in the active site. Together, this reduces the rate of succinate/fumarate interconversion, as reflected in the kinetic measurements.

**Formation of the covalent FAD linkage.** The covalent flavinylation reaction (Fig. 3b) is mechanistically distinct from fumarate reduction (Fig. 3a). If covalent flavinylation is autocatalytic and proceeds via a quinone:methide intermediate16, the reaction likely involves: (1) deprotonation of the \( \alpha \)-carbon of FAD; (2) resonance rearrangement stabilized by a positive charge near the FAD N1/C2; and (3) attack of the deprotonated \( \alpha \)-carbon by a histidyl side chain (E. coli FrdA H44).

This FrdA-SdhE structure contains the FrdA\(^{H44}\)-FAD covalent linkage. This represents the product of the flavinylation reaction, making it useful for inferring the roles of side chains during the reaction. The first step of the covalent flavinylation reaction involves a side chain that can extract a proton from the \( \alpha \)-carbon of FAD. Analysis of the structure suggests that one likely residue for proton abstraction from free FAD is FrdA H355 (Fig. 3b, c). Consistent with this proposal, when FrdA H355 is mutated, covalent flavinylation levels are statistically similar to those observed with the negative control 14. Removal of this proton to solvent may require a proton shuttle. One possibility would be proton transfer between FrdA H355, FrdA R287, and FrdA E245. The latter two residues are proposed to facilitate catalytic proton extraction and proton shuttling during the fumarate reduction reaction and have pKa values consistent with this function. When these
residues are mutated, covalent flavinylation is statistically similar to the negative control in each case. As these residues are both located on the capping domain, their involvement in covalent flavin attachment would also explain the observation that capping domain alignment appears important for flavin attachment.

Next, resonance rearrangement localizes the negative charge across the FAD C1/N2, which may be stabilized by the close proximity of positive charge. Here, a subtle architectural change, potentially facilitated by the rotation of the bound dicarboxylate, places the positively charged side chain of FrdAR390 0.6 Å nearer the negatively charged region of the FAD near the N1/C2 atoms (Fig. 3b, c). Consistent with an essential role in promoting formation of the quinone methide intermediate, mutation of FrdAR390 eliminates detectable covalent flavinylation.

If this mechanistic proposal for covalent flavinylation is correct, we would anticipate that the FrdA-SdhE assembly intermediate would bind with reasonable affinity to biologically relevant dicarboxylates. To validate this aspect of the mechanism, we therefore measured how SdhE impacts the affinity between E. coli FrdA or SdhA subunits and dicarboxylate. We took advantage of the fact that binding of a dicarboxylate ligand near the flavin in FrdA alters the optical properties of the cofactor and used optical difference spectroscopy to measure affinity of the four-carbon fumarate and three-carbon malonate to FrdA/SdhA subunits in the presence and absence of SdhE (Fig. 5a, b, Table 2). The apparent K_{fum} decreased 2- to 3-fold and the K_{mal} increased 5- to 7-fold in both FrdA and SdhA subunits. Thus, the shifted architecture of SdhE-bound FrdA maintains dicarboxylate binding, supporting our mechanism.

The final requirement for covalent flavinylation is the nucleophilic attack of the deprotonated α-carbon of FAD by a histidyl side chain (E. coli FrdA{H44}), forming the covalent bond and completing the reaction. In contrast to all chemical steps for succinate/fumarate interconversion and the preceding steps of covalent flavinylation, this reaction now occurs on the si-face of the FAD, making the active site of covalent flavinylation spatially distinct from that of succinate/fumarate interconversion. Here, the direct hydrogen-bonding interaction between FrdA{H44} N(1) and SdhE{G16} suggests that in the unflavinylated FrdA, FrdA{H44} is deprotonated and carries the lone pair at N(3). This interaction also rotates the imidazole ring of the FrdA{H44} histidyl ligand by 23° as compared to its orientation in assembled FrdABCD complexes (Figs. 1a, 5c, Supplementary Figure 7c). In the presence of the non-covalent FAD substrate, this could optimize the geometry of the FrdA{H44} N(3) nucleophile for attack. In the product complex containing covalent FAD, the rotation changes the N(3) bond angles from ~130°/120°/120° to ~160°/70°/120° (Fig. 5c, Supplementary Movie 1). This is anticipated to place strain across this bond, which may facilitate SdhE release from the covalently flavinylated FrdA subunit.

**Table 2 Effect of SdhE on the kinetic parameters of isolated FrdA and SdhA subunits**

|       | \( K_{fum} \) (µM) | \( K_{mal} \) (µM) | \( K_d \) (µM) | IC_{50} (µM) |
|-------|-------------------|-------------------|---------------|-------------|
| FrdA  | 150 ± 15          | 26 ± 3            | 0.7 ± 0.1     | 1.2 ± 0.1   |
| FrdA + SdhE | 70 ± 7          | 180 ± 20          |               |             |
| SdhA  | 242 ± 16          | 11 ± 1            | 1.5 ± 0.2     | 1.5 ± 0.2   |
| SdhA + SdhE | 64 ± 4          | 60 ± 5            |               |             |

*The IC_{50} for SdhE inhibition of FrdA and SdhA activity was monitored by inhibition of succinate:DCIP reductase reaction of the flavoproteins in the presence of SdhE.

**Discussion**

Cofactor-assisted enzymatic reactions are critical for life; however, our understanding of cofactor assembly has previously been hampered by the single-turnover nature of cofactor attachment combined with the transient nature of the likely intermediates. This gap in understanding cofactor assembly is perfectly illustrated in the case of covalent flavin, a relatively common enzyme-attached cofactor that imparts stability, prevents cofactor

### Figure 5

**Effect of SdhE on the requirements for covalent flavinylation activity of FrdA.**

- **a.** Optical difference spectra measuring the binding of a 5 mM fumarate or b 2 mM malonate. Spectra were collected using the same protocol as for Fig. 4a with 6.6 µM of the FrdA subunit. Insets show the difference spectra (FrdA–ligand complex minus free FrdA) for each corresponding complex. The difference spectra reflect a change in the FAD environment and were used to calculate the K_{i} value. Data are representative traces from three or more analyses.
- **b.** An overlay FrdA{H44} from the FrdA–SdhE assembly intermediate (cyan) with that from mature FrdABCD (gray) highlights at 23° rotation of the FrdA{H44} ligand. This is associated with a reduced distance to FAD C8α and a change in angles of the imidazole N(3).
- **c.** An overlay of FrdA{H44} catalyzed by SdhE and SdhE with flavin.
loss, and enhances the redox potential. Indeed, despite covalent flavin being discovered in mammalian Complex II over 60 years ago, it was only recently that additional protein flavinylation factors were identified, and controversy remains on how these assist the process of covalent attachment.

Characterized accessory proteins that contribute to covalent flavinylation have previously been suggested to fall into two broad classes: flavin transferases and assembly chaperones. The role of flavin transferases is perhaps best understood for the bacterial flavin trafficking protein (formerly called ApbE). The flavin trafficking protein assists in covalent attachment of FMN to the NqRc subunit of bacterial Na⁺–translocating NADH:quinone oxidoreductase. X-ray crystallography revealed that FAD binds directly to the flavin trafficking protein, while biochemical investigations suggested that this assembly factor performs Mg²⁺-dependent cleavage of FAD and then transfers and links FMN to a conserved threonine in the NqRc subunit. SdhE does not appear to act as a flavin transferase. Indeed, both the crystal structure presented here (Fig. 1) and prior nuclear magnetic resonance titration analyses indicate that SdhE does not directly interact with FAD.

In contrast, chaperones traditionally interact with hydrophobic residues of a partially folded, or partially assembled, protein in a way that prevents aggregation during maturation. For example, in Saccharomyces cerevisiae, the chaperonin-like Tcm62 responds to heat stress and has been shown to directly bind and stabilize Complex II subunits, which may facilitate assembly. The binding of SdhE between the domains of the unassembled FrdA subunit may exhibit some chaperone-like activity because this interaction shields a hydrophobic surface of the FrdA subunit from solvent during maturation, which could prevent aggregation.

However, SdhE likely has the greatest effect on covalent flavinylation by a third mechanism: shifting the conformational equilibrium of the malleable flavoprotein subunit toward an architecture that favors covalent flavinylation (Figs. 1–3). In this way, SdhE may almost be best classified as a regulatory subunit (or β-subunit) of the FrdA enzyme. SdhE and the Fe:S subunit bind to the same surface of the Complex II flavoprotein subunit (Fig. 1c), hinting at the likelihood that these each promote a distinct biological activity. Indeed, the data presented here identify that SdhE inhibits succinate/fumarate interconversion by FrdA/SdhA subunits, while prior studies evaluating covalent flavinylation in either ΔsdhEΔfrdAB or ΔfrdB/ΔsdhE strains indicate that SdhE is more efficient at promoting flavin attachment than the FeS subunit. Comparison of the FrdA-SdhE structure to the mature FrdABCD structure identifies three major differences in the FrdA subunit that could explain the how the binding partners promote different substrate selectivity and mechanisms. First, while the capping domain position of the FrdA subunit is stabilized by both SdhE and the FeS subunit, the positions are distinct (Fig. 1d). Second, SdhE binding is associated with an active site tunnel, and mutations designed to eliminate this tunnel reduce covalent flavinylation (Fig. 2). Together, these two conformational changes adjust catalytic residues and dicarboxylate on the re-face of FAD, which optimizes a different chemical reaction in each case. Similarly, binding of the FeS subunit and the hydrogen bond to SdhE bind differently the destination ligand, FrdA on the si-face of the FAD (Figs. 1a, 5c). In the case of the SdhE-bound FrdA subunit, the rotation of the FrdA imidazole likely optimizes the approach of N(3) toward the deprotonated 8k-carbon of FAD, facilitating nucleophili attack.

These findings identify that the two regulatory subunits of the Complex II flavoprotein promote distinct substrate selectivity and enzymatic mechanisms. Consistent with this proposal are prior observations that ΔsdhE/ΔfrdAF2 strains and cell lines have reduced, but not eliminated, covalent flavinylation. Here, inherent malleability of the FrdA/SdhA subunits of Complex II enzymes would be required if these are to occasionally sample the conformation required for covalent flavinylation spontaneously. The interaction with SdhE enhances this reaction by shifting the conformational equilibrium to favor an active site architecture supporting flavinylation.

While speculative, this also suggests one plausible mechanism for any enzyme to exhibit substrate and mechanistic diversity, and an intriguing general route for the evolution of modern enzyme superfamilies from a primordial, multifunctional ancestor. One could envision that if different regulatory subunits can modulate the substrate selectivity or mechanism of an enzyme scaffold, these could eventually fuse, resulting in a more selective enzyme. A multidomain architecture with a conserved catalytic domain and a variable regulatory domain has been noted as a hallmark of several major superfamilies, including those that, like FrdA/SdhA, are arranged around a Rossmann fold. A fusion with SdhE or the Fe:S subunit is not anticipated for the Complex II family because correct function requires that both the flavinylation reaction and the succinate/fumarate reaction proceed sequentially in the same molecule.

This proposal for covalent flavinylation also explains the seemingly contradictory reports that the SdhE assembly factor, dicarboxylates, and the Fe:S subunit can enhance covalent flavinylation. While the Fe:S subunit appears to preferentially support succinate/fumarate interconversion, the stabilization of the capping domain may enhance covalent flavinylation above the level found in isolated FrdA subunits. Dicarboxylates have also been shown to stimulate covalent flavinylation. While SdhE and the Fe:S subunit clearly do not work together to assist covalent flavinylation, the structure of the FrdA-SdhE assembly intermediate suggests that dicarboxylates work synergistically with SdhE. Bound dicarboxylate, represented by malonate in the structure of the E. coli FrdA-SdhE complex, may organize the active site side chains to optimize the covalent flavinylation reaction or contribute to proton shuttling.

One previous question in the field was whether dicarboxylate turnover accompanied covalent flavinylation. Prompting this proposal was a combination of the stimulating nature of dicarboxylates and the observation that many missense mutations associated with loss of covalent flavin involve residues important for dicarboxylate turnover. However, there are missense mutations of active site residues that are required for fumarate reduction, but do not impact flavinylation. For example, FrdAH23 helps orient the substrate during fumarate reduction, but has no proposed role in covalent flavinylation. Its mutation in E. coli results in loss of fumarate reduction and succinate oxidation activity, but retention of covalent FAD. Similarly, FrdA24 stabilizes the transition state during fumarate reduction but is excluded from the active site during covalent flavinylation; its mutation in E. coli FrdABCD or SdhABCD substantially impacts catalytic efficiency but does not affect covalent FAD.

Steps of assembly following covalent flavinylation include the disassociation of the FrdA-SdhE complex, the interaction with FrdB, the assembly into the full membrane-spanning complex, and the reoxidation of the FAD. The timing of each of these steps is not currently known. The disassociation of SdhE from the FrdA/SdhA flavoprotein is anticipated to fold the loop regions, close the active site tunnel, release the capping domain and liberate the binding surface for the FrdB subunit such that assembly of the FrdABCD complex can proceed. In eukaryotes, an additional assembly factor termed Sdh8 in yeast (SdhAF4 in humans) is suggested as modest enhancer of Complex II flavinylation and could facilitate this process. No sequence or functional
homologs of SdhB/SdhAF4 have yet been identified in bacteria. It is not yet clear whether bacteria use as-yet undiscovered functional homologs of SdhaF4 to facilitate SdhE release or whether the bacterial assembly intermediate has lower affinity than the human homologs. While this remains to be determined, one possibility is that following the formation of the 8r-N(3)-histidyldisulfide linkage (Fig. 5c), the strain across the newly formed covalent bond helps to dissociate the FrdA-SdhE assembly intermediate. Another aspect of the final maturation of Complex II enzymes is reoxidation of the FAD. Indeed, the product of the attack of the histidyl Nε on the 8-methylene is the reduced FrdAΔ444-FAD cofactor, which requires reoxidation to function. This could proceed by many routes, including a direct interaction with O2, a single-turnover reduction of fumarate, or (if reoxidation follows assembly) the shuttling of e− to the FeS center.

In conclusion, the ability of the FrdA subunit to catalyze both the oxido-reduction of succinate and fumarate (Fig. 3a) and covalent fлавилин (Fig. 3b) reflects substrate and mechanistic promiscuity. In proposing how one enzyme can perform two such distinct reactions, our studies suggest that SdhE acts as a transient avinylation mechanism. This also explains the previous enigmatic and chemical mechanisms.

Methods

Expression and purification of E. coli FrdA and SdhE. Isolated E. coli FrdA subunits were expressed at 37 °C overnight in E. coli DE3 CP-2 cells. Also termed RP437ΔfrdΔsdh, this strain was developed in our laboratory from RP437 with the genes for FrdABCD and SdhABCD disrupted19,46. In this strain, plasmid-encoded RP437 regulatory subunit that pushes the conformational equilibrium of distinct reactions, our studies suggest that SdhE acts as a transient avinylation mechanisms. In addition, it demonstrates how one enzyme can be tuned to catalyze reactions with distinct substrates and chemical mechanisms.

FrdA-SdhE complex formation and purification. The FrdA-SdhE assembly intermediate was formed and purified with a modification of the previously reported protocol19. Purified SdhE-pBzF8 was added to lysate containing FrdA and illuminated with UV radiation for 3 h at 4 °C. The FrdA-SdhE assembly intermediate was then purified by Ni2+ affinity chromatography, which resulted in copurification of isolated FrdA subunits. The complex was therefore additionally purified via size exclusion chromatography in buffer containing 50 mM HEPES pH 7.4.

Crystallization and structure determination. The FrdA-SdhE crosslinked assembly intermediate was crystallized using the hanging drop vapor diffusion method by mixing 1 μL of the FrdA-SdhE crosslinked assembly intermediate (30 mg/mL) in 50 mM HEPES pH 7.4 and 1 μL reservoir solution (50 mM Bis-Tris pH 5.5, 100 mM NaCl, 0.05% PEG 10,000 and 50 mM Na Malonate) and equilibrating over reservoir solution at 22 °C. Crystals were cryoprotected in a solution containing 60% reservoir solution and 40% of 1:1 mix of ethylene glycol and glycerol and then flash cooled by plunging into liquid nitrogen. Data were collected at SRL beamline 9-2 using a Pilatus detector. Unit cell parameters and data collection statistics are listed in Table 1.

The structure was determined using the Phaser47 subroutine in Phenix48 with the isolated flavin-binding domain of the E. coli FrdA subunit (residues 0–232 and 352–375) excised from PDB entry 1KFe9 as a search model. This procedure placed two copies of the flavin-binding domain in each asymmetric unit. Additional molecular replacement searches using the capping domain or SdhE in conjunction with this fixed, partial solution failed to identify solutions for these components.

Nevertheless, inspection of the maps calculated after molecular replacement with the isolated flavin-binding domain revealed electron density consistent with the presence of the capping domain (FrdA residues 233–351). The isolated capping domain from PDB entry 1KFe9 was placed by hand into the maps by superpositioning an intact FrdA subunit onto the flavin-binding domain and performing a rigid-body real-space fit. Electron density calculated after addition of the capping domain to the model showed the presence of density consistent with SdhE. Coordinates for monomeric SdhE from PDB entry 1X6I were placed by hand into the model and the position optimized using a rigid-body real-space fit.

At this point, refinement proceeded by standard methods using alternating rounds of model building in Coot50 and refinement in Phenix48. Final refinement statistics are listed in Table 1.

Detection of in vivo covalent flavilylation. Wild-type or variant FrdA subunits were expressed in aerobic or anaerobic conditions in ΔfrdABCDΔsdbhABCD or ΔfrdABCDΔsdhEABCD strains of E. coli. Cells were harvested by centrifugation and the A260 was used to normalize the protein load for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the whole-cell lysate. The FrdAΔSdhE variant expressed at levels similar to wild-type (Supplementary Table 1). Following separation of the proteins by SDS-PAGE, the gel was incubated for 5 min in 5% (w/v) trichloroacetic acid and then illuminated with UV light to visualize flavin fluorescence covalently associated with the 67 kDa FrdA band.

Spectroscopic analysis of dicarboxylate binding. Studies were performed using a HP8453 UV/Vis spectrophotometer (Agilent, Santa Clara, CA). Binding experiments were carried out in 50 mM Bis-Tris-Propane (pH 8.0) at 25 °C. The isolated flavoproteins were added to a cuvette and titrated by the sequential addition of malonate. The proteins were monitored at 600 nm in 50 mM Bis-Tris-Propane (pH 8.0) at 30 °C in the presence of 10 mM succinate and 50 mM sodium fumarate (i.e., fumarate reduction) was determined by monitoring reoxidation of the previously oxidized QFR, the wavelength used for fumarate was 509 nm, for malonate was 495 nm, for succinate was 454 nm, and for malonate was 454 nm. The isolated capping domain from PDB entry 1KFe9 was placed by hand into the maps by superpositioning an intact FrdA subunit onto the flavin-binding domain and performing a rigid-body real-space fit. Electron density calculated after addition of the capping domain to the model showed the presence of density consistent with SdhE. Coordinates for monomeric SdhE from PDB entry 1X6I were placed by hand into the model and the position optimized using a rigid-body real-space fit.

At this point, refinement proceeded by standard methods using alternating rounds of model building in Coot50 and refinement in Phenix48. Final refinement statistics are listed in Table 1.

Kinetic analysis. Succinate oxidation with dichlorophenolindophenol (DCIP) was monitored at 600 nm in 50 mM Bis-Tris-Propane (pH 8.0) at 30 °C in the presence of 10 mM succinate and 50 μM DCIP. The rate of the flavin reduction was also determined directly following the decrease of FAD absorption at 460 nm upon addition of 5 mM succinate. The rate of FADH2 oxidation by fumarate (i.e., fumarate reduction) was determined by monitoring reoxidation of the flavin following the addition of 5 mM fumarate.

Data availability. Coordinates and structure factors have been deposited with the RCSB Protein Data bank with accession code 6B58. Raw diffraction images have been deposited with SBGrid and can be accessed at doi:10.15785/SBGRID/497. Other data are available from the corresponding authors upon reasonable request.
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**Author contributions**
P.S. purified and crystallized the FrdA-SdhE crosslinked complex, determined the structure, and performed flavinylation analysis of the disulfide-trapped variant. E.M. developed expression protocols and performed kinetic and binding analyses. G.C. and T. M.I. designed and guided the study. The manuscript was written with input from all authors.

**Additional information**
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