Binding of the Covalent Flavin Assembly Factor to the Flavoprotein Subunit of Complex II

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Escherichia coli harbors two highly conserved homologs of the essential mitochondrial respiratory complex II (succinate:ubiquinone oxidoreductase). Aerobically the bacterium synthesizes succinate:quinone reductase as part of its respiratory chain, whereas under microaerophilic conditions, the quinol:fumarate reductase can be utilized. All complex II enzymes harbor a covalently bound FAD co-factor that is essential for their ability to oxidize succinate. In eukaryotes and many bacteria, assembly of the covalent flavin linkage is facilitated by a small protein assembly factor, termed SdhE in E. coli. How SdhE assists with formation of the covalent flavin bond and how it binds the flavoprotein subunit of complex II remain unknown.

Using photo-cross-linking, we report the interaction site between the flavoprotein of complex II and the SdhE assembly factor. These data indicate that SdhE binds to the flavoprotein between two independently folded domains and that this binding mode likely influences the interdomain orientation. In so doing, SdhE likely orients amino acid residues near the dicarboxylate and FAD binding site, which facilitates formation of the covalent flavin linkage. These studies identify how the conserved SdhE assembly factor and its homologs participate in complex II maturation.

Succinate:quinoine oxidoreductase (SQR) is commonly referred to as complex II of the mitochondrial and bacterial respiratory chains. The succinate dehydrogenase activity of the enzyme is essential for the proper functioning of the tricarboxylic acid cycle. In Escherichia coli, SQR is expressed under aerobic conditions, whereas its homolog quinol:quinone reductase (QFR) is expressed under anaerobic or microaerophilic conditions when fumarate is used as an electron acceptor in respiration (1). Membrane-bound SQR and QFR from bacteria and mitochondrial complex II enzymes show a remarkable degree of structural and functional conservation (1–10). Mitochondrial complex II and E. coli SQR and QFR are composed of four distinct protein subunits. The largest subunit (~64–70 kDa) is termed SdhA (in SQR) or FrdA (in QFR), contains the dicarboxylate binding site, and covalently binds a FAD co-factor. The remainder of the enzyme complex is composed of the ~27-kDa SdhB/FrdB subunit, which contains three distinct iron-sulfur clusters, and two membrane spanning subunits, SdhC/FrdC and SdhD/FrdD, which together form the quinone binding site.

Although a considerable amount is known about the structure and catalytic function of complex II less is known about the assembly of the mature enzyme complex. In Saccharomyces cerevisiae, the gene encoding Tcm62 was suggested to be involved in assembly of complex II (11), although its specific role may be to support mitochondrial protein stability under stress conditions (12). Additional complex II assembly factors have been identified in eukaryotes (13–15). One of these, termed SdhAF4 in humans (Sdh8 in yeast), binds to the covalently flavinylated SdhA subunit and promotes assembly of complex II through an unknown mechanism (15). In addition to the assembly factors that interact with the flavoprotein, subunit members of the LYR protein family have been shown to be involved in stabilization and maturation of the SdhB iron-sulfur subunit of complex II (13, 14, 16).

A specific protein assembly factor has been identified that is required for the assembly of the covalent flavin linkage to the SdhA subunit of complex II (17). This assembly factor, termed Sdh5 in yeast and SdhAF2 in humans, is a small protein (mature form, ~119–137 amino acids) found in the mitochondrial matrix (17). In E. coli, a similar protein that has been termed SdhE (88 amino acids) promotes covalent flavinylation of the flavoprotein (SdhA or FrdA) subunits of complex II homologs (18, 19). SdhE was previously termed YgF and is part of the ygfYX operon in E. coli (20). Immunoprecipitation studies show that Sdh5 and SdhE bind to the complex II flavoprotein subunit from both mitochondrial and bacterial sources (17–19).
Although it has previously been suggested that the Sdh5/SdhE flavin assembly factor could play a role in delivery of FAD to the flavoprotein subunit (18) or alternatively that it could act as a chaperone (17), its exact function in the assembly of the covalent flavin linkage or its specific binding site to SdhA/FrdA has not been unambiguously established. Nevertheless SdhE (or its homologs) seems to be required in eukaryotes and many bacteria for covalent flavinylation.

To investigate the molecular mechanism of assembly of the essential covalent flavin linkage in complex II, we developed methods to stabilize the interaction of SdhE with SdhA/FrdA in E. coli. Combining in vivo and in vitro photoaffinity cross-linking methods with mass spectrometry analysis, we identify the surfaces of SdhE and SdhA/FrdA that interact. This allows us to propose the approximate orientation between the proteins. We show that the SdhE binding site is similar to that where the N terminus of the iron-sulfur subunit (SdhB/FrdB) would bind to the flavoprotein in the mature SQR or QFR heterotetramer. The data suggest a model where SdhE and dicarboxylate substrates act in concert to alter the conformation of the flavoprotein subunit (18) or alternatively that it could act as a chaperone (17), its exact function in the assembly of the covalent flavin linkage or its specific binding site to SdhA/FrdA has not been unambiguously established. Nevertheless SdhE (or its homologs) seems to be required in eukaryotes and many bacteria for covalent flavinylation.

Experimental Procedures

Strains, Plasmids, and Bacterial Growth—E. coli strains and plasmids used in this study are described in Table 1. E. coli RP437 is the parent strain for several of the derivatives used in this work. E. coli strain RP-2 (ΔsdhCDAB, ΔfrdABCD) lacks all subunits of SQR or QFR. Its construction was previously described, where its previous designation was RP437ΔsdhΔfrd (21). To construct the triple deletion strain RP-3 (ΔsdhΔfrdΔsdhE), P1 transduction of RP-2 was done following a standard protocol as previously described (21). Briefly, the insertion of the kanamycin resistance gene in the position corresponding to sdhE in the ygfY-ygfX operon was verified by PCR amplification using a forward primer complement to a sequence of the beginning of kanamycin resistance gene (TAT GTC CTG ATA GCG GTC CGC C) and a reverse primer complement to the last 21 base pairs of the ygfX gene (TTA TCT TTG CGT CTC TTG TTG). Then the amplified PCR product (of the expected length) was purified and sequenced with the ygfX reverse primer. DNA sequencing shows insertion of the kanamycin resistance gene in the correct position and confirms deletion of sdhE. A similar procedure was followed to produce the singly knocked out ΔsdhE strain RP-E from RP437 (see Table 1).

The pEVOL-pBpF (catalog no. 31190) and pEVOL-pAzF (catalog no. 31186) vectors were obtained from Addgene (Cambridge, MA). To create pQE-SdhE, the E. coli ygfX operon was synthesized (GenScript) with a 5’ BamHI site and a 3’ Sall site to facilitate cloning into the pQE-80L (Qiagen) vector. A stop codon was introduced after sdhE to prevent synthesis of ygfX. Plasmids pSdhA or pFrdA were constructed by introducing a stop codon after the sdhA or frdA genes from the previously described pFAS (22) and pH3 (23) plasmid vectors, respectively. pQE-SdhE/SdhA and pQE-SdhE/FrdA were constructed for dual expression of SdhE and the flavoprotein subunit of the complex II orthologs. The SdhE protein was expressed from the T5 promoter in pQE-80, and SdhA or FrdA was constitutively expressed by the frd promoter. The constructs were made by a double digestion of pSdhA (see Table 1) with Zral and PshAI restriction enzymes, which yield a blunt end fragment harboring the sdhA gene under control of the Pfrd promoter. This fragment was inserted into pQE-SdhE that had been digested with Zral, yielding the pQE-SdhE/SdhA construct with the correct orientation as verified by sequencing. The FrdA construct was made by removing the SdhA coding sequence from pQE-SdhE/SdhA by digestion with Eco47III and Clal and then inserting the Pfrd promoter and coding sequence for FrdA from pH3 by digestion with Psil and ClaI. The resulting plasmid is termed pQE-SdhE/FrdA and encodes an isopropyl β-D-thioglactopyranoside (IPTG)-inducible SdhE gene with an N-terminal His6 tag and a constitutively expressed native sequence FrdA protein.

Site-directed Variants—Site-directed variants were constructed using the QuikChange II XL site-directed mutagenesis kit (Agilent) with appropriate primers obtained from Eurofins MWG Operon (Huntsville, AL). All mutations and constructs were verified by DNA sequencing (Sequestech, Mountain View, CA).

In Vitro Photo-cross-linking—RP-3 cells were co-transformed with pQE-SdhE/SdhA and pEVOL-pBpF. A single colony was grown overnight in 10 ml of LB medium in a 250-ml flask supplemented with 100 μg/ml ampicillin and 20 μg/ml chloramphenicol at 37 °C and shaken at 225 rpm in a gyrotory shaker. The following morning the culture was diluted to 50 ml with LB supplemented with para-benzoyl-l-phenylalanine (BpF) to a final concentration of 0.2 mM. After continued growth for 90 min, SdhE was induced at 30 °C by the addition of 0.1 mM IPTG and 0.2 mM arabinose. RP-3 cells transformed with pFrdA were grown under microaerophilic conditions for 17 h at 37 °C and 160 rpm. The cells were harvested by centrifugation, washed once with 20 mM potassium phosphate (pH 7.5), and stored at −80 °C. Fractions from cell lysates were obtained after thawing the frozen cells and resuspension in 3 ml of 20 mM potassium phosphate (pH 7.5) with an EDTA-free protease inhibitor mixture (Roche). The suspension was briefly sonicated, and the cytoplasmic fraction was obtained by centrifugation (120,000 × g for 40 min). Photoaffinity cross-linking was performed between soluble fractions of RP-3 cell lysates containing the SdhE amber variants, and SdhA and/or FrdA was placed in 96-well plates (sample volume 100 μl) on ice and exposed to 365-nm light for 1–1.5 h by placement under a Black-Ray 100 Watt UV lamp (UVP). The plate was gently mixed every 15 min, and ice was added as needed to control temperature. As a control, parallel culture samples were left under ambient light for the same time at room temperature. After separation of proteins by SDS-PAGE and blot transfer using a Trans-Blot Turbo transfer system (Bio-Rad), the cross-linked complexes were detected by using murine monoclonal anti-His6 antibody (Aviva Systems Biology; catalog no. OAE00010). For in vivo cross-linking experiments, the freshly collected cells were resuspended in buffer and exposed to UV-light as described above. Where appropriate, similar studies to those described above were done using azidophenylalanine (AzF) and the pEVOL-pAzF amber suppression system.
Expression and Purification of the Hexahistidine FrdA Protein—RP-2 cells were transformed with pQTF-FrdA to produce holo-FrdA. Cells were grown overnight in LB medium containing 100 μg/ml ampicillin at 37 °C with mixing at 225 rpm in a gyrotyor shaker. The following morning, the culture was diluted 10-fold with LB, and growth continued as above for 90 min. Expression of FrdA was induced by the addition of 0.1 mM IPTG, growth continued for 3 h, and then the cells were harvested by centrifugation. The cells were then suspended in 25 mM HEPES (pH 7.5), 30 μM imidazole, 0.2 mM NaCl, and an EDTA-free protease inhibitor mixture. The cell lysate was obtained as above by sonication and applied to a Ni-NTA (Qiagen) gravity column. The column was washed with 25 mM HEPES (pH 7.5), 30 μM imidazole, 0.5 mM NaCl, and the FrdA was eluted with 25 mM HEPES (pH 7.5), 300 μM imidazole, 0.1 mM NaCl. The eluted fractions containing FrdA were concentrated on a Centriprep 30 kDa (Millipore) centrifugal filter following manufacturer's instructions. The concentrated protein was then diluted 10-fold with 25 mM HEPES (pH 7.5), 50 mM NaCl, 5% (v/v) glycerol and concentrated one additional time as above. To remove the N-terminal His₆ tag from FrdA, the purified protein was treated overnight with TEV protease at 10 °C, and the FrdA was collected by passage through a Ni-NTA column as above. The protein was stored at −80 °C.

Analysis of Cross-linked Products by Mass Spectrometry—The samples containing cross-linked products between His₆-tagged SdhE-BpF variants and FrdA were purified on a Ni-NTA mini-column as described above except for the TEV treatment. The ~75-kDa band corresponding to the cross-linked complex of SdhE-R8BpF-FrdA was excised and subjected to in gel trypsin digestion, whereas complexes arising from the SdhE-M17BpF-FrdA were subjected to in-gel chymotrypsin digestion. The resulting peptides were analyzed by a 90-min data-dependent LC-MS/MS analysis. Briefly, peptides were auto-sampled onto a 200 mm × 0.1 mm (Jupiter 3 micron, 300A) self-packed analytical column coupled directly to an LTQ Orbitrap-Velos mass spectrometer (Thermo Fisher) using a nanoelectrospray source and resolved using an aqueous to organic gradient. A series of high resolution Orbitrap full scans followed by five data-dependent MS/MS spectra were collected using both ion trap collision-induced dissociation and Orbitrap higher energy collisional dissociation. Dynamic exclusion was enabled to minimize acquisition of redundant peptide spectra. MS/MS spectra were searched via SEQUEST against an E. coli database that also contained a reversed version for each of the entries. Identiﬁcations were filtered and collated at the protein level using Scaffold (Proteome Software) and confirmed the presence of the specific complex II flavoprotein and SdhE within the excised bands. To identify cross-linked peptides, spectra were extracted into MGF format using MSConvert and probed for BpF cross-links using the StavroX program. Extracted ion chromatograms and modeling of predicted isotopic proportions were performed using Skyline.

Molecular Docking Analysis—Docking was performed in two stages. Initial docking between the E. coli FrdA subunit of QFR (PDB code 1KF6) (24) and SdhE (PDB code 1X6I) (25) with both the distal N and C termini removed was performed using ZDOCK (26). Initial docking calculations constrained SdhE-R8, SdhE-M17, FrdA-M176, and FrdA-E460 as part of the binding surfaces. The top 10 poses were manually curated; the pose that most closely satisfied the cross-linking distances was selected for use as a starting point for high resolution docking in Rosetta v2015.19 (27).

Docking in Rosetta (27) included distance restraints derived from the cross-linking data (with a minimum distance of 9 Å and a maximum distance of 13 Å) between the β-carbons of relevant SdhE-FrdA β-carbons. For the distance between SdhE-R8 and FrdA-M176, this was a single constraint. Because the precise site of cross-linking to the FrdA-456-GLAMEEG-462 peptide could not be unambiguously determined, a series of calculations used each amino acid (excluding the glycines) to estimate the likelihood that the cross-link could be formed at that position. Docking was performed with and without the capping domain of FrdA. The final calculations were performed with the restraint between FrdA-E160 and SdhE-R17, which resulted in 168 poses that satisfied these restraints, all of which effectively formed a single cluster.

In Gel FAD Detection—E. coli RP-3 cells were co-transformed with either pQE-SdhE/FrdA (to express FrdA) or pQE-SdhE/SdhA (to express SdhA) and pEVOL-pBpF. The resulting transformants constitutively express either FrdA or SdhA under microaerophilic growth conditions. The 1-mL cultures of cells were grown overnight in the presence of ampicillin (100 μg/ml) and chloramphenicol (35 μg/ml) in LB medium supplemented with 0.2 mM BpF, 0.2 mM arabinose, and 5 mM IPTG, at 37 °C under microaerophilic conditions. The cells were harvested by centrifugation, resuspended in 80 μl of SDS loading buffer (Bio-Rad), and boiled for 10 min, and then 15 μl of each sample was separated by SDS-PAGE using Bio-Rad Any kDa gels. The gel was incubated in 10% acetic acid, 20% methanol for 5 min for fluorescent detection of covalent FAD. FAD fluorescence was detected using a Safe Imager 2.0 blue light transilluminator (Thermo Fisher Scientific). The ImageJ (National Institutes of Health, Bethesda, MD) program was used for quantification of FAD levels. A similar growth and protein separation protocol was used for Western blots except for the last step of incubation of the gel in the acetic acid/methanol mixture.

Circular Dichroism Spectroscopy—Wild-type SdhE and the SdhE-R8BpF and SdhE-M17BpF variants were expressed and purified by a procedure similar to that for the FrdA-His₆ protein described earlier. Briefly, protein lysate was applied to a Ni-NTA (Qiagen) gravity column followed by gel filtration on a Superdex 200 Increase 10/300 GL column (GE Healthcare). The eluted protein was concentrated with an Amicon ultracentrifugal 3-kDa filter (Millipore) to 0.15 mg/ml in 25 mM potassium phosphate (pH 7.4). Far-UV CD spectra were collected on a Jasco J-810 spectropolarimeter using the Spectra analysis program. Spectra were collected from 190 to 260 nm at 20 °C using a 0.1-cm path length quartz cuvette, with a data point collection interval of 1 degree. Secondary structure was analyzed using the DichroWeb analysis server (28). The data are the averages of two runs and have been corrected for the subtraction of buffer spectra.

Analytical Methods—Protein concentration was determined by the BCA protein assay kit from Pierce. Proteins in
SDS-PAGE gels were stained with Coomassie Blue G (Sigma-Aldrich).

Results

A promising experimental approach toward understanding the mechanism of SdhE-mediated flavinylation and its binding site is to map the interacting surfaces in SdhE and the flavoprotein subunit of complex II. We chose for study the small globular SdhE protein (88 amino acids) to map the sites of SdhE-flavoprotein interaction using surface-exposed residues that can be modified using a TAG Amber codon expression system (29). This system allows genetic incorporation of an unnatural amino acid, such as BpF, which is capable of photo-cross-linking through its photoreactive benzophenone group to interacting protein partners.

Interaction between SdhE-BpF Variants and the SdhA or FrdA Subunits—To investigate the specific site for interaction of SdhE with the flavoprotein subunit of the complex II, site-directed variants of SdhE were constructed. Photoactivatable amino acids were introduced into specific positions of SdhE using suppressor tRNAs encoded by the pEVOL-pBpF and pEVOL-pAzF plasmids (29). The specific sites on SdhE where the photoactivatable amino acids were incorporated are shown in Fig. 1A, with the amino acid numbering used from PDB file (1X6I) for the x-ray structure of SdhE (25). The library of 16 amber variants listed in the legend to Fig. 1 targeted residues of SdhE that are surface exposed in the x-ray crystal structure (25).

This library included amino acids distributed over the entire surface of the protein with a focus on residues within the N terminus, which contains amino acids previously identified to be important for covalent flavinylation (17, 18, 30). Thus, to examine the potential for cross-linking to the SdhA subunit of complex II, the SdhE amber variants were incorporated into plasmid pQE-SdhE/SdhA (Table 1), which contains the sdhA gene constitutively expressed from the FRD promoter (22) under microaerophilic conditions and SdhE expressed from the T5 promoter with an N-terminal His tag. It is known that an N-terminal His tag to SdhE does not interfere with covalent attachment of FAD to SdhA (18).

Each of the pQE-SdhE-Amber/SdhA plasmids was co-transformed with pEVOL-pBpF into E. coli RP-3, which is a triple deletion of sdhCDAB, frdABCD, and sdhE. Following induction (as described under “Experimental Procedures”) of the 16 BpF variants, only the SdhE-A7BpF variant showed consistently impaired protein expression, although it was still capable of supporting covalent flavinylation of SdhA (Fig. 1B). SdhE-H10BpF, SdhE-W11BpF, and SdhE-R14BpF also had a reduction in expression from the IPTG-inducible vector, although only the SdhE-W11BpF variant affected covalent flavinylation (Fig. 1B). The latter result is in agreement with findings from yeast Sdh5, where the loosely conserved similarly positioned residue (Sdh5-Y71 equivalent to SdhE-W11) was substituted with an aspartate residue, which also affected covalent flavinylation in yeast complex II (31). The other 13 amber variants were expressed at levels estimated between 50 and 100% of wild type SdhE based on detection by antibody directed against the N-terminal His tag on SdhE (Fig. 1B). The insertion of BpF into the other SdhE amber variants did not disrupt the formation of the covalent FAD linkage to SdhA with the exception of the SdhE-E19BpF variant (Fig. 1, B and C). This finding is consistent with previous results (30), where it was shown that a SdhE-E19A substitution affects flavinylation to SdhA (Fig. 1C). It can also be observed in Fig. 1B from the expression data on the SdhE-BpF variants that the majority of the proteins show only a single band reactive with the His tag antibody. It is only when wild-type and the SdhE-BpF variants are highly overexpressed

![Fig. 1](https://example.com/fig1.png)

**FIGURE 1.** A, ribbon representation of SdhE (PDB file 1X6I, (25)). The surface amino acids SdhE-N5, -A7, -H10, -W11, -R14, -M17, -R18, -E19, -E29, -H30, -E48, -F55, -H61, -A67, and -R80 were individually substituted with photoactivatable BpF and are shown as red spheres. The conserved N-terminal region is shown in blue. The positions in SdhE where BpF was introduced and proved positive for cross-linking to FrdA are represented as sticks. B, expression levels of SdhE-BpF variants and covalent FAD levels in RP-3 cells co-transformed with pQE-SdhE/SdhA and pEVOL-pBpF. The top panel shows the level of SdhE-XBpF detected by His tag antibody (Ab) following induction of SdhE as described under “Experimental Procedures.” Whole cell lysates equal to 20 μg of protein were used for comparison. The arrow represents full-length SdhE-XBpF. Lanes on the right labeled 51, 61, 67, and 81 were from a different gel treated in an identical manner to those on the left. The lower panel shows the SdhE-XBpF fluorescence of SdhA in the RP-3 cells as described under “Experimental Procedures.” Whole cell lysates equal to 20 μg of protein were used for comparison. %: left, functional evaluation of SdhE-BpF variants for their ability to assist with covalent FAD incorporation into SdhA, functional evaluation of SdhE-BpF variants for their ability to assist with covalent FAD incorporation into SdhA.
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from the IPTG-inducible promoter that a lower molecular weight band is observed, indicating some level of proteolysis of the expressed protein.

Consistent with previous results (18) using a different \( \Delta \text{sdhE} \) strain for expression of \( E. \) coli \( \text{SdhA} \), RP-3 cells showed low levels (~5%) incorporation of covalent FAD into \( \text{SdhA} \) or FrdA (Fig. 1C). When RP-3 whole cells or cell extracts of the \( \text{SdhE-BpF} \) variants were exposed to UV light for 1 h and the proteins were separated by SDS-PAGE, no cross-linking to \( \text{SdhA} \) was observed (data not shown). Because the \( \text{SdhA} \) and FrdA subunits from \( E. \) coli have ~60% sequence homology and incorporation of the covalent FAD into either has been reported to be \( \text{SdhE} \) dependent (18, 19), the studies were repeated using FrdA as the partner to investigate interaction with \( \text{SdhE} \). FrdA can be expressed in both apo and holo forms; however, the apo form is very sensitive to proteolysis under the same conditions where the holo form is stable. Therefore, the cross-linking studies were repeated in RP-2 cells expressing holo-FrdA. As a simple test of the ability to form cross-links with the \( \text{SdhE} \) amber variants, cell lysates expressing holo-FrdA were mixed with lysates from cells expressing the \( \text{SdhE-BpF} \) variants. The soluble cell lysates were then exposed with or without UV light for 1 h at 4 °C. As shown in Fig. 2A, immunoblot analysis using a primary antibody against the His\(_6\) tag identified that the 15 \( \text{SdhE} \) amber variants tested; only the \( \text{SdhE-R8BpF} \) variant shows significant amounts of the ~75-kDa immunoreactive product, consistent with cross-linking to FrdA. To confirm that the cross-link is a result of the direct interaction between \( \text{SdhE} \) and FrdA without any other adapter protein, the same reaction was performed between the isolated proteins. Fig. 2B shows that when holo-FrdA is purified from \( E. \) coli RP-2 cells, the protein cross-links in vitro with purified \( \text{SdhE-R8BpF} \).

It has been previously observed that an \( \text{SdhE-E19A} \) variant affects flavinylation in \( \text{SdhA} \) (30) as confirmed in Fig. 1C (right panel). This same substitution, however, did not affect flavinylation of FrdA (Fig. 1C) (19). To address whether or not the \( \text{SdhE-BpF} \) substitutions of the conserved N-terminal region of \( \text{SdhE} \) also affect FrdA flavinylation, we co-transformed RP-3 cells with \( \text{SdhE-Amber/FrdA} \) grown in the presence of IPTG to induce \( \text{SdhE} \) with FrdA constitutively expressed. As shown in Fig. 1C (left panel), \( \text{SdhE-R14BpF} \) decreases flavinylation of FrdA but not \( \text{SdhA} \). However, when the \( \text{SdhE-R14A} \) variant was tested, no effect on FAD incorporation was observed with either flavoprotein (Fig. 1C, right panel). Overall, these data suggest that these \( \text{SdhE-BpF} \) variants are correctly folded. The reduced FrdA flavinylation observed in \( \text{SdhE-R14BpF} \) (Fig. 1C) suggests that BpF in the \( \text{SdhE-R14} \) position may be oriented toward FrdA and interfere with protein interaction, whereas Arg in this position is not critical for flavinylation because substitution with an alanine residue has no effect on flavinylation.

It should be noted that none of the 16 \( \text{SdhE-AzF} \) variants showed photo-cross-linking to FrdA or \( \text{SdhA} \) under identical conditions. This may result from the brief reaction time (~10^(-4) s) of the nitrene reactive group for the aryl azides (32, 33) compared with benzophenone derivatives. As a result, all subsequent studies were done exclusively with the \( \text{SdhE-BpF} \) variants.

\( \text{SdhE-R8BpF} \) Covalently Cross-links to FrdA-M176—To reveal the identity of the specific amino acid in FrdA that forms a covalent bond to \( \text{SdhE-R8BpF} \), the cross-linked product formed after exposure of cell extracts to UV light was purified on Ni-NTA-agarose (Fig. 2B). In gel, proteolysis was performed on the ~75-kDa protein, which showed the presence of covalent FAD. High resolution full scan (MS1), low resolution collision-induced dissociation, and high resolution higher energy collisional dissociation were collected over the course of a 90-min LC-MS/MS experiment using an LTQ Orbitrap-Velos. These tandem spectra were probe for the presence of BpF cross-linked peptides using the StavroX program. In results from the MS/MS spectra, of the 21 cross-linked peptide candidates that exceeded the 5% FDR threshold, 17 showed a cross-link between the A[BpF]IHWABR peptide of \( \text{SdhE} \) and the GLVAMNMEGTLYQIR peptide of FrdA. There was not unanimity on the site of localization of the cross-link in the second peptide with FrdA-M176 and FrdA-E177 showing similarly plausible cross-links among the highest scoring peptides. (Amino acid numbering for \( E. \) coli FrdA is based on PDB entry 1KF6 (24).) A representative cross-link with FrdA-M176 is shown in Fig. 2A and mass spectral analytic data in Fig. 3 (B and C).

To distinguish between the two possible sites of cross-linking, FrdA-M176 and FrdA-E177 were substituted with Ala (Fig. 4, A and B). The FrdA-M176A variant eliminates the cross-link, whereas the cross-link is still present in the FrdA-E177A vari-

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### Table 1

E. coli strains and plasmids used in study

| Strains/plasmid | Genotype/description | References |
|----------------|----------------------|------------|
| **Strains** | | |
| JW2865-3 | \( \text{rrnB3} \ \Delta\text{lacZ}A74787 \text{IodR514} \Delta(\text{araBAD})567 \Delta(\text{rhaBAD})568 \text{rph-1} \Delta\text{yggY} \) | Ref. 58 |
| RP437 | \( \text{his thr leu met e thi eda rpsL} \) wild type for chemotaxis | Ref. 59 |
| RP-E | \( \text{his thr leu met e thi eda rpsL} \Delta\text{sdhE} \) | This study |
| RP-2 | \( \text{his thr leu met e thi eda rpsL} \Delta\text{sdhA} \) | Ref. 21 |
| **Plasmids** | | |
| pH3 | \( \text{FrdABC}D, \text{frdC} \text{D}^\ast \text{A}^\ast \text{B}^\ast \) operon with natural frd promoter, pBR322 derivative, Amp\( ^\beta \) | Ref. 23 |
| pFAS | \( \text{SdhA} \) expression plasmid, pFAS derivative; \( \Delta\text{sdhCD} \text{sdhA} \Delta\text{sdhB} \), Amp\( ^\beta \) | Ref. 22 |
| pFrdA | \( \text{FrdA} \) expression plasmid, \( \Delta\text{frdBCD} \), Amp\( ^\beta \) | This study |
| pSdhA | \( \text{SdhA} \) expression plasmid, pFAS derivative; \( \Delta\text{sdhCD} \text{sdhA} \Delta\text{sdhB} \), Amp\( ^\beta \) | This study |
| pQE-80L | Expression vector for N-terminal 6 × His protein, Amp\( ^\beta \) | Qiang |
| pQE-SdhE | \( \text{SdhE} \) expression vector, N-terminal 6 × His, pQE-80L-based, Amp\( ^\beta \) | This study |
| pQE-SdhE/SdhA | \( \text{SdhE/SdhA} \) expression vector, N-terminal 6 × His, \( \text{T5} \) promoter for \( \text{SdhE} \), \( \text{SdhA} \), frd promoter, Amp\( ^\beta \) | This study |
| pQE-SdhE/FrdA | \( \text{SdhE/FrdA} \) expression vector, N-terminal 6 × His, \( \text{T5} \) promoter for \( \text{SdhE} \), \( \text{FrdA} \), frd promoter, Amp\( ^\beta \) | This study |
| pQT-FrdA | \( \text{FrdA} \) expression vector, N-terminal 6 × His, TEV proteolytic site, pQE-80L derivative, Amp\( ^\beta \) | This study |
| pQT-SdhA | \( \text{SdhA} \) expression vector, N-terminal 6 × His, TEV proteolytic site, pQE-80L derivative, Amp\( ^\beta \) | This study |

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A.

![Image of diagram](image-url)

**FIGURE 2.** A, photo-cross-linking of SdhE-8BpF to FrdA. Cell lysates containing FrdA and individual SdhE-BpF variants at the indicated position were exposed to UV light for 90 min on ice (+) or left under ambient light (−) under identical conditions. SdhE was detected by Western blotting with anti-His6 antibodies. The −75-kDa band corresponds to the specific FrdA cross-linked product. B, cross-linking between purified SdhE-8BpF and FrdA. Purified holo-FrdA generated in RP2 cells with the His tag cleaved with TEV protease was used for these studies. Isolated proteins were exposed to UV light for 45 min in 25 mM HEPES (pH 7.5). The top panel shows an immunoblot against the His6 tag of SdhE cross-linked to FrdA. The middle panel shows covalent FAD fluorescence where it is evident that the cross-linked product also contains covalent FAD. The bottom panel is the Coomassie Blue-stained gel. The asterisk indicates the cross-linked protein band that was excised from the SDS gel and characterized by mass spectral analysis. The −50 kDa protein that can be seen below native FrdA is a proteolytic fragment of FrdA.

This result supports a defined location for the cross-link between SdhE-R8BpF and FrdA-M176 and suggests that these two residues are adjacent in the biological complex.

**Formation of a Covalent Cross-link between SdhE-R8BpF and SdhA**—The flavoprotein subunits FrdA and SdhA are highly conserved structurally, but the amino acid sequence identity is only ~44% (2, 4, 10, 34). Therefore we wanted to determine whether the lack of cross-linking of SdhE-8BpF to SdhA in our initial experiments reflected the differences in absolute sequence of SdhA and FrdA. For example, both structure and sequence alignment suggests that the residue corresponding to FrdA-M176 is SdhA-E186 (Fig. 4A). Moreover, the photoreactive benzophenone (pBpF) group does not react avidly with all amino acids but shows enhanced reactivity with Met residues (35). Because no cross-linking with SdhA had been observed with SdhE-8BpF, the SdhA-E186 and SdhA-T187 residues were substituted with Met. As seen in Fig. 4B when cellular extracts harboring these variants were exposed to UV light, only the SdhA-E186M variant cross-linked to SdhE-R8BpF. These data indicate that SdhE-R8BpF binds at a similar location in SdhA (E186 in wild type) and FrdA (M176 in wild type). The data also confirm the mass spectral analysis showing that the primary cross-link between SdhE-R8BpF and FrdA is at the FrdA-M176 residue (Fig. 3). The amino acid substitutions in SdhA and FrdA also did not interfere with the ability of the flavoproteins to incorporate FAD covalently (Fig. 4C). When both the SdhA-E186M and SdhE-8BpF proteins were co-expressed from the pQE-SdhE/SdhA plasmid and whole cells were exposed to UV light, photo-cross-linking was also observed in vivo (Fig. 4D).

**Additional Points of Contact between SdhE and FrdA**—To identify additional points of contact between SdhE and FrdA, the strategy of introducing Met residues into selected positions was utilized. Met is expected to have increased efficiency of cross-linking with BpF as compared with other amino acids (35). Because the x-ray structures of SdhE (PDB code 1X6I (25)) and FrdA (PDB code 1KF6 (24)) are known, it is possible to use in silico modeling to suggest other plausible sites for interaction between SdhE and FrdA. Fig. 5A shows a model where SdhE-R8 and FrdA-M176 are in close proximity as would be expected if they form a cross-link. Based on the preliminary modeling, sites for introducing Met residues into FrdA were chosen (FrdA-K130M, FrdA-G206M, and FrdA-S239M) (Fig. 5B). These sites are spatially separated in the x-ray structure (PDB code 1KF6) by ~19 Å between FrdA-K130 and the region of FrdA encompassing FrdA-G206 and FrdA-S239, and 12 Å between FrdA-G206 and FrdA-S239, and 12 Å between FrdA-G206 and FrdA-S239. All Met variants of FrdA had covalently incorporated FAD when grown in *E. coli* RP2 cells (i.e. in presence of chromosomal SdhE), indicating that these substitutions did not interfere with the functional association of SdhE and FrdA (Fig. 5D). Surprisingly, when the SdhE-M17BpF was probed against these FrdA Met-variants, all three of the variant proteins were found to photo-cross-link (Fig. 5C) despite being at positions distributed broadly on the surface of FrdA. To investigate whether the SdhE-BpF variants that proved positive for cross-linking to FrdA were correctly folded, these variants were purified and analyzed by CD spectroscopy (Fig. 5E). It can be seen that the SdhE-R8BpF and SdhE-M17BpF variant proteins showed a spectra similar to wild-type SdhE. This is consistent with a largely α-helical protein as it has been determined by the x-ray and NMR structures of SdhE and yeast Sdh5, respectively (25, 31).

The location of the cross-link between the FrdA-G206M, S239M variants and SdhE-M17BpF was determined by mass spectrometry. In gel chymotrypsin digestion and subsequent mass spectrometry analysis revealed that both FrdA-G206M and FrdA-S239M formed a photo-cross-link to the same FrdA-456-GLAMEEG-462 peptide (Fig. 6, A and B). In FrdA, this
FIGURE 3. Mass spectrometric identification of the photo-cross-linked residue in SdhE-R8BpF/FrdA. A, diagram of cross-linked peptides. BpF substituted amino acid represented as x. The presence of various b and y ions of different charge states within the spectrum is shown with the relative intensity of those fragments indicted by the color spectrum. B, full scan of predicted and actual isotopic peaks for cross-linked peptide. The stacked bar chart represents the predicted and observed proportions of the isotopic peaks. C, higher energy collisional dissociation spectrum with zoomed and annotated insets showing fragment ions corresponding to the cross-linked model shown in A.

FIGURE 4. FrdA and SdhA cross-linking to SdhE-R8BpF. A, ribbon diagram of flavoprotein subunit of complex II. Overlay of the E. coli SdhA subunit (PDB code 1NEK (4)) is shown in gray, and the FrdA subunit (PDB code 1KF6 (24)) is shown in cyan. The region where SdhE-R8BpF cross-links to the wild-type FrdA and SdhE-E186M is shown in the inset. B, SdhE-R8BpF cross-linking to FrdA and SdhA. Immunoblot of the 75-kDa region of SDS-PAGE gels with anti-His6 antibodies. Cell lysates containing SdhA and FrdA wild-type and variant proteins were incubated with lysates from cells harboring SdhE-R8BpF with (+) or without (−) exposure to UV light as indicated in Fig. 1. C, FAD fluorescence of SdhA and FrdA variants. The covalent flavin fluorescence of WT and the SdhA and FrdA variants in cell lysates of RP-2 cells are shown. D, in vivo photo-cross-linking in RP-3 cells expressing His6-SdhE-R8BpF and the SdhA-E186M variant. Immunoblots using anti-His6 tag antibody shows the formation of the cross-linked product in cells that constitutively express SdhA-E186M and IPTG-induced His6-SdhE-R8BpF. The cells from three separate clones were exposed to UV light for 1 h prior to harvesting for immunoblot analysis. The lane with the molecular weight marker proteins between the UV exposed samples (+) and control (−) is removed for clarity because of the excessive signal of the marker proteins.
FIGURE 5. FrdA cross-linking to SdhE-M17BpF. A, molecular model showing juxtaposition of SdhE-R8 (gold sphere) and FrdA-M176 (cyan sphere). The panel on the right is rotated 90°. The FAD co-factor is shown as yellow sticks. B, residues in FrdA chosen for site-directed substitution to methionine. FrdA is positioned to show the proposed interacting surface with SdhE. The three amino acid residues proposed as potential sites for cross-linking to SdhE-BpF variants are shown as orange spheres. Also shown is the FrdA-M176 residue (cyan sphere) demonstrated to cross-link to SdhE-R8BpF. C, cross-linking of FrdA variants to SdhE-R8BpF and SdhE-M17BpF. Extracts from cells expressing wild-type FrdA or its variants were combined with purified SdhE-R8BpF or SdhE-M17BpF and exposed to UV light for 1 h as described in Fig. 2A. D, covalent FAD fluorescence of FrdA variants. The flavin fluorescence of FAD covalently bound to FrdA is shown from the experiment described in C. E, far UV CD spectra of purified SdhE-WT enzyme, and SdhE-R8BpF and SdhE-M17BpF variants. SdhE-WT enzyme data are shown as blue squares, SdhE-R8BpF data are shown as red triangles, and SdhE-M17BpF data are shown as black circles.

FIGURE 6. Mass spectrometric identification of the photo-cross-linked FrdA peptide to the SdhE-M17BpF variant. A, diagram of cross-linked peptides to FrdA-S239M. BpF substituted amino acid represented as x. The presence of various b and y ions of different charge states within the spectrum is shown with the relative intensity of those fragments indicated by the color spectrum. B, full scan of predicted and actual isotopic peaks for cross-linked peptide. The stacked bar chart represents the predicted and observed proportions of the isotopic peaks.
sequence is surface exposed and is a part of a helical bundle that is reasonably spatially close to FrdA-G206 and FrdA-S239 but distant from FrdA-K130. Interestingly, all three FrdA Met variants show a significantly decreased reaction with the SdhE-R8BpF construct (Fig. 5C). This difference in photo-cross-linking reactivity of the FrdA Met variants with SdhE-R8BpF and SdhE-M17BpF indicates that each of these amino acids is likely within the SdhE-FrdA binding interface, and the introduction of the mutation affects the orientation of the two proteins such that the reaction with the relevant amino acid within the 456-GLAMEEG-462 sequence was optimized for chemical attack. The data also suggest that the interaction between SdhE and FrdA or SdhA is dynamic. Thus, SdhE interacts with specific regions of the flavoprotein subunit rather than requiring specific amino acid contacts to induce covalent flavinylation.

**Modeling of the FrdA/SdhE Interaction Based on Two Restraints**—As a result of the screening approach used above, two points of interaction between SdhE and FrdA were identified. SdhE-R8 cross-links with FrdA-M176, and SdhE-M17 cross-links with a residue between FrdA-456–462. Analysis of x-ray structures indicates that in each interaction partner, these positions are anticipated to be surface exposed and are ~20 Å apart. Moreover, the residues in SdhE are within a region that has been shown to harbor residues important for covalent flavinylation (17, 30).

Molecular modeling in the presence of spatially separated distance restraints can suggest both the reciprocal binding surfaces and the relative orientation of the two binding partners. We used two complementary algorithms to calculate possible interaction modes between SdhE and FrdA. The program ZDOCK was first used. ZDOCK allows the choice of specific side chains as a part of the protein-protein interface but does not allow distance restraints between these residues. In the calculations, SdhE-R8, SdhE-M17, and FrdA-M176 were all constrained as being part of the binding surface. The top scoring docks covered the same binding surface on both SdhE and FrdA. The results were manually curated to select the pose that most closely satisfied the cross-linking data. Rosetta was used as a complement to ZDOCK because Rosetta allows the introduction of specific distance restraints. The lengths of the distance restraints for each cross-link were set as 9–13 Å, a value estimated by measuring Cβ-Cβ distance in the crystal structure of the BpF cross-linked complex of the liver oncoprotein gankyrin and C-terminal domain of the S6 proteasomal protein (36). Distance restraints between the β-carbons of SdhE-R8 and FrdA-M176 and the β-carbons of SdhE-M17 and each of the amino acids in the FrdA-456-GLAMEEG-462 peptide were included. These initial docking calculations resulted in few poses. Careful inspection of the ZDOCK model suggested that the position of the capping domain of the FrdA flavoprotein in the crystal structure might prevent satisfaction of the distance restraints. Accordingly, the capping domain of FrdA (residues 234–352 (2)) was removed from subsequent docking calculations. The greatest number of poses was based upon a calculation that used a restraint between SdhE-M17 and FrdA-E460 of the FrdA-456-GLAMEEG-462 peptide, which resulted in 168 poses. These models generated by Rosetta were of striking similarity and effectively formed a single cluster. The effectively single solution may arise from having two widely spaced constraints to define the binding location and lends confidence to the identification of both the interacting surfaces and the orientation of the two proteins in the complex. It is notable that in all of the docked poses, the conserved surface patch of SdhE (RGXXE motif) (30) was oriented in a manner suggesting an interaction with FrdA (Fig. 7A) consistent with previously reported mutagenesis (19, 30). The docking poses also suggest that SdhE interacts with the same surface of the flavoprotein subunit as does the N-terminal domain of the iron-sulfur protein subunit in intact complex II (Fig. 7B).
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**Discussion**

Succinate dehydrogenase was the first example of a protein shown to harbor a covalent flavin linkage (37). For many years the formation of the 8α-N(3)-histidyl-FAD covalent linkage was considered to be an autocatalytic process. Therefore it was of considerable interest when it was first shown for eukaryotes that a specific protein assembly factor is needed to assist with covalent flavinylation in complex II (17). More recently, a bacterial ortholog of the eukaryotic assembly factor that has been termed SdhE was shown to be present in *E. coli* (18). It has been shown that both the eukaryotic and prokaryotic covalent FAD assembly factor associates with the SdhA or FrdA flavoprotein subunit of complex II (17–19, 38). It is not known, however, where the assembly factor binds to the flavoprotein subunit or how the protein assists with covalent flavinylation.

In the present communication, we have determined two different sites where SdhE interacts with the flavoprotein subunit of complex II. This was accomplished by utilizing site-specific *in vivo* incorporation of the artificial amino acid BpF followed by photo-cross-linking to SdhA or FrdA. When substituted with BpF, the residues in SdhE (SdhE-R8 and SdhE-M17) that form covalent cross-links to the flavoprotein are separated by ~19–22 Å. SdhE-R8BpF forms a covalent cross-link to FrdA-M176/SdhA-E186M and SdhE-M17BpF cross-links to the FrdA-M176GLAMEEG_{252} peptide. It should be noted that the SdhE-R8 and -M17 positions flank the conserved region in SdhE that has been shown to be important for covalent flavinylation of complex II flavoproteins (17, 19, 30). Thus, our data are in agreement with an orientation of this conserved region directed toward the flavoprotein subunit. The finding of two separate interacting regions also provides a good opportunity to identify the reciprocal contact surface of the proteins. The region of SdhA/FrdA where SdhE binds is in a helical domain of the flavoprotein subunit (Fig. 8A). When compared with the fully assembled complex II structures, it is apparent that SdhE occupies an area similar to the N-terminal domain of the iron-sulfur protein subunit in the SQR and QFR complexes. Overall, the data presented herein allow suggestion of how the assembly factor assists with formation of the covalent flavin linkage.

A mechanism of how the covalent FAD bond is formed in complex II enzymes was proposed many years before the identification of an associated assembly factor (39, 40). This self-catalytic mechanism was supported by mutagenesis and structural studies with other covalent flavoproteins (41–46). It was also shown recently that covalent FAD attachment to complex II in some hyperthermophilic organisms is self-catalytic and does not require a homolog of the SdhE assembly factor (47). In hyperthermophilic bacteria, the presence of dicarboxylates facilitated formation of the covalent FAD linkage even in the absence of additional protein assembly factors (47). Redox-induced Fourier transform infrared spectroscopy, however, shows that in thermophilic complex II enzymes, the flavin catalytic center has a significantly higher degree of hydrophobicity and structural stability than found in mesophiles such as the *E. coli*, yeast, or mammalian enzymes (48). This suggests that the increased stability of the flavoprotein subunit in hyperthermophiles may contribute to the relative ease of formation of the covalent FAD linkage. In complex II enzymes from both prokaryotes and eukaryotes, the critical amino acid residues thought to be important for catalysis and formation of the covalent FAD bond are conserved (1, 8, 49). Using *E. coli* SdhA as an example (50), SdhA-H45 is where the covalent FAD linkage is formed to the C(8) position of the isoalloxazine ring of FAD. The conserved SdhA-R399 positioned near the C(2) carbonyl of the isoalloxazine ring is thought to stabilize the electrophilic iminoquinone methide (40), and a base near SdhA-H45 activates the C(8α) position. The protein environment near C(8α) involving the dicarboxylate substrates, conserved SdhA-H354, and conserved catalytic residues SdhA-R286, SdhA-E255, and SdhA-R258 all contribute to proper orientation and activation of the flavin isoalloxazine ring to facilitate formation of the covalent FAD bond (51).
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provides tight association between the FAD and capping domains. It is reasonable to assume that during assembly of the complex II flavoprotein subunit like in LASPO, the domain structures of the apo-proteins are preorganized and that FAD binding induces closure of the capping domain (Fig. 8). The suggestion that the FrdA or SdhA subunits are folded prior to flavinylation is also in agreement with circular dichroism analysis of apo- and holo-SdhA from Serratia 39006 (30).

Modeling of the flavoprotein/SdhE interaction, based on the two restraints, positions SdhE at the site where the N-terminal domain of the iron-sulfur protein subunit resides in the fully assembled complex II structure (Fig. 7B). In this position, SdhE would make most of its contacts with a helical region of the flavin domain of the flavoprotein (Fig. 7, A and B). The least number of clashes are observed when the capping domain is in the closed conformation with both FAD and dicarboxylate present. The “open” state of the capping domain is anticipated when flavin is not bound, similar to that seen in LASPO (Fig. 8A). The presence of dicarboxylate substrates or inhibitors also affects the orientation of the capping and flavin domains (6, 7, 55). Indeed, the binding of dicarboxylates to the flavoprotein is known to facilitate both covalent flavinylation and capping domain closure (42, 49, 55). It is apparent that the open position of the capping domain in the apo-flavoprotein would cause a steric clash with SdhE and may negatively affect SdhE binding. Thus, the basic mechanism for how SdhE assists with covalent flavinylation may be described as diagrammed in Fig. 9. The apo-flavoprotein is in equilibrium with FAD, and SdhE binds to and stabilizes the flavoprotein-FAD complex. The binding occurs at a surface helical region of the flavin domain of the flavoprotein (Figs. 7 and 9). We suggest, therefore, that upon binding of SdhE to the flavoprotein, the assembly factor acts as a wedge that locks the capping domain over the dicarboxylate (Fig. 9). This closure of the capping domain may facilitate formation of the covalent flavin linkage by orienting amino acid side chains and protein helices to allow self-catalytic formation of the covalent FAD linkage consistent with a quinone methide mechanism (40, 50, 51).

An intriguing aspect of complex II maturation is that assembly of the membrane-bound enzyme does not require the presence of SdhE or its homologs. It is also apparent that the absence of SdhE also does not affect the expression levels of the complex. In such cases, the mature complexes are formed with tightly bound noncovalent FAD but are incapable of catalyzing succinate oxidation (Fig. 9) (17, 18, 41). As suggested from the model in Fig. 7B because the N-terminal domain of the iron-sulfur protein subunit of complex II binds in a similar region of the flavoprotein as to what we find for SdhE, it might be thought that binding of the iron-sulfur protein to the flavoprotein subunit might be sufficient to induce formation of the covalent flavin linkage. This is clearly not the case because a significant percentage of complex II enzymes require the assistance of the SdhE or its orthologs to induce covalent flavinylation (17, 18, 38, 57). Because stable complexes with noncovalent FAD accumulate in both eukaryotic and prokaryotic cells, it is apparent that once assembled with noncovalent FAD, the flavinylation turnover is inhibited in these complexes. It might be suggested that the structural tuning for the flavoprotein upon binding SdhE or the iron-sulfur subunit may be different. In the former case, the flavoprotein/SdhE interaction is primed for maximal efficiency for the flavinylation reaction. In the latter case, the flavoprotein/iron-sulfur protein interaction has adapted for optimal electronic communication between the FAD and the proximal [2Fe-2S] cluster of the iron-sulfur protein. This is analogous to suggestions for the role of the cytochrome subunit of p-cresol methylhydroxylase from Pseudomonas putida where association of the cytochrome subunit with the apo-flavin subunit facilitates formation of the 8α-O-tyrosyl-FAD covalent linkage in that enzyme (43). In addition, for the P. putida enzyme it was shown that association of the cytochrome subunit with the flavin subunit results in subtle changes in the flavoprotein side chains (44), which would be consistent with what is shown in the model (Fig. 9).

The interaction of the flavoprotein with the iron-sulfur protein subunit (Fp/Ip) or SdhE (Fp/SdhE) can be inferred from the x-ray structures of complex II (Fp/Ip) or by limited co-purification and immunoprecipitation assays (Fp/SdhE) (2, 4, 17, 18, 20). These data suggest that the binding of flavoprotein to the iron-sulfur protein is significantly stronger than that for SdhE.
This raises the question of what prevents premature binding of the iron-sulfur protein to the flavoprotein, which might inhibit the formation of the covalent linkage. One possibility is that SdhE is recruited to the flavoprotein, whereas it is still bound to another assembly chaperone. This could be the general bacterial or mitochondrial chaperone systems (14, 38) or another unknown factor. At least one newly discovered mitochondrial protein termed SdhAF4 was shown to facilitate association of the flavoprotein and the iron-sulfur protein in yeast (15). Whether such a protein is present in bacteria remains to be shown.

Conclusions

We have shown where SdhE interacts with the flavoprotein subunit of complex II by photo-cross-linking. The data suggest that SdhE, along with dicarboxylate substrates, locks the capping and flavin domains to position the FAD co-factor and amino acid side chains into an orientation to facilitate covalent bond formation. Although these studies were done with the E. coli complex II flavoprotein(s) SdhA and FrdA and the SdhE assembly factor, they are likely applicable to eukaryotic complex II. X-ray (25) and NMR (31) structures of SdhE and yeast Sdh5, respectively, show that the proteins adopt identical folds and key residues are conserved. In addition, bacterial and eukaryotic complex II flavoproteins have highly similar x-ray structures (2–6), so it is reasonable to suggest that the binding of SdhE or the eukaryotic Sdh5/SdhAF2 assembly factors to the flavoprotein of complex II and the mechanism by which they work would be similar to what is shown in this communication.

Author Contributions—E. M. designed and carried out experiments and helped write the paper. S. R., C. A. S., W. H. M., and A. K. carried out the experiments described in the paper. M. E. helped edit the paper and oversaw some of the genetic constructions. T. M. I. and G. C. designed experiments, oversaw all aspects of the work, and wrote the manuscript.

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References

1. Cecchini, G. (2003) Function and structure of complex II of the respiratory chain. Annu. Rev. Biochem. 72, 77–109
2. Iverson, T. M., Luna-Chavez, C., Cecchini, G., and Rees, D. C. (1999) Structure of the Escherichia coli fumarate reductase respiratory complex. Science 284, 1961–1966
3. Lancaster, C. R., Kröger, A., Auer, M., and Michel, H. (1999) Structure of fumarate reductase from Wolinella succinogenes at 2.2 A resolution. Nature 402, 377–385
4. Yankovskaya, V., Horzfeld, R., Törnroth, S., Luna-Chavez, C., Miyoshi, H., Léger, C., Byrne, B., Cecchini, G., and Iwata, S. (2003) Architecture of succinate dehydrogenase and reactive oxygen species generation. Science 299, 700–704
5. Sun, F., Huo, X., Zhai, Y., Wang, A., Xu, J., Su, D., Bartlam, M., and Rao, Z. (2005) Crystal structure of mitochondrial respiratory membrane protein complex II. Cell 121, 1043–1057
6. Huang, L. S., Shen, J. T., Wang, A. C., and Berry, E. A. (2006) Crystallographic studies of the binding of ligands to the dicarboxylate site of Complex II and the identity of the ligand in the “oxaloacetate-inhibited” state. Biochim. Biophys. Acta 1757, 1073–1083
7. Huang, L. S., Sun, G., Cobessi, D., Wang, A. C., Shen, J. T., Tung, E. Y., Anderson, V. E., and Berry, E. A. (2006) 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. J. Biol. Chem. 281, 5965–5972
8. Lancaster, C. R. (2002) Succinate-quinone oxidoreductases: an overview. Biochim. Biophys. Acta 1553, 1–6
9. Maklashina, E. and Cecchini, G. (2010) The quinone-binding and catalytic site of complex II. Biochim. Biophys. Acta 1797, 1877–1882
10. Iverson, T. M., Maklashina, E., and Cecchini, G. (2012) Structural basis for malfunction in complex II. J. Biol. Chem. 287, 35430–35438
11. Dibrov, E., Fu, S., and Lemire, B. D. (1998) The Saccharomyces cerevisiae TCM62 gene encodes a chaperone necessary for the assembly of the mitochondrial succinate dehydrogenase (complex II). J. Biol. Chem. 273, 32042–32048
12. Klanner, C., Neupert, W., and Langer, T. (2000) The chaperon-related protein Tcm62p ensures mitochondrial gene expression under heat stress. FEBS Lett. 470, 365–369
13. Na, U., Yu, W., Cox, J., Bricker, D. K., Brockmann, K., Rutter, J., Thummel, C. S., and Winge, D. R. (2014) The LYR factors SDAF1 and SDAF3 mediate maturation of the iron-sulfur subunit of succinate dehydrogenase. Cell Metab. 20, 253–266
14. Van Wranen, J. G., Na, U., Winge, D. R., and Rutter, J. (2015) Protein-mediated assembly of succinate dehydrogenase and its cofactors. Crit. Rev. Biochem. Mol. Biol. 50, 168–180
15. Van Wranen, J. G., Bricker, D. K., Dephoure, N., Gygi, S. P., Cox, J. E., and Fineran, P. C. (2014) Succinate dehydrogenase assembly factor, SDAF4 promotes mitochondrial succinate dehydrogenase activity and prevents neurodegeneration. Cell Metab. 20, 241–252
16. Ghezzi, D., Goffrini, P., Uziel, G., Horvath, R., Krajewski, W., D’Adamo, P., Gasparini, P., Strom, T. M., Proksich, H., Invernizzi, F., Ferrero, I., and Zeviani, M. (2009) SDAF1, encoding a LYR complex-II specific assembly factor, is mutated in SDH-defective infantile leukoencephalopathy. Nat. Genet. 41, 654–656
17. Hao, H., Khalmichuk, O., Schraders, M., Dephoure, N., Bayley, J. P., Kunst, H., Devilee, P., Cremers, C. W., Schiffman, J. D., Bentz, B. G., Gygi, S. P., Winge, D. R., Kremer, H., and Rutter, J. (2009) SDH5, a gene required for flavination of succinate dehydrogenase, is mutated in paraganglioma. Science 325, 1139–1142
18. McNeil, M. B., Clulow, J. S., Wilf, N. M., Salmond, G. P., and Fineran, P. C. (2012) SdhE is a conserved protein required for flavinylation of succinate dehydrogenase in bacteria. J. Biol. Chem. 287, 18418–18428
19. McNeil, M. B., Hampton, H. G., Hards, K. J., Watson, B. N., Cook, G. M., and Fineran, P. C. (2014) The succinate dehydrogenase assembly factor, SdhE, is required for the flavinylation and activation of fumarate reductase in bacteria. FEBS Lett. 588, 414–421
20. Masuda, H., Tan, Q., Awano, N., Yamaguchi, Y., and Inouye, M. (2012) A novel membrane-bound toxin for cell division, CptA (YgJX), inhibits polymerization of cytoskeleton proteins, FtsZ and MreB, in Escherichia coli. FEMS Microbiol. Lett. 328, 174–181
21. Cohen-Ben-Lulu, G. N., Francis, N. R., Shimoni, E., Noy, D., Davidov, Y., Prasad, K., Sagi, Y., Cecchini, G., Johnston, R. M., and Eisenbach, M. (2008) The bacterial flagellar switch complex is getting more complex. EMBO J. 27, 1134–1144
22. Maklashina, E., Berthold, D. A., and Cecchini, G. (1998) Anaerobic expression of Escherichia coli succinate dehydrogenase: functional replacement of fumarate reductase in the respiratory chain during anaerobic growth. J. Bacteriol. 180, 5998–5996
23. Westenberg, D. J., Gunsalus, R. P., Ackrell, B. A., Sices, H., and Cecchini, G. (1993) Escherichia coli fumarate reductase frdC and frdD mutants. Identification of amino acid residues involved in catalytic activity with quinones. J. Biol. Chem. 268, 815–822
24. Iverson, T. M., Luna-Chavez, C., Croal, L. R., Cecchini, G., and Rees, D. C. (2002) Crystallographic studies of the Escherichia coli quinol-fumarate reductase with inhibitors bound to the quinol-binding site. J. Biol. Chem. 277, 16124–16130
25. Lim, K., Doseeva, V., Demirkan, E. S., Pullalarevu, S., Krajewski, W., Galkin, A., Howard, A., and Herzberg, O. (2005) Crystal structure of the YgfY from Escherichia coli, a protein that may be involved in transcrip-
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M. Mewies, W. S. McIntire, and N. S. Scrutton (1998) Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide to proteins: assembly, regulation and role in human disease. FEBS Lett. 393–401

J. Rutter, D. R. Winge, and J. D. Schiffman (2010) Succinate dehydrogenase: assembly, regulation and role in human disease. FEBS Lett. 588, 1058–1063

M. Melin, M. Noor, M. R. Pardeis, E. Boulmedais, F. Banhart, F. Cecchini, G. Soulimane, and T. Hellwig (2014) Investigating the thermostability of succinate: quinone oxidoreductase enzymes by direct electrochemistry at SWNTs-modified electrodes and FTIR spectroscopy. Chemphyschem 15, 3572–3579

M. Iverson, M. Luna-Chavez, C. Schroeder, I. Cecchini, G. Rees, D. C. (2000) Analyzing your complexes: structure of the quinol-fumarate reductase respiratory complex. Curr. Opin. Struct. Biol. 10, 448–455

C. Cecchini, M. Maklashina, E. Iverson, and T. M. (2013) Succinate dehydrogenase (complex II) and fumarate reductase. In Handbook of Flavoproteins. Vol. 2 (Hille, R. Miller, S. and Palfrey, B., eds.) pp. 141–164, Walter de Gruyter GmbH, Berlin

D. Heuts, P. Scrottun, W. S. McIntire, and M. W. (2009) What's in a covalent bond?: on the role and formation of covalently bound flavin cofactors. FEBS J. 276, 3405–3427

T. Tomasiak, T. Maklashina, E. Cecchini, G. Iverson, and T. M. (2008) A threonine on the active site loop controls transition state formation in the attachment of flavin to complex II. J. Biol. Chem. 283, 15460–15468

M. Mattevi, A. Tedeschi, G. Bacchella, L. Coda, A. Negri, A. and R. (1999) Structure of 1-aspartate oxidase: implications for the succinate dehydrogenase/fumarate reductase oxidoreductase family. Structure 7, 745–756

R. Bossi, R. Negri, A. Tedeschi, G. and Mattevi, A. (2002) Structure of FAD-bound 1-aspartate oxidase: insight into substrate specificity and catalysis. Biochemistry 41, 3018–3024

C. Lancaster, C. Gross, R. and Simon, J. (2001) A third crystal form of Wolinella succinogenes quinol:fumarate reductase reveals domain closure at the site of fumarate reduction. Eur. J. Biochem. 268, 1820–1827

D. Robinson, K. M., and Lemire, B. D. (1996) A requirement for matrix processing peptidase but not for mitochondrial chaperonin in the covalent attachment of FAD to the yeast succinate dehydrogenase flavoprotein. J. Biol. Chem. 271, 4061–4067

M. McNeil, M. B., and Fineran, P. C. (2013) Prokaryotic assembly factors for the attachment of flavin to complex II. Biochim. Biophys. Acta 1827, 637–647

B. Baba, A. Ara, T. Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. 2, 2006.0008

J. Parkinson, J. S. (1978) Complementation analysis and deletion mapping of Escherichia coli mutants defective in chemotaxis. J. Bacteriol. 135, 45–53