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Redox State of Flavin Adenine Dinucleotide Drives Substrate Binding and Product Release in *Escherichia coli* Succinate Dehydrogenase

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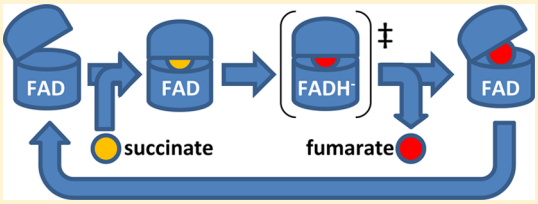
Supporting Information

ABSTRACT: The Complex II family of enzymes, comprising respiratory succinate dehydrogenases and fumarate reductases, catalyzes reversible interconversion of succinate and fumarate. In contrast to the covalent flavin adenine dinucleotide (FAD) cofactor assembled in these enzymes, soluble fumarate reductases (e.g., those from *Shewanella frigidimarina*) that assemble a noncovalent FAD cannot catalyze succinate oxidation but retain the ability to reduce fumarate. In this study, an SdhA-H45A variant that eliminates the site of the 8α-N3-histidyl covalent linkage between the protein and FAD was examined. Variants SdhA-R286A/K/Y and -H242A/Y that target residues thought to be important for substrate binding and catalysis were also studied. The variants SdhA-H45A and -R286A/K/Y resulted in the assembly of a noncovalent FAD cofactor, which led to a significant decrease (−87 mV or more) in its reduction potential. The variant enzymes were studied by electron paramagnetic resonance spectroscopy following stand-alone reduction and potentiometric titrations. The "free" and "occupied" states of the active site were linked to the reduced and oxidized states of FAD, respectively. Our data allow for a proposed model of succinate oxidation that is consistent with tunnel diode effects observed in the succinate dehydrogenase enzyme and a preference for fumarate reduction catalysis in fumarate reductase homologues that assemble a noncovalent FAD.



Succinate dehydrogenase (Sdh, SdhCDAB), also known as mitochondrial Complex II or succinate:quinone oxidoreductase, is an essential Krebs cycle enzyme that couples succinate oxidation to ubiquinone (UQ) reduction. X-ray crystallographic structures of Sdh from *Sus domesticus*,[1] *Gallus gallus*,[2,3] and *Escherichia coli*,[4,5] as well as those of homologous fumarate reductases (Frd) from *Wolinella succinogenes*[6] and *E. coli*,[7−9] show remarkable conservation of tertiary architecture in the Complex II family of enzymes. Succinate oxidation is initialized by a hydride transfer mechanism that donates 2 electrons to a covalent flavin adenine dinucleotide (FAD) cofactor in SdhA.[10] The electrons are then individually funneled through three iron−sulfur clusters in SdhB ([2Fe-2S], [4Fe-4S], and [3Fe-4S]) toward the quinone binding site, where UQ is reduced to ubiquinol (UQH_2). The *E. coli*, avian, and mammalian Sdh enzymes also contain a heme *b* moiety in the membrane anchor domain that is not required for catalysis.[11,12]

In *E. coli*, the paralogues Sdh and Frd play key roles in aerobic and anaerobic respiration, respectively. Although Sdh is optimized for succinate oxidation and Frd for fumarate reduction, the overexpressed enzymes can functionally replace each other in vivo.[13,14] In the *E. coli* paralogues, there is remarkable sequence conservation of residues surrounding FAD, except for the presence of a conserved Gln (Q50) residue in Sdh enzymes that is replaced by a conserved Glu (E49) residue in Frd enzymes, suggesting that directionality of catalysis is partially governed by Coulombic effects.[15] Other important determinants of functional directionality include the electrochemical profile of electron transfer through redox cofactors and the type of quinone species preferentially utilized.[16] In addition, FAD reduction potentials (E_m) may also impact the directionality of the reaction. In the soluble *Shewanella frigidimarina* fumarate reductase, the noncovalent FAD has an $E_{m,7}$ value of −152 mV, and the enzyme can only catalyze fumarate reduction.[17,18] In *E. coli* Sdh and Frd, the FAD cofactor is covalently bound to SdhA-H45 and FrdA-H44, respectively, via an 8α-N3-histidyl linkage.[19,20] Variants of *E. coli* Frd (FrdA-H44Y/C/S/R) and *Saccharomyces cerevisiae* Sdh (Sdh1-H90S) with noncovalently bound FAD lose succinate dehydrogenase activity but retain fumarate reductase activity.[21,22] In a FrdA-H44S variant, the $E_{m,7}$ value of the noncovalent FAD was determined to be −134 mV using protein film voltammetry.[23] The increased $E_{m,7}$ values observed in enzymes with covalently attached FAD (−55 and −79 mV in Frd and Sdh, respectively[24,25]) are believed to be a critical determinant in their ability to catalyze succinate oxidation as

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well as fumarate reduction.[26] In vanillyl-alcohol oxidase, covalent attachment elevates the E_m value of FAD by ~100 mV and increases the rate of catalysis by 1 order of magnitude.[27] These observations support the hypothesis that covalent attachment is responsible for the high E_m values reported for the FAD in both Sdh and Frd, which allows these enzymes to catalyze succinate oxidation.

The specific mechanism of covalent FAD attachment to SdhA remains elusive but is believed to be autocatalytic.[28−30] Proper folding of the apoprotein and the presence of citric acid cycle intermediates appear to be prerequisites for covalent flavinylation.[31] However, recent experiments have shown that recruitment of FAD to the apoenzyme is chaperone-mediated. The Sdh5 protein in *S. cerevisiae*, and subsequently SdhE (previously named YgfY) in bacteria, was the first chaperone identified that is absolutely required for covalent FAD attachment to Sdh and Frd in vivo.[32−34] In humans, individuals carrying germline mutations in the *SDHAF2* gene (equivalent to *Sdh5* in yeast) exhibit a loss-of-function phenotype and have a tendency to develop paragangliomas or pheochromocytomas.[33]

The dicarboxylate binding site is situated adjacent to the FAD molecule and can bind a range of substrates and inhibitors, including succinate, fumarate, oxaloacetate (OAA), malonate, citrate, and 3-nitropropionate (3-NP). A compilation of X-ray crystallographic structures of Sdh with different inhibitors bound reveals a diversity of SdhA-R286 and -H242 side chain conformations, whereas those of other residues show little to no variability. A study of the *W. succinogenes* Frd enzyme showed that the positive charge of FrdA-R301 (equivalent to SdhA-R286) is important for catalysis and covalent FAD attachment.[35] The *E. coli* FrdA-H232S variant (equivalent to SdhA-H242) is unable to oxidize succinate but retains the ability to reduce fumarate.[36] Herein, we examined substrate binding, catalysis, and the importance of covalent flavinylation by studying variants of SdhA-R286, -H242, and -H45 (Figure 1). Using redox potentiometry and electron paramagnetic resonance (EPR) spectroscopy, we report a definitive E_m value for noncovalent FAD in Sdh. We also show that the EPR spectrum of the [2Fe-2S] cluster is sensitive to both inhibitor/substrate binding and the redox state of FAD.

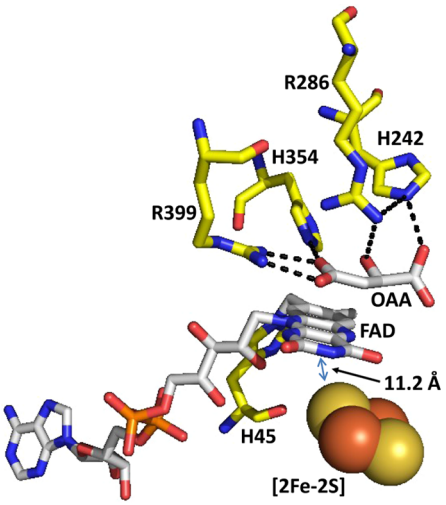

Figure 1. Overview of the dicarboxylic acid binding site in *E. coli* succinate dehydrogenase (PDB entry 2WDQ).[68] Residues that H bond to the cocrystallized inhibitor oxaloacetate (OAA) are shown. SdhA-H45 is the site of covalent FAD attachment. The distance between the isoalloxazine ring and the [2Fe-2S] cluster located in the SdhB subunit is also indicated.

MATERIALS AND METHODS

Strains, Plasmids, and Protein Preparation. The *E. coli* strain TG1 (*supE hsd*Δ5 *thi*Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15], GE Healthcare) was used for mutagenesis. The *E. coli* strain DW35 (Δ*frdABCD*, *sdhC*:*kan*)[37] transformed with pFAS plasmids[14] carrying mutant *sdhCDAB* constructs was used for protein expression and growth studies. Mutations were constructed using primers from Integrated DNA Technologies and the QuikChange protocol from Stratagene. All recombinant plasmids were verified by DNA sequencing. Variant enzymes, isolated as the major component of the cytoplasmic membrane fraction, were malonate-activated as previously described.[11,38] Additionally, the membranes were subsequently pelleted by centrifugation at 100000*g* and resuspended in either 100 mM MOPS/5 mM EDTA (pH 7) or 100 mM tricine/5 mM EDTA (pH 8) to remove the malonate.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE), Covalent Flavin Visualization, and Flavin Estimation. Protein concentrations were estimated using a modified Lowry method[39] by incorporating 1% (w/v) sodium dodecyl sulfate in the incubation mixture to solubilize membrane proteins.[40] To analyze enzyme expression and covalent FAD attachment, we resolved 30 μg of protein on a 12% SDS−PAGE gel followed by visualization using UV fluorescence and Coomassie Blue staining. The unstained gel was washed three times for 2 min in H_2O followed by incubation in 10% acetic acid at pH 3 before visualization by UV irradiation. The intensities of the SdhA bands from Coomassie Blue staining and UV fluorescence were quantified using ImageJ.[41] Fluorometric quantitation of covalent flavin was also carried out in triplicate as previously described.[42] To estimate the relative amounts of assembled noncovalent FAD, 10 μL of 55% trichloroacetic acid was added to 100 μL of membrane preparation containing ~2 μg of protein. After incubation on ice for 15 min, the samples were centrifuged at 10000*g* for 3 min, and the supernatant fractions were collected. Fluorescence intensities at pH 7.0 and 3.3 were used to calculate the relative amounts of noncovalent FAD in each preparation.[42]

Enzyme Assays. Succinate-dependent reduction of 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2*H*-tetrazolium bromide (MTT, ε = 17 mM^{-1} cm^{-1}) was measured spectrophotometrically at 570 nm in the presence of 750 μM phenazine methosulfate (PMS) and 0.1% Triton X-100 as previously described.[43] Fumarate-dependent oxidation of reduced benzyl viologen (BV, ε = 7.8 mM^{-1} cm^{-1}) was monitored at 570 nm with excess sodium dithionite present at the beginning of the assay as previously described.[44]

Redox Titrations and EPR Spectroscopy. Redox titrations were carried out anaerobically under argon at 25 °C on SdhCDAB-enriched membranes in 100 mM MOPS/5 mM EDTA (pH 7). The following redox mediators were used at a concentration of 25 μM: 2,6-dichloroindophenol, toluylene blue, phenazine methosulfate, thionine, methylene blue, resorufin, indigotrisulfonate, indigocarmine, phenosafranine, and neutral red. EPR spectra were recorded using a Bruker Elexsys E500 spectrometer. For studies on the [2Fe-2S] cluster, the sample cavity was cooled to 40 K using an Oxford Instruments ESR900 flowing helium cryostat. For studies on

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the flavosemiquinone and ubisemiquinone radicals, a Bruker liquid nitrogen-evaporating cryostat (Bruker ER4111 VT) was used to maintain the temperature at 150 K. In both cases, a microwave power of 20 mW, a microwave frequency of 9.38 GHz, and a modulation frequency of 100 kHz were used. Modulation amplitudes of 10 and 1 G_{pp} were used for spectra of the [2Fe-2S] cluster and flavosemiquinone, respectively. For preparation of EPR samples, dithionite was used at a final concentration of 2.5 mM succinate plus OAA. Incubations were carried out anaerobically at 23 °C under an argon atmosphere. When appropriate, incubations with succinate and OAA were carried out for 4 min prior to dithionite addition for a subsequent 2 min.

RESULTS

Expression and Flavinylation of Sdh Variants. Using gel electrophoresis, we confirmed that all overexpressed variant enzymes assembled correctly to the cytoplasmic membrane (Figure 2, top). The presence of covalently bound FAD was evaluated by UV transillumination of SDS−PAGE protein gels prior to Coomassie Blue staining (Figure 2, bottom). Protein gels containing the wild-type enzyme show intensely fluorescence bands corresponding to the position of the SdhA subunit, whereas virtually no signal is observed for the SdhA-H45A variant (an enzyme predicted to lack covalent FAD on the basis of studies of variants of *E. coli* Frd and *S. cerevisiae* Sdh).[21,22] Although the SdhA polypeptides in the SdhA-R286A/K/Y variants are clearly visible following staining with Coomassie Blue, their UV fluorescence intensities are negligible compared to that of the wild-type enzyme. The SdhA-H242A/Y variants both exhibit significant fluorescence at the position corresponding to SdhA along with a faint signal from a proteolytic fragment that migrates at a lower molecular weight. In the SdhA-H45A and -R286A/K/Y variant enzymes, which do not assemble covalent FAD, intense fluorescence signals are observed at the dye front, which we interpret as arising from labile noncovalent FAD. Table 1 shows quantitation of the SdhA bands observed in the Coomassie Blue-stained gel presented in Figure 2 as well as relative quantitation of covalent and noncovalent FAD by fluorimetry.

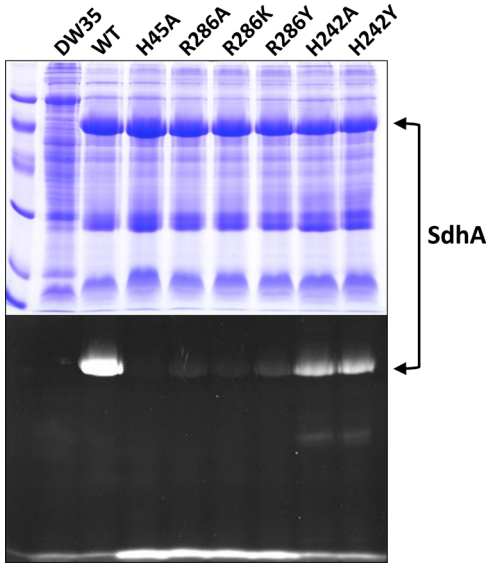

Figure 2. Enzyme assembly and covalent FAD incorporation. Proteins were separated on a 12% SDS−PAGE gel and visualized by either Coomassie Blue staining (top) or UV excitation (bottom). Samples from left to right: low molecular weight standards followed by enriched membrane preparations containing no Sdh (*E. coli* DW35), wild-type Sdh (WT), and SdhA-H45A, -R286A, -R286K, -R286Y, -H242A, and -H242Y enzymes.

As described above, the SdhA-H45A variant has no detectable covalent FAD, whereas the SdhA-R286 variants each contain <4% of covalent FAD. Trichloroacetic acid (TCA) precipitation results in the release of noncovalent FAD from the SdhA-H45A and -R286A/K/Y variants. In the SdhA-H242A and -H242Y variants, ∼30% of the assembled enzyme contains covalently bound FAD, whereas the remainder (∼15−18%) is assembled in the noncovalent form. The data also suggest that a significant number of the SdhA-H242A and -H242Y variant enzymes are assembled with no FAD. Overall, these results suggest that SdhA-R286 plays an essential role in the mechanism of covalent flavinylation and that SdhA-H242 plays a role in its efficiency and completeness.

Impact of Flavinylation on Enzyme Catalysis. In vitro succinate:PMS/MTT and BV:fumarate assays were used to measure the rates of succinate oxidation and fumarate reduction at the dicarboxylate binding site, respectively. All substitutions at SdhA-R286 and -H242, with the exception of the SdhA-H242A variant, essentially abolish succinate oxidase and fumarate reductase activities (Table 1). The SdhA-H242A variant enzyme has BV:fumarate activity that is ∼50% of that of the wild-type enzyme despite showing negligible succinate:PMS/MTT activity. This is similar to the results obtained for an FrdA-H232S variant.[36] The SdhA-H45A variant with noncovalent FAD shows depressed rates of succinate/fumarate interconversion with succinate oxidation affected (79%

Table 1. Assembly and Enzyme Activity of Variant Enzymes

	SdhA[a] (%)	covalent FAD[b] (%)	noncovalent FAD[b] (%)	succinate:PMS/MTT[c] (μmol min^{-1} mg^{-1})	BV:fumarate[c] (μmol min^{-1} mg^{-1})
DW35	0	0	0	0.13	0.07
SdhCDAB	100	100	0	2.40	2.48
H45A	103	0	100	0.52	1.03
R286A	96	3	93	0.19	0.11
R286K	94	2	85	0.12	0.07
R286Y	84	4	77	0.10	0.07
H242A	84	32	18	0.13	1.27
H242Y	74	31	15	0.10	0.15

[a]The relative amounts of SdhA Coomassie Blue staining were quantified from Figure 1 using ImageJ software. [b]The amounts of covalent and noncovalent FAD relative to SdhCDAB and SdhA-H45A, respectively, were quantified by fluorimetry. Standard error is <5% of reported values. [c]Standard error is ±0.05 μmol min^{-1} mg^{-1}.

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decrease) to a larger degree than fumarate reduction (60% decrease). This observation is consistent with flavinylation increasing the $E_{m,7}$ of FAD and rendering it more likely to participate in succinate oxidation.

$E_{m,7}$ of Noncovalent FAD and the [2Fe-2S] Cluster in Wild-Type and Variant Enzymes. Although it is widely assumed that the E_m value of a noncovalent FAD is lower than that of its covalent form, this has never been directly demonstrated in *E. coli* Sdh. We addressed this question using redox potentiometry followed by EPR spectroscopy. In the wild-type enzyme, covalent FAD titrates with an $E_{m,7}$ value of −100 mV (Table 2). In the SdhA-H45A variant, titration of

Table 2. Midpoint Potentials of FAD, the [2Fe-2S] Cluster, and the Occupied/Free Transition at pH 7[a]

	FAD $E_{m,7}$[b] (mV)	[2Fe-2S] $E_{m,7}$[c] (mV)	$E_{FAD\text{-}FS1}$[d] (mV)
wild-type Sdh	−100	−15	−125
SdhA-H45A	−187	−15	−195
SdhA-R286A	−209	−18	−255
SdhA-R286K	−266	−15	−230
SdhA-R286Y	−258	+45	NA
SdhA-H242A	−202	−10	ND
SdhA-H242Y	−207	+40	NA

[a]Samples (200 μL) poised at varying reduction potentials were frozen with liquid nitrogen-chilled ethanol and analyzed by EPR spectroscopy. The error in E_m values is approximately ±10 mV. [b]The 2-electron FAD → $FADH^-$ transition gave rise to a transient radical species at g = 2.00 that was fitted according to methods of Hastings et al.[67] [c]The g_z = 2.03 component was fitted to the Nernst equation to determine the midpoint potential of the [2Fe-2S] cluster. [d]$E_{FAD\text{-}FS1}$ was determined by plotting the height difference between the g = 1.92 "free" component and the g = 1.91 "occupied" component as in Figure 7. NA = not applicable. ND = not determined.

noncovalent FAD yields an $E_{m,7}$ value of −187 mV, a decrease of 87 mV compared to that of covalent FAD in the wild-type enzyme. Titrations of the noncovalent FAD cofactor in the SdhA-R286A/K/Y variants also yield $E_{m,7}$ values that are significantly lower than that of the wild-type enzyme (Table 2). Specifically, the $E_{m,7}$ values of noncovalent FAD in SdhA-R286K and -R286Y are ∼160 mV lower than that of covalent FAD in wild-type Sdh. Titrations of the SdhA-H242A and -H242Y variants yield apparent FAD $E_{m,7}$ values of −202 and −207 mV, respectively (Table 2). However, the intensities of the respective EPR signals in potentiometric titrations are much lower than those obtained for wild-type Sdh (Supplemental Figure 1 in the Supporting Information), suggesting that the $E_{m,7}$ being measured is that of the small amount of noncovalent FAD found in the SdhA-H242 variants with the proportion of covalent flavosemiquinone in these enzymes likely being thermodynamically unstable and thus essentially EPR invisible. Flavosemiquinone instability also likely explains the low signal intensities observed in potentiometric titrations of the SdhA-R286A variant. To gauge the quality of our redox titration data, the E_m values of the ubisemiquinone species were examined as an internal control. In all of the variant enzymes studied, the appearance of the ubisemiquinone species was aligned at an almost identical potential, indicating good reproducibility of the conditions among the titrations (Supplemental Figure 1 in the Supporting Information). Overall, our data demonstrate that noncovalent FAD has an $E_{m,7}$ value that is lower than that of the covalently bound form in Sdh.

The [2Fe-2S] cluster lies in close proximity (∼12 Å) to the isoalloxazine ring of FAD. We therefore explored the possibility that variants of residues surrounding the isoalloxazine might elicit changes in [2Fe-2S] redox chemistry. In agreement with previous studies,[45,46] we obtained a [2Fe-2S] cluster $E_{m,7}$ of approximately −15 mV in the wild-type enzyme. The majority of the variants studied herein exhibited $E_{m,7}$ values that did not differ significantly from that of the wild-type (Table 2). Exceptions to this are the Tyr substitutions of Arg286 and His242, which exhibit $E_{m,7}$ values of +45 and +40 mV, respectively. These results indicate that in some cases, changes in the environment of the FAD can be propagated to the [2Fe-2S] cluster, suggesting that these two centers are electrostatically or conformationally linked in Sdh.

Communication between the FAD and the [2Fe-2S] Cluster. Given the proximity of the FAD isoalloxazine ring to the [2Fe-2S] cluster and the effects of the SdhA-R286Y and -H242Y substitutions on the E_m of the latter, we hypothesized that substrate or inhibitor binding might elicit changes in the EPR line shape of the cluster. Recently, a study of the *Thermus thermophilus* Sdh enzyme showed that reduction of the enzyme by succinate yielded a reduced [2Fe-2S] EPR signal with higher rhombicity at the g_{xy} feature.[47] Figure 3 shows that when

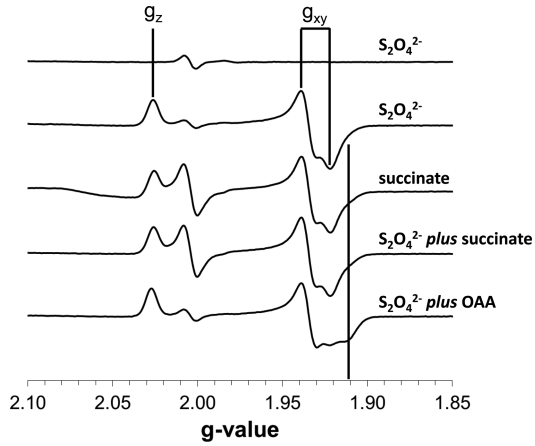

Figure 3. The EPR line shape of the [2Fe-2S] cluster changes upon inhibitor binding. Spectra were of the following samples (as labeled in the figure): dithionite reduction of membranes prepared from DW35 cells lacking SdhCDAB; membranes enriched in wild-type SdhCDAB reduced with dithionite, succinate, dithionite plus succinate, and dithionite plus OAA. The g = 1.91 feature is marked for comparison.

reduced with dithionite, the *E. coli* SdhCDAB [2Fe-2S] cluster EPR signal is comprised of a peak at g_z = 2.03 and a multicomponent peak-trough at g_{xy} = 1.94 and 1.92. When the enzyme was solely reduced by succinate, or by dithionite in the presence of succinate, a small shoulder at g = 1.91 became apparent. When the enzyme was reduced with dithionite in the presence of OAA, the g = 1.91 feature became more prominent and in fact became a local trough, giving the g_{xy} component a more rhombic feature that is reminiscent of the spectrum of the succinate-reduced [2Fe-2S] cluster in the *T. thermophilus* enzyme.

Figure 4 shows the reduced EPR spectra of the [2Fe-2S] clusters in the variant enzymes studied herein. The SdhA-H45A variant gave rise to a noticeable change in the EPR line shape of the [2Fe-2S] cluster; it results in the disappearance of the g = 1.92 trough, which is replaced by a trough at g = 1.91. Similarly, when the SdhA-H45A variant is reduced by succinate or by

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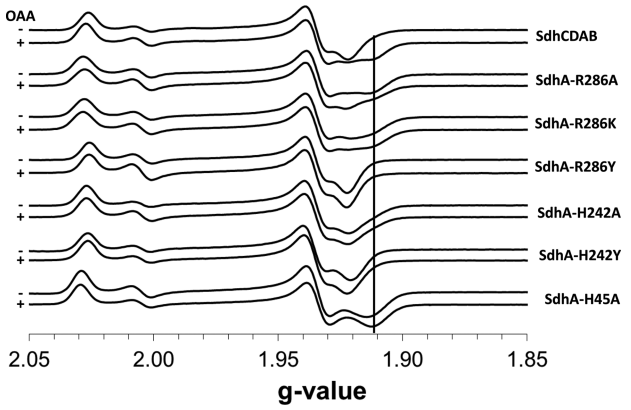

Figure 4. EPR line shapes of [2Fe-2S] clusters in variant enzymes. Each pair of spectra were obtained by reducing the indicated enzymes with 2.5 mM dithionite in the absence (−, top) or presence (+, bottom) of 25 mM oxaloacetate (OAA). The g = 1.91 feature is marked for comparison.

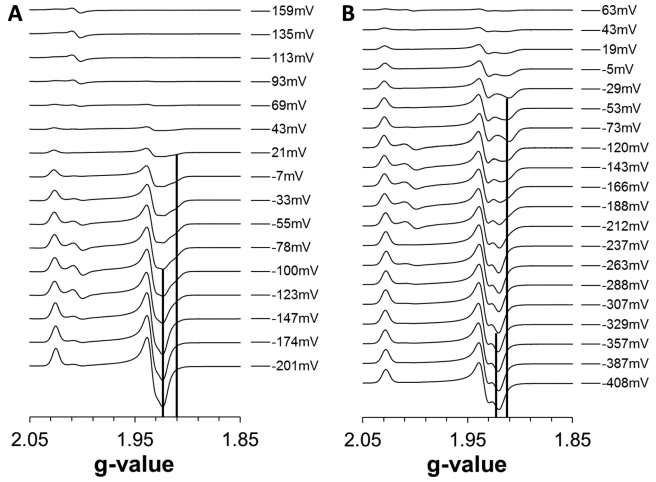

Figure 5. Redox titrations of the [2Fe-2S] cluster. Representative EPR spectra from redox titrations of the (A) wild-type SdhCDAB enzyme and (B) SdhA-H45A variant are shown. The positions of the g = 1.91 and 1.92 troughs are indicated by vertical lines.

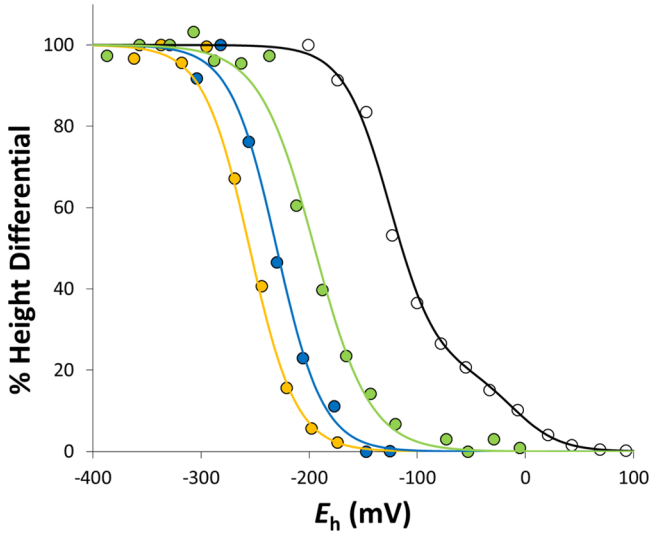

Figure 6. Redox-dependent interplay between the g = 1.91 and 1.92 components of the [2Fe-2S] cluster EPR spectrum. The height differentials between the g = 1.91 and 1.92 components of the [2Fe-2S] cluster EPR signal as a function of ambient potential were plotted for wild-type SdhCDAB (black), SdhA-H45A (green), SdhA-R286A (yellow), and SdhA-R286K (blue) and fitted to the Nernst equation. For titration of the wild-type SdhCDAB enzyme, a second component corresponding to reduction of the [2Fe-2S] cluster itself was also modeled.

dithionite in the presence of either succinate or OAA, a trough is observed at g = 1.91, and the g = 1.92 trough is absent (Figure 4, Supplemental Figures 2 and 3 in the Supporting Information). In the case of the SdhA-R286A and -R286K variants, dithionite reduction results in a spectrum with a significant g = 1.91 feature that appears to be unaffected by the presence of succinate or OAA (Figure 4, Supplemental Figures 2 and 3 in the Supporting Information). The [2Fe-2S] cluster in the SdhA-H242A variant behaves in a similar fashion, but the magnitude of the g = 1.91 feature is moderated such that a steady slope is observed under all reducing conditions. In stark contrast to this, the EPR line shape of the reduced [2Fe-2S] cluster in the SdhA-R286Y and -H242Y variants shows absolutely no change under the different reducing conditions, and the appearance of the g = 1.91 feature could not be manifested using succinate or OAA (Figure 4, Supplemental Figures 3 and 4 in the Supporting Information).

Surprisingly, the interplay between the native g = 1.92 trough and the inducible g = 1.91 trough is also present during redox titrations in which substrates and inhibitors are absent. As noted above, the g = 1.91 feature appears when the wild-type enzyme is reduced in the presence of substrate or inhibitor. In redox titrations of the wild-type enzyme, the magnitudes of the g = 1.92 and 1.91 features are potential dependent (Figure 5A). The height differences between these two trough signals can be plotted against the ambient potential to yield a Nernstian relationship with a midpoint potential ($E_{FAD\text{-}FS1}$) of −125 mV (Figure 6, Table 2). Interestingly, in the SdhA-H45A variant, this value decreases to −195 mV (Figure 5B, Table 2). In the SdhA-R286A and -R286K variants, the $E_{FAD\text{-}FS1}$ value decreases even further to −255 and −230 mV, respectively (Table 2). In redox titrations of the paralogous enzyme FrdABCD, the transition between g = 1.92 and 1.91 is not observed (Supplemental Figure 4 in the Supporting Information).

DISCUSSION

In the Complex II family of enzymes, the covalent attachment of a FAD moiety via an 8α-N3-histidyl linkage is absolutely conserved. How is the FAD cofactor recruited to the apoenzyme, and how does flavinylation proceed thereafter? For SdhCDAB, it was recently discovered that the SdhE chaperone directly interacts with the SdhA subunit to mediate flavinylation,[32,48] and the absence of the equivalent SdhAF2 chaperone in humans results in paraganglioma tumorigenesis.[33] Following the insertion of FAD into its binding pocket and repositioning of the capping domain, covalent attachment is thought to be an autocatalytic process, a common theme that is conserved in other flavoenzymes.[49−51] In this study, we show that the SdhA-R286 residue is absolutely essential for flavinylation. In a prior study on *W. succinogenes* fumarate reductase, covalent attachment of FAD was abolished in an FrdA-R301E variant (equivalent to residue SdhA-R286) but was retained in an FrdA-R301K variant.[35] Preliminary studies on an FrdA-R287K variant suggested that covalent attachment of FAD was retained (unpublished data, Maklashina et al.). Interestingly, all three substitutions of SdhA-R286 studied

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herein resulted in assembly of noncovalent FAD, including the Lys variant. It has been proposed that the guanidinium side chain of the conserved SdhA-R286 residue can H bond to and stabilize the substrate in the dicarboxylate binding site and act as a proton donor for fumarate reduction and proton acceptor during succinate oxidation.[9,15,35,52] For comparison, the imidazole ring of SdhA-H242 plays a more limited role by H bonding to the substrate and is not believed to be involved in proton shuttling during catalysis. The observation that the SdhA-H242Y variant enzyme does not turn over but can still incorporate a covalent flavin suggests that substrate binding (see below for discussion on substrate binding), but not enzyme turnover, is necessary for flavinylation. A study on the *Bacillus subtilis* Sdh enzyme has also shown that residues not involved in catalysis or substrate binding can hinder FAD recruitment and flavinylation.[53]

Removal of the covalent linkage via substitution of the His residue results in elimination of succinate dehydrogenase activity and partial retention of fumarate reductase activity.[21,22] It is widely believed that covalent attachment increases the E_m value of the FAD cofactor by ~100 mV to facilitate electron transfer between its isoalloxazine ring and succinate/fumarate.[16,21,54] This assertion is based on two key observations. First, mutational studies on other flavoenzymes, such as vanillyl-alcohol oxidase[27] and cholesterol oxidase,[55] demonstrated that removal of the covalent linkage resulted in a ~100 mV decrease in the E_m value of the FAD. Second, in the homologous soluble fumarate reductase from *Sh. frigidimarina*, the FAD is assembled in the noncovalent form and has an E_m value that is ~100 mV lower than that of the covalent FAD in *E. coli* Frd.[56] In this study using redox potentiometry on the SdhCDAB system, we show definitively that noncovalent FAD in an SdhA-H45A variant does indeed have a decreased midpoint potential ($E_{m,7}$ = −187 mV) relative to that of the wild-type SdhCDAB enzyme ($E_{m,7}$ = −100 mV). We also showed that the E_m value of the covalent FAD can be lowered via SdhA-H242A and -H242Y mutations. In agreement with similar studies, the SdhA-H45A and -H242A variants show that decreasing the E_m value of the FAD results in loss of succinate oxidase and fumarate reductase activities with the former affected to a greater degree. When we examined whether the [2Fe-2S] cluster can be reduced by succinate, we noted that there was enough succinate oxidase activity to elicit significant reduction (Supplemental Figure 2 in the Supporting Information). In the case of the SdhA-H242Y variant, the lack of succinate-dependent [2Fe-2S] cluster reduction may be partially explained by its E_m value being increased relative to that of the wild-type enzyme (+40 mV versus −15 mV, respectively).

Our observations that substrates and inhibitors can alter the EPR line shape of the reduced [2Fe-2S] cluster enabled us to explore how it communicates with the substrate binding site. A recent study on *T. thermophilus* Sdh showed that specific reduction of the enzyme by succinate caused the g_{xy} component of the [2Fe-2S] cluster signal to become more rhombic.[47] Using the variant enzymes herein, we extended this study to include OAA. As detailed in the Results, a feature at g = 1.91 became apparent when succinate was used as the sole reductant or was used in conjunction with dithionite to reduce the enzyme. This trough feature became even more apparent when the enzyme was preincubated with a strong inhibitor such as OAA (K_i = 70 nM)[15] (Figure 3). In agreement with the *T. thermophilus* study, we believe also that the EPR line shape changes are suggestive of two distinct conformations of the enzyme: an "occupied" state with substrate/inhibitor bound (g = 1.91 trough) and a "free" state without substrate/inhibitor bound (g = 1.92 trough), which are in rapid equilibrium. This is consistent with the hypothesis that structural changes, especially of the hinge region and the capping domain, occur to allow for opening and closing of the active site during catalysis.[5,9,26,57]

Reduction of the SdhA-R286A, -R286K, and -H242A variants using dithionite alone elicited the appearance of the g = 1.91 feature, suggesting that these variant enzymes are at least partially present in a state that mimics the occupied state of the wild-type enzyme. Additionally, the lack of change observed in their EPR spectra when succinate and OAA were added before reduction by dithionite also indicates that these variant enzymes are in the occupied state, and that they are tightly locked in this conformation. Interestingly, Tyr substitutions of both SdhA-R286 and -H242 resulted in the absence of the g = 1.91 feature under all conditions. One possible explanation to account for this observation is that the Tyr side chain forms a strong H bond with SdhA-E255, preventing entry of the substrate into the active site and locking it in the free state. But, given that substrate binding is absolutely necessary for meaningful flavinylation and that ~31% of the assembled SdhA-H242Y variant contained covalently attached FAD (Table 1), it is possible that only this proportion of enzyme was in a substrate binding competent comformation during maturation. Indeed, OAA binding was observed, but to a lesser extent, in an FrdA-H232S variant (equivalent to SdhA-H242).[36] A more likely explanation is that the Tyr side chain raises the pK_a of the g_{xy} components of the [2Fe-2S] cluster EPR signal. In *T. thermophilus* Sdh, the axial signal representing the free state occurs when pH < pK_a, and the rhombic signal representing the occupied state occurs when pH > pK_a.[47] If the pK_a were indeed raised by Tyr substitutions of SdhA-R286 and -H242, then the equilibrium at any given pH would be shifted toward the free state, which is the form that we observed exclusively for these two Tyr variants. By analogy, because the g = 1.91 trough was the only one observed for the SdhA-H45A variant, the noncovalent FAD must decrease the pK_a of the "free" ↔ "occupied" transition such that the "occupied" form becomes dominant at pH 7 in our experiments. Thus, one important role of protein flavinylation is to maintain the pK_a of the free/occupied transition near physiological pH to allow enzyme turnover in vivo.

The free/occupied transition became even more intriguing when we examined the EPR spectra from redox titrations. In the wild-type SdhCDAB enzyme, a hint of the g = 1.91 feature was noticeable at potentials above approximately −150 mV. When the ambient potential was further decreased, this feature slowly disappeared concurrent with an increase in signal intensity of the trough at g = 1.92. The height difference between the g = 1.91 and 1.92 troughs can be fitted to a Nernstian function with a midpoint potential of −125 mV (Figure 6 and Table 2). Thus, the switch between the occupied (g = 1.91) and free (g = 1.92) states for the wild-type SdhCDAB enzyme occurs at −125 mV, which we have termed $E_{FAD\text{-}FS1}$. In the SdhA-H45A variant with a noncovalent FAD, the $E_{FAD\text{-}FS1}$ value was decreased to −195 mV, which approximately equals the decrease observed for the $E_{m,7}$ value of a noncovalent FAD versus its covalent form. Similarly, in the SdhA-R286A and -R286K variants, the $E_{FAD\text{-}FS1}$ values were further decreased, which is also consistent with the decreases

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observed for their noncovalent FAD moieties. Altogether, the EPR data suggest that the $E_{FAD\text{-}FS1}$ value, which reports the transition between the "occupied" and "free" states of the active site, is dependent on the redox state of the FAD cofactor.

On the basis of this newfound knowledge, we constructed a simple model to visualize succinate oxidation at the FAD binding domain (Figure 7). First, the oxidized enzyme assumes

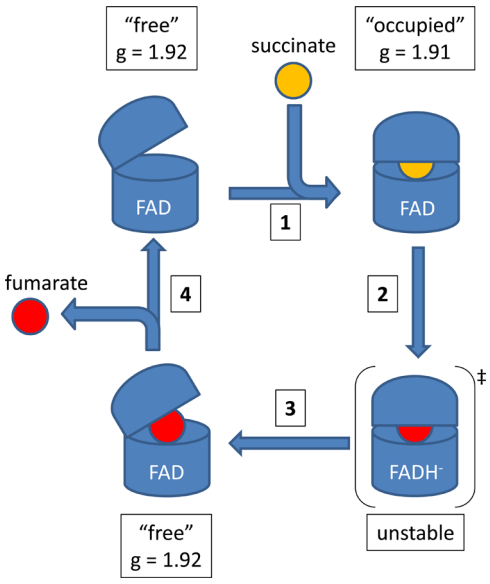

Figure 7. Proposed model for succinate oxidation and fumarate release. (1) Succinate binds to the enzyme to induce a conformational change from the "free" to the "occupied" form. (2) Succinate is oxidized, and FAD is reduced to $FADH^-$. (3) The high energy "occupied"/$FADH^-$ transition state drives the conformational change to the "free" form. (4) Fumarate is released from the "free" form, and electrons are passed to SdhB.

a conformation in which the dicarboxylic acid binding site is "free" (g = 1.92). Next, succinate binds and induces the "occupied" conformation (g = 1.91). Succinate is then oxidized to fumarate, and 2 electrons are transferred to the FAD via a hydride transfer. The combination of the "occupied" form and a reduced $FADH^-$ molecule represents a high energy transition state that can drive conformational change back to the "free" form, resulting in product (fumarate) release. Alternatively, oxidation of $FADH^-$ by the [Fe-S] clusters in SdhB may also induce a conformational change to the "free" form. In *E. coli* Sdh, movements of the capping and FAD binding domains are important for activating the C2−C3 bond of fumarate/succinate to attain the transition state.[5,9] In X-ray crystallographic studies of *W. succinogenes* fumarate reductase, the capping domain has been captured at different positions relative to the FAD binding domain, hinting at structural changes that occur during enzyme turnover.[35,58] In spite of this, the isoalloxazine ring always assumes a planar conformation in all available X-ray crystal structures of Complex II enzymes, indicating that the reduced $FADH^-$ form is highly unstable. In studies of FAD in solution and in other flavoenzymes, reduction of the isoalloxazine ring (involving the N5−C4a−C10a−N1 atoms) results in a bent structure in solution or leads to a conformational change in the host protein.[59−63] A previous study has shown that the biophysical properties of FAD were altered depending on the redox state of the [2Fe-2S] cluster.[25] The EPR data on the [2Fe-2S] cluster presented in this study provides complementary data that strongly suggests that a conformational shift between the "free" and "occupied" states occurs when the redox state of FAD is altered.

The proposed mechanism of succinate oxidation, similar to the one proposed in 1992,[64] has important ramifications for enzyme catalysis. First, another succinate molecule cannot bind until the $FADH^-$ cofactor has distributed its electrons to the iron−sulfur clusters in SdhB. However, this should not limit the rate of catalysis because electron transfer is orders of magnitude faster than the rates of fumarate release and succinate binding. Second, the gating mechanism presented by the redox state of the FAD cofactor implies that the reversible reactions of succinate oxidation and fumarate reduction do not occur as mirror images of each other. In the voltammetry studies performed on the SdhAB dimer, the soluble enzyme exhibits tunnel diode behavior in the presence of fumarate (the rate of fumarate reduction actually decreases with increasing driving force).[64−66] Further, the studies showed that the dissociation constant for the substrate−FrdAB complex when FAD is reduced is highest compared to those of its semiquinone and quinone forms.[66] Although the data presented herein do not directly explain the tunnel diode mechanism, there is undeniably a strong connection: both the $E_{FAD\text{-}FS1}$ transition and the E_{switch} potential at which the tunnel diode effect is turned on closely match the E_m of the FAD.

The model presented in Figure 7 incorporates these findings and aligns with tunnel diode behavior; that is, when the flavin is reduced (i.e., higher driving force), the equilibrium is shifted toward the "free" state, preventing fumarate binding and its subsequent reduction. In variant enzymes (and soluble *Sh. frigidimarina* fumarate reductase) that assemble a noncovalent FAD, two factors come into play. First, the E_m value of the FAD cofactor is decreased, resulting in lower occupancy of FAD by electrons, which makes fumarate binding and reduction favorable. Second, although the "free" state with oxidized FAD also makes succinate binding more favorable, the succinate/fumarate coupling of +30 mV is energetically incapable of driving FAD reduction (−185 mV or lower for noncovalent FAD). This explains why fumarate reductase activity but not succinate dehydrogenase activity is preferentially retained when the E_m value of the FAD is decreased.

SUMMARY

Through a series of EPR experiments, we have provided, for the first time, a definitive midpoint potential of the noncovalent FAD cofactor in the SdhCDAB enzyme. Noncovalent FAD does indeed have an $E_{m,7}$ value that is decreased ($\Delta E_{m,7}$ = −87 mV) compared to that of its covalent counterpart in the wild-type enzyme. Substitutions made at the SdhA-R286 position resulted in enzymes that assembled noncovalent FADs and were incapable of catalysis. EPR spectroscopy also suggested the existence of two conformational states, "free" and "occupied", that are tightly coupled to the redox state of the FAD and reduction of the [2Fe-2S] cluster. A model for succinate oxidation was also presented that takes into account the tunnel diode behavior of Sdh and the preference for fumarate reduction in variant enzymes that lack covalent flavin.

ASSOCIATED CONTENT

Supporting Information

Redox titrations of variant enzymes, EPR line shapes of [2Fe-2S] clusters with and without dithionite, and EPR spectra from

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redox titration of FrdABCD. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

FAD, flavin adenine dinucleotide; Frd, fumarate reductase; OAA, oxaloacetate; Sdh, succinate dehydrogenase; SdhCDAB, succinate:ubiquinone oxidoreductase (bacterial, e.g., *E. coli*); UQ, ubiquinone; UQH_2, ubiquinol

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