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The Conserved RGxxE Motif of the Bacterial FAD Assembly Factor SdhE Is Required for Succinate Dehydrogenase Flavinylation and Activity

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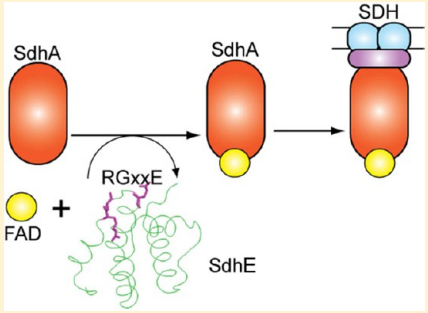
Supporting Information

ABSTRACT: Succinate dehydrogenase (SDH) is an important respiratory enzyme that plays a critical role in the generation of energy in the majority of eukaryotes, bacteria, and archaea. The activity of SDH is dependent on the covalent attachment of the redox cofactor FAD to the flavoprotein subunit SdhA. In the Gram-negative bacteria *Escherichia coli* and *Serratia* sp. ATCC 39006, the covalent attachment of FAD to SdhA is dependent on the FAD assembly factor SdhE (YgfY). Although mechanisms have been proposed, experimental evidence that elucidates the molecular details of SdhE-mediated flavinylation are scarce. In this study, truncation and alanine swap mutagenesis of SdhE identified a highly conserved RGxxE motif that was important for SdhE function. Interestingly, RGxxE site-directed variants were not impaired in terms of protein folding or interactions with SdhA. Purification and analysis of SdhA from different mutant backgrounds demonstrated that SdhE interacts with and flavinylates folded SdhA without a requirement for the assembly of the entire SDH complex. SdhA was also partially active in the absence of SdhE, suggesting that SdhA is able to attach FAD through an inefficient autocatalytic mechanism. The results presented are of widespread relevance because SdhE and SDH are required for bacterial pathogenesis and mutations in the eukaryotic homologues of SdhE and SDH are associated with cancer in humans.



Succinate dehydrogenase (SDH), also known as complex II or succinate-ubiquinone oxidoreductase, is an important respiratory enzyme in the majority of eukaryotes, bacteria, and archaea that participates in both the electron transport chain and tricarboxylic acid cycle.[1] SDH consists of four distinct protein subunits that function by coupling the oxidation of succinate to fumarate with the reduction of the mobile electron carrier ubiquinone to ubiquinol.[1,2] The catalytic flavoprotein subunit, termed SdhA, contains a covalently bound redox cofactor flavin adenine dinucleotide (FAD) that is required for enzyme activity.[2−4] Under aerobic conditions, the FAD moiety catalyzes succinate oxidation and is itself reduced to $FADH_2$. Differences in redox potential move electrons from the reduced $FADH_2$ through Fe−S clusters in SdhB to ultimately reduce ubiquinone at the ubiquinone binding site shared by SdhC and SdhD.[2,5]

Many flavoproteins that covalently bind FAD do so through an autocatalytic mechanism that is not dependent on any accessory proteins or assembly factors.[6−11] However, work in yeast examining the function of a conserved hypothetical protein, termed Sdh5, demonstrated that Sdh5 was required for the activation and flavinylation of the SDH flavoprotein subunit homologue, Sdh1.[3] Subsequent analysis led to the identification of homologues first in bacteria[4] and later in plant species,[12] termed SdhE and SdhAF2, respectively, which are also required for the flavinylation of SDH. Consequently, this protein family represents the first example of a family of FAD assembly factors. SdhE proteins are associated with bacterial pathogenicity,[4] root elongation in *Arabidopsis thaliana*,[12] and cancer in human populations,[3,13,14] demonstrating their cellular importance. Phylogenetic analysis of these proteins has led to the model in which they arose only once in an ancestral α-proteobacterium prior to the evolution of mitochondria and are functionally conserved in eukaryotic and bacterial descendants.[4,15]

Biochemical analysis of SdhE in the Gram-negative bacterium *Serratia* sp. ATCC 39006 (*Serratia* 39006) and Sdh5 in the yeast *Saccharomyces cerevisiae* demonstrated that they interact with the flavoprotein subunits SdhA and Sdh1, respectively.[3,4] Recent analysis of this interaction in *S. cerevisiae* has led to the suggestion that Sdh5 may bind to two specific Arg residues in the C-terminal tail of Sdh1 at a location distal to the FAD binding site.[16] Furthermore, in *Serratia* 39006, SdhE bound FAD in multiple *in vitro* assays, suggesting that SdhE presents FAD to SDH as part of the flavinylation process.[4] However, no change in the NMR spectra of the eukaryotic homologue Sdh5 was detected in the presence of FAD, suggesting that Sdh5 does not bind FAD.[17] From these combined results, it has been proposed that SdhE/5 interacts with SdhA/1 to mediate flavinylation, yet the molecular details of this process remain unresolved.[15,18]

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Table 1. Bacterial Strains and Plasmids Used in This Study

strain	genotype/phenotype	ref
	Escherichia coli	
CC118 λpir	*araD*, Δ(*ara, leu*), Δ*lacZ74, phoA20, galK, thi-1, rspE, rpoB, argE, recA1, λpir*	37
DH5α	F-, Δ80Δ*dlacZ*M15, Δ(*lacZYA*−*argF*)*U169, endA1, recA1, hsdR17* ($r_K^- m_K^+$), *deoR, thi-1, supE44*, λ^-, *gyrA96, relA1*	Gibco/BRL
HH26	marker exchange mobilization strain for conjugal transfer	38
S17-1 λpir	*recA*, *pro*,*hsdR*, *recA*::RP4−2-Tc::Mu, *λpir*, Tmp^R, Sp^R, Sm^R	39
SM10 λpir	*thi-1, thr, leu, tonA, lacY, supE, recA*::RP4−2-Tc::Mu, λpir, Km^R	39
	Serratia sp. ATCC 39006	
LacA (parental strain; WT)	Lac^- derivative of *Serratia* sp. ATCC 39006, made by EMS mutagenesis	40
Δ*sdhE*	clean chromosomal deletion mutant of *sdhE*	4
Δ*sdhCDAB*::Kan	chromosomal deletion mutant of *sdhCDAB* replaced with a kanamycin resistance marker	this study
Δ*sdhA*	unmarked chromosomal deletion mutant of *sdhA*	this study
sdhA(H44S)	chromosomal swap mutant of SdhA to SdhA(H44S)	this study

plasmid	description	ref
pQE-80L	protein expression vector	QIAGEN
pQE-80LoriT	protein expression vector derivative of pQE-80L with RP4 *oriT*, Ap^R	23
pMAT15	protein expression vector derivative of pQE-80L with RP4 *oriT*, Cm^R	4
pBAD30	arabinose-inducible expression vector, ori 15A replicon, Ap^R	41
pBluescript II KS(+)	cloning vector, ColE1 replicon, Ap^R	Stratagene
pKNG101	marker exchange suicide vector, *sacBR*, mobRK2, oriR6K, Sm^R	42
pNJ5000	mobilizing plasmid used in marker exchange, Tc^R	38
pBluescript II KS(+) (132)	pBluescript II KS(+) with Δ*sdhCDAB*::kan^R, Ap^R, Kan^R	this study
pKNG101 (132)	pKNG101 with Δ*sdhCDAB*::kan^R deletion construct, Sm^R, Kan^R	this study
pBluescript II KS(+) (67)	pBluescript II KS(+) with Δ*sdhA* markerless, Ap^R	this study
pKNG101 (67)	pKNG101 with Δ*sdhA* markerless deletion construct, Sm^R	this study
pBluescript II KS(+) (89)	pBluescript II KS(+) with *sdhA*(H44S), Ap^R	this study
pKNG101 (89)	pKNG101 with *sdhA*(H44S) swap construct, Sm^R	this study
pTA71	*Serratia* SdhE, pQE-80L, Ap^R	4
pMAT7	SdhE(WT)-FLAG, pBAD30, Ap^R	4
pMAT10	His-SdhE(WT), pMAT15, Cm^R	4
pMAT32	His-SdhA, pQE-80LoriT, Ap^R	4
pMAT34	His-SdhA, pMAT15, Cm^R	4
pMAT35	His-SdhE(G16R), pMAT15, Cm^R	4
pMAT47	untagged SdhE(R15A), pQE-80oriT, Ap^R	this study
pMAT48	untagged SdhE(G16A), pQE-80oriT, Ap^R	this study
pMAT49	untagged SdhE(E19A), pQE-80oriT, Ap^R	this study
pMAT50	untagged SdhE(D21A), pQE-80oriT, Ap^R	this study
pMAT51	untagged SdhE(F27A), pQE-80oriT, Ap^R	this study
pMAT52	untagged SdhE(D51A), pQE-80oriT, Ap^R	this study
pMAT53	untagged SdhE(G16R), pQE-80oriT, Ap^R	this study
pMAT54	His-SdhE(R15A), pMAT15, Cm^R	this study
pMAT56	His-SdhE(E19A), pMAT15, Cm^R	this study
pMAT57	SdhE(R15A)-FLAG, pBAD30, Ap^R	this study
pMAT59	SdhE(G16R)-FLAG, pBAD30, Ap^R	this study
pMAT60	SdhE(E19A)-FLAG, pBAD30, Ap^R	this study
pMAT63	His-SdhA(H44S), pQE-80oriT, Ap^R	this study
pMAT64	untagged SdhE(Δα1), pQE-80oriT, Ap^R	this study
pMAT65	untagged SdhE(Δα1.5), pQE-80oriT, Ap^R	this study
pMAT66	untagged SdhE(Δα12), pQE-80oriT, Ap^R	this study
pMAT67	untagged SdhE(Δα123), pQE-80oriT, Ap^R	this study
pMAT68	untagged SdhE(Δα45), pQE-80oriT, Ap^R	this study
pMAT69	untagged SdhE(Δα5), pQE-80oriT, Ap^R	this study
pMAT70	SdhE(Δα1)-FLAG, pBAD30, Ap^R	this study
pMAT71	SdhE(Δα1.5)-FLAG, pBAD30, Ap^R	this study
pMAT72	SdhE(Δα12)-FLAG, pBAD30, Ap^R	this study
pMAT73	SdhE(Δα123)-FLAG, pBAD30, Ap^R	this study
pMAT74	SdhE(Δα45)-FLAG, pBAD30, Ap^R	this study
pMAT75	SdhE(Δα5)-FLAG, pBAD30, Ap^R	this study
pMAT77	untagged SdhE(E19D), pQE-80oriT, Ap^R	this study

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To examine the molecular mechanism of SdhE-dependent flavinylation of SdhA, this study describes truncation and site-directed mutagenesis of residues conserved across bacterial homologues of SdhE. Using phenotype rescue assays, we demonstrated that a highly conserved RGxxE motif is required for flavinylation and activation of SDH activity. Evidence that bacterial SdhE interacts with and flavinylates prefolded SdhA in the absence of other SDH subunits is also presented. Given the functional interkingdom conservation of SdhE homologues, these results have implications not only for bacterial physiology but also for an improved understanding of mitochondrial physiology and cancer pathology.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. Bacterial strains and plasmids are listed in Table 1. *Serratia* 39006 and *E. coli* strains were grown at 30 and 37 °C, respectively, in Luria broth (LB) (5 g/L yeast extract, 10 g/L Bacto tryptone, and 5 g/L NaCl), minimal medium [0.1% $(NH_4)_2SO_4$, 0.41 mM $MgSO_4$, 40 mM K_2HPO_4, 14.7 mM KH_2PO_4, and 0.2% (w/v) glucose or 0.4% (w/v) succinate] at 180 rpm, or LB agar or minimal agar supplemented with 1.5% (w/v) agar. Minimal succinate medium was buffered with 75 mM HEPES (pH 6.9–7.1). For growth in minimal medium, strains were first grown overnight in LB, subcultured overnight into minimal medium with glucose (0.2%), and then used to start growth in minimal medium in flasks. Bacterial growth was measured at an optical density of 600 nm (OD_{600}) in a Jenway 6300 spectrophotometer. When required, media were supplemented with antibiotics at the following concentrations: 50 μg/mL kanamycin, 50 μg/mL streptomycin, 100 μg/mL ampicillin, and 25 μg/mL chloramphenicol.

DNA Manipulation and Sequence Analysis. Molecular biology techniques, unless stated otherwise, were performed using standard techniques.[19] Oligonucleotides are listed in Table S1 of the Supporting Information. DNA sequencing was performed at the Allan Wilson Centre Sequencing Service and analyzed using Chromas and BLAST2. Plasmids constructed in this study were confirmed by sequencing, and pQE-80LoriT derivatives were introduced into *Serratia* 39006 by conjugation using either *E. coli* SM10 λpir or S17-1 λpir using minimal medium to counterselect the donors and appropriate antibiotics to select for the transconjugants. Other plasmids were introduced into *E. coli* or *Serratia* 39006 by transformation. Protein sequence alignments were performed using ClustalW2[20] and the output generated using Geneious (version R6) created by Biomatters. Secondary structure analysis was performed using Jpred[21] with *Serratia* 39006 SdhE as the input query and the output drawn manually.

Construction of Δ*sdhCDAB*::Kan and Δ*sdhA* Mutants. *Serratia* 39006 chromosomal mutants that had the entire SDH biosynthetic operon (Δ*sdhCDAB*::Kan) or the flavoprotein subunit (Δ*sdhA*) deleted were constructed using allelic exchange and homologous recombination. Construction details are provided in the Supporting Information.

Construction of SdhE Truncation and Alanine Swap Variants. Plasmids that expressed untagged and tagged versions of truncation and alanine swap variants of SdhE were constructed using overlap extension polymerase chain reaction.[22] Construction details are provided in the Supporting Information.

Stability of Truncation and Alanine Swap Variants. The stability of C-terminal FLAG-tagged truncation and alanine swap variants was assessed by Western blotting. Briefly, cultures were inoculated from an overnight culture into LB at a ratio of 1:50. Cultures were grown to an OD_{600} of 0.4 and induced with 0.2% arabinose. Cultures were grown for an additional 4 h and samples collected. Twenty five microliters of the OD_{600}-adjusted culture was loaded per lane and analyzed by Western blotting as described below.

Phenotype Rescue Assays. To assess the function of SdhE truncation and alanine swap variants, a Δ*sdhE* strain of *Serratia* 39006 expressing the appropriate SdhE variant was grown in minimal medium with 0.4% succinate as the sole carbon source. Cultures were inoculated at a starting OD_{600} of 0.05, and plasmids were induced with 0.1 mM IPTG at time zero. OD_{600} was measured at the stated time points. All results are the average of three biological replicates ± the standard deviation.

His-Tagged Protein Purification. All His-tagged proteins were purified using Ni-NTA agarose as previously described.[4] Briefly, strains were grown overnight in LB and used to inoculate 100 mL of LB in a 1 L flask at a ratio of 1:50. Cultures were grown to an OD_{600} of ∼0.6, induced with 1 mM IPTG, and grown overnight. Harvested cultures were resuspended in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole (pH 8.0)] with 10 μL of a protease inhibitor mixture (Sigma) and 5 μL of DNase (Promega) and lysed by sonication (six times 10 s on and off, on ice). Cellular debris was removed following lysis by centrifugation at 9000 rpm for 30 min.[4] Proteins were purified using Ni-NTA and gravity feed columns using the batch purification protocol as described previously.[4] Proteins were eluted using an imidazole gradient from 45 to 250 mM imidazole. The protein concentration was determined using the Thermo Scientific BCA assay.

Western Blotting. Western blotting following sodium dodecyl sulfate–polyacrylamide gel electrophoresis was conducted using a Tris/glycine buffer system following standard protocols.[23] Membranes were analyzed using mouse monoclonal anti-His (Sigma) or mouse monoclonal anti-FLAG (Sigma) antibodies and, as a secondary antibody, goat anti-mouse IgG horseradish peroxidase (Santa Cruz Biotechnology). Blots were developed using the Supersignal West Pico chemiluminescent substrate kit (Thermo Scientific) and were visualized by either the ChemiDoc imaging system (Bio-Rad) or X-ray film.

FAD Binding Assays, FAD-UV Gel Assays, and Excitation and Emission Spectra of FAD. FAD binding assays and FAD-UV gel assays were performed as previously described.[4] Samples were split into two and separated on additional gels for visualization by Coomassie Blue staining or Western blotting. For further spectral analysis of covalently bound FAD, 100 μg of purified protein was precipitated with 3% trichloroacetic acid as previously described.[4] Following centrifugation, samples were resuspended in 50 μL of 6 M guanidine hydrochloride [dissolved in 0.1 M $NaPO_4$ (pH 7)]. Resuspended protein samples were aliquoted into a 96-well microtiter plate and excited at 450 nm, and the emission between 500 and 600 nm was measured.[24] Measurements were performed in either an Infinite 200 PRO series (Tecan) or a Varioskan Flash Multimode Reader (Thermo Scientific) plate reader.

Biological Membrane Preparation and Succinate Dehydrogenase Assays. Cultures of *Serratia* 39006 strains expressing proteins of interest were grown overnight and used to inoculate 100 mL of LB at a ratio of 1:50 in a 1 L flask.

Plasmids were induced with 0.1 mM IPTG at 0 h and were left to grow overnight (growth for approximately 15 h). The preparation of membrane and soluble fractions was performed as described previously.[4,23] The enzymatic activity of SDH was determined by monitoring the decrease in the absorbance of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm over a period of 10 min. Reaction mixtures consisted of 0.05 mM DCPIP (extinction coefficient of 22 mM^{-1} cm^{-1}), 50−100 μg of biological membranes, 0.1 M sodium succinate (pH 7.5) (Sigma), and 0.1 M $NaPO_4$ (pH 7) in a total volume of 1 mL. SDH activity was expressed relative to the background reduction of DCPIP (i.e., membranes without succinate). All results are the average of three replicates ± the standard deviation.

Copurification. Copurification experiments were performed using either a Ni-NTA or anti-FLAG agarose as previously described.[4,25] Briefly, for anti-FLAG co-immunoprecipitation, strains co-expressing SdhE-FLAG (Bait) and His-SdhA (Prey) were grown in 50 mL of LB. Plasmids were induced with 1 mM IPTG and 0.2% arabinose at an OD_{600} of 0.4. Cells were harvested after being grown for an additional 4 h. Cells were resuspended in lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 (pH 7.4)] and lysed as described previously,[25] with the exception that lysis included an additional sonication step (six times 10 s on and off). For Ni-NTA copurification, His-SdhA (Bait) and SdhE-FLAG (Prey) co-expressing strains were grown as described for the purification of His-tagged proteins with 0.2% arabinose to induce expression of protein from pBAD30. Lysates were passed through a Ni-NTA column, washed six times with 20 mM imidazole wash buffer, and eluted on an imidazole gradient as described for the purification of His-tagged proteins. Proteins were visualized by Western blotting.

Circular Dichroism. Purified His-SdhE (variants) or His-SdhA was concentrated to 0.3 mg/mL in 20 mM $NaPO_4$ buffer (pH 7.0). Far-UV circular dichrosim (CD) spectra were recorded on an Olis DCM-10 CD spectrophotometer using the Olis Globalworks software. Spectra were obtained at 20 °C from 260 to 190 nm with a total of 50 collection points in a 0.2 cm path length quartz cuvette. Protein secondary structure was analyzed using the Olis Globalworks software. Data are the average of three runs corrected for the subtraction of the buffer spectra and presented in units of degrees square centimeter per decimole.

RESULTS

SdhE Flavinylates SdhA in the Absence of Other SDH Complex Proteins. SDH can assemble in the absence of covalently bound FAD.[4] However, in bacteria, it is unknown whether the flavinylation of SdhA is dependent on the other SDH subunits. To investigate this, His-SdhA was purified from wild-type (WT), Δ*sdhE*, and a complete SDH deletion mutant (Δ*sdhCDAB*::Kan) in which a functional copy of *sdhE* is still present. His-SdhA purified from WT *Serratia* 39006 contained covalently bound FAD that comigrated at the same molecular weight as His-SdhA when they were analyzed by FAD-UV (Figure 1A). Consistent with previous data, His-SdhA purified from Δ*sdhE* did not contain covalently bound FAD (Figure 1A).[4] His-SdhA that was purified from Δ*sdhCDAB*::Kan contained covalently bound FAD (Figure 1A).[16,26] In conclusion, SdhE flavinylates SdhA in the absence of other SDH complex proteins.

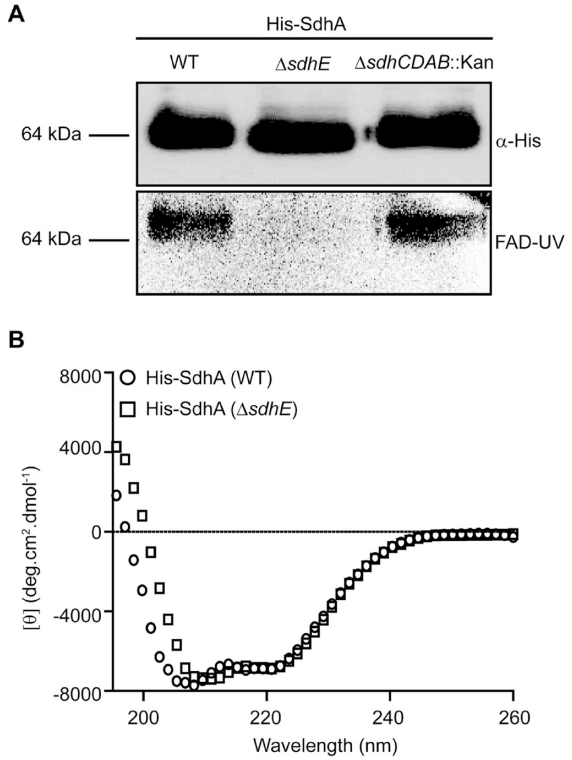

Figure 1. SdhA flavinylation is not dependent on other SDH subunits. (A) His-SdhA (pMAT32) purified from either WT, Δ*sdhE*, or Δ*sdhCDAB*::Kan *Serratia* 39006 was assessed for FAD incorporation by FAD-UV (bottom) normalized to total protein (1 μg per lane). Western blotting against the His tag (top). (B) Far-UV range (190−260 nm) of His-SdhA (pMAT32) purified from WT or Δ*sdhE Serratia* 39006 analyzed by CD.

SdhA Is Folded Prior to Flavinylation. It has been hypothesized that eukaryotic Sdh1 is folded into an intermediate or fully folded form prior to flavinylation and interaction with Sdh5.[18] To investigate this hypothesis in bacteria, His-SdhA was purified from WT and Δ*sdhE Serratia* 39006, and the degree of protein folding was estimated using circular dichrosim (CD) in the far-UV range (190−260 nm). Consistent with previous structural characterization of *E. coli* SDH,[2] His-SdhA purified from WT *Serratia* 39006 had a largely α-helical secondary structure (Figure 1B). His-SdhA purified from Δ*sdhE* (i.e., lacks covalently bound FAD) had a spectrum above 210 nm similar to that of His-SdhA from WT *Serratia* 39006. Interestingly, below 210 nm, the spectrum of His-SdhA with or without FAD diverged (Figure 1B). This subtle divergence was reproduced in multiple experiments. In conclusion, there are no large secondary structure changes in His-SdhA with and without covalently attached FAD, suggesting that SdhA is folded prior to flavinylation and interactions with SdhE.

SdhA Retains Partial Activity in the Absence of SdhE. The SDH deletion mutant (Δ*sdhCDAB*::Kan) had a slight, yet statistically significant, reduction in SDH activity relative to that of Δ*sdhE* (i.e., 93% reduction compared with a 90% reduction) (Figure 2A). We hypothesized that, in the absence of SdhE, a small proportion of SdhA proteins might be inefficiently flavinylated by an autocatalytic mechanism or a redundant assembly factor, resulting in partial SDH activity. In this scenario, the overexpression of SdhA in a Δ*sdhE* background should increase the number of SdhA proteins that were

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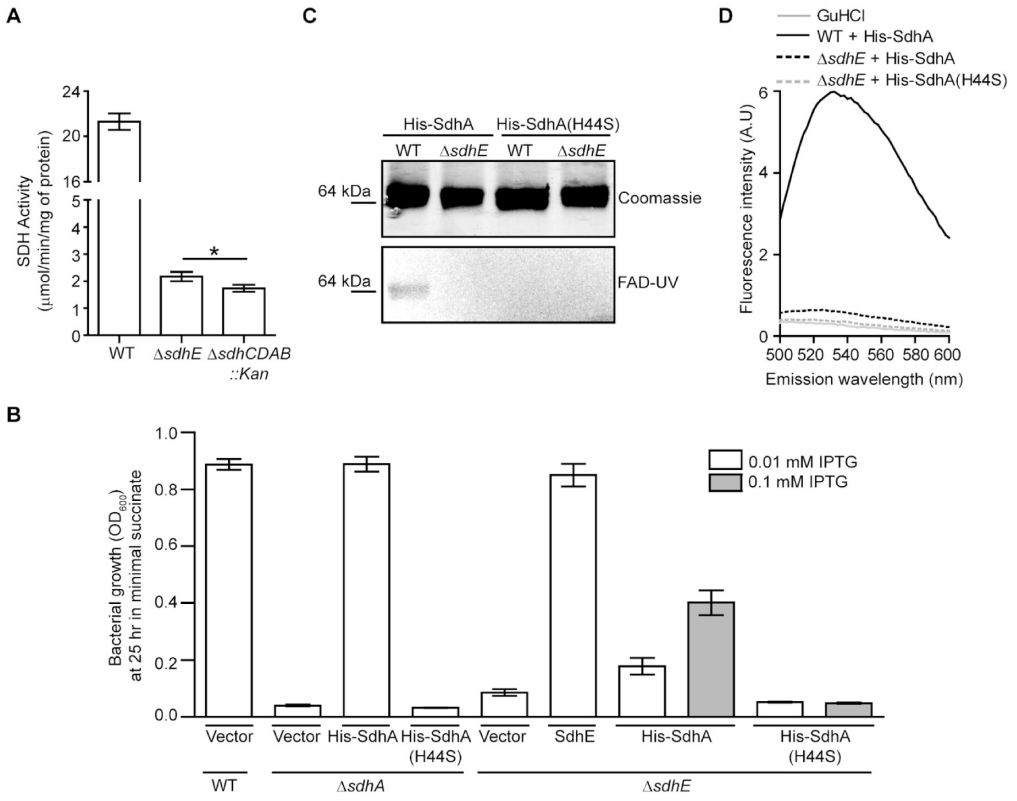

Figure 2. SdhA is partially active and flavinylated in the absence of SdhE. (A) SDH assays were performed on membranes of WT, Δ*sdhE*, and Δ*sdhCDAB*::Kan *Serratia* 39006. *$p < 0.05$ as determined using a Student's *t* test. (B) Phenotype rescue assays of WT, Δ*sdhE*, and Δ*sdhA Serratia* 39006 with a vector control (pQE-80oriT) or expressing SdhE (pTA71), His-SdhA (pMAT32), or His-SdhA(H44S) (pMAT63). Strains were induced with either 0.01 or 0.1 mM IPTG at 0 h and were grown in minimal medium with succinate (0.4%) as the sole carbon source. (C and D) His-SdhA (pMAT32) and His-SdhA(H44S) (pMAT63) were purified from WT or Δ*sdhE Serratia* 39006. Purified His-SdhA (WT or H44S) was assessed for FAD incorporation by (C) FAD-UV (bottom) normalized to total protein (5 μg per lane). Coomassie staining (top). (D) Excitation–emission scan of 100 μg of protein from panel C precipitated with trichloroacetic acid. Results are the average of two technical replicates.

autocatalytically flavinylated and partially compensate for the loss of SdhE. To test this hypothesis, Δ*sdhE* expressing His-SdhA *in trans* to various levels (i.e., 0.01 and 0.1 mM IPTG) was grown in medium with succinate as the sole carbon source. WT *Serratia* 39006 grew, while the loss of SdhA (Δ*sdhA*) prevented growth in minimal succinate medium (Figure 2B) but could be complemented by the low-level expression of His-SdhA (i.e., 0.01 mM IPTG) (Figure 2B). Consistent with previous results,[4] Δ*sdhE* exhibited impaired growth when succinate was used as the sole carbon source, but this could be complemented by the *in trans* expression of SdhE (Figure 2B). Interestingly, low-level expression of His-SdhA (i.e., 0.01 mM IPTG) partially restored growth to Δ*sdhE* (Figure 2B). Increasing the level of expression (i.e., 0.1 mM IPTG) resulted in ∼50% complementation (Figure 2B). The covalent linkage of FAD to a completely conserved His residue in SdhA (His44 in *Serratia* 39006) is essential for SDH activity.[2,27] To determine if this partial activity of SdhA was dependent on the FAD binding residue, an H44S mutant was constructed. The His-SdhA(H44S) mutant did not complement Δ*sdhA*, demonstrating the functional requirement of covalent FAD attachment (Figure 2B). Furthermore, His-SdhA(H44S) was unable to partially complement Δ*sdhE* when induced with either 0.01 or 0.1 mM IPTG (Figure 2B). In conclusion, SDH has a low basal activity in the absence of the FAD assembly factor SdhE. This basal activity can be partially overcome by expression of high levels of SdhA and is dependent on the FAD binding residue His44.

Low-Level Covalent Flavinylation of SdhA in the Absence of SdhE. We hypothesized that the partial SDH activity in the absence of SdhE was due to the covalent flavinylation (i.e., covalent attachment of FAD) of a small proportion of SdhA proteins through an inefficient autocatalysis. However, His-SdhA purified from Δ*sdhE* does not contain detectable covalently attached FAD when previously analyzed using in-gel analysis (i.e., FAD-UV) (Figures 1A and 2C).[4] To investigate a low-level covalent flavinylation of His-SdhA purified from Δ*sdhE*, we utilized a more sensitive spectral analysis following acid precipitation. Under these experimental conditions, only covalently bound FAD will coprecipitate with His-SdhA and be detected, while noncovalent FAD would be released into the supernatant. Excitation of coprecipitating FAD at 450 nm will produce an emission spectrum with a maximum at ∼530 nm.[24] Consistent with in-gel FAD-UV analysis (Figure 2C), precipitated His-SdhA purified from WT *Serratia* 39006 produced an emission spectrum consistent with covalently bound FAD (Figure 2D). The His-SdhA(H44S) flavinylation mutant when purified from either WT or Δ*sdhE Serratia* 39006 did not contain detectable covalently attached FAD when analyzed by either FAD-UV or spectral analysis (Figure 2C,D). Consistent with previous observations, His-SdhA purified from Δ*sdhE* did not contain detectable covalent FAD upon being analyzed by FAD-UV (Figure 2C).[4] Interestingly, precipitated His-SdhA purified from Δ*sdhE* had a small FAD spectrum upon being analyzed by excitation and emission that was greater than the buffer control and absent from the His-SdhA(H44S)

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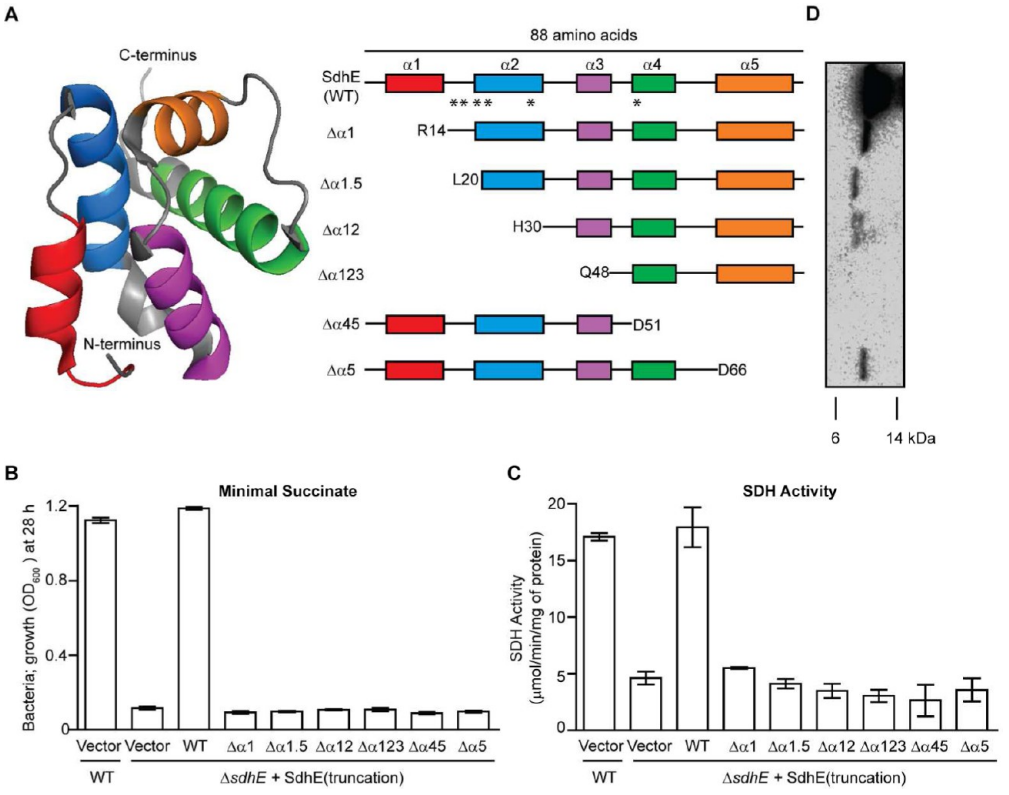

Figure 3. SdhE truncation variants are inactive. (A) Progressive N- and C-terminal truncation variants were constructed. Asterisks denote the location of residues that were individually mutated (Figure 4A). Individual α-helices are color-coded and correspond to the α-helices in the crystal structure of the *E. coli* homologue (left; Protein Data Bank entry 1X6J). (B) Phenotype rescue assays of untagged SdhE truncation variants expressed from pQE-80oriT (pMAT63-pMAT69) in Δ*sdhE* grown with succinate as the sole carbon source. (C) SDH assays of strains performed on membranes of Δ*sdhE Serratia* 39006 used in panel B. (D) WT *Serratia* 39006 expressing C-terminally FLAG-tagged SdhE truncation variants (pMAT70−75). SdhE(WT)-FLAG (pMAT7) was used as an expression control.

flavinylation mutant (Figure 2D). In conclusion, a small proportion of SdhA proteins are covalently flavinylated in the absence of SdhE.

The α-Helices of SdhE Are Required for Function. SdhE consists of five α-helices (Figure 3A). Structural analysis of SdhE from *E. coli* and various homologues suggests that a highly conserved surface-exposed region, consisting predominately of residues from α-helices 1−3, is required for interaction with SdhA.[15,17,18] To investigate this hypothesis, N- and C-terminal truncation variants of SdhE were constructed to progressively remove individual α-helices (Figure 3A). In phenotype rescue assays, all untagged truncation variants were nonfunctional and unable to complement growth of a Δ*sdhE* mutant grown in minimal succinate (Figure 3B) or restore SDH activity to membranes prepared from the Δ*sdhE* mutant (Figure 3C). To test the stability of these truncation variants, we added a C-terminal FLAG tag. The combined loss of α-helices 1−3 or α-helices 4 and 5 produced unstable proteins that were not detected by Western blotting (Figure 3D). The remaining truncation variants were detected by Western blotting, but at reduced levels relative to that of WT SdhE-FLAG, suggesting that these truncation variants have reduced stability (Figure 3D). This reduced stability might contribute to the loss of function. In conclusion, all five α-helices of SdhE are required for function by contributing to the structural integrity and stability and/or SdhE-mediated activation of SDH.

Selection of Residues for Alanine Swap Mutagenesis of SdhE. The loss of function associated with SdhE truncation variants suggested that functional motifs or residues might exist within these α-helices and that a more subtle mutagenesis approach was required. We hypothesized that residues conserved across bacterial homologues would be important for function. A sequence alignment of SdhE homologues from various α-, β-, and γ-proteobacterial species identified conserved residues that were clustered in the loop between α-helices 1 and 2 and within α-helix 2 (Figure 4A,B). On the basis of structural data from various homologues, it has been hypothesized that R15, G16, and E19A form an important RGxxE motif.[15,17] Crystal structure data demonstrate that D21 and D51 form electrostatic interactions with R15.[28] On this basis, R15, G16, E19, D21, and D51 were chosen for alanine swap mutagenesis. These residues are clustered in a highly conserved surface-exposed region of SdhE (Figure 4C).[15,17] A structurally buried F27 was also chosen on the basis of conservation across a wide range of homologues (Figure 4A,B). Other conserved hydrophobic residues were not mutated as they were hypothesized to be important structural residues internal to the protein.[17,28,29] The G78R (equivalent to G16 in SdhE) mutation in eukaryotic Sdh5 homologues is associated with cancer.[3,14] The equivalent mutation in *Serratia* 39006 SdhE, G16R, was also included to gain further molecular insights into this pathological mutation. Eletsky and colleagues[17] recently demonstrated that R68E, Y71D, and W113A variants of Sdh5 (equivalent to R8, W11, and P52 in SdhE, respectively) (Figure 4B) were impaired for flavinylation of SDH.[17] These residues were not chosen for mutagenesis because they are not strictly conserved in bacteria (Figure 4A).

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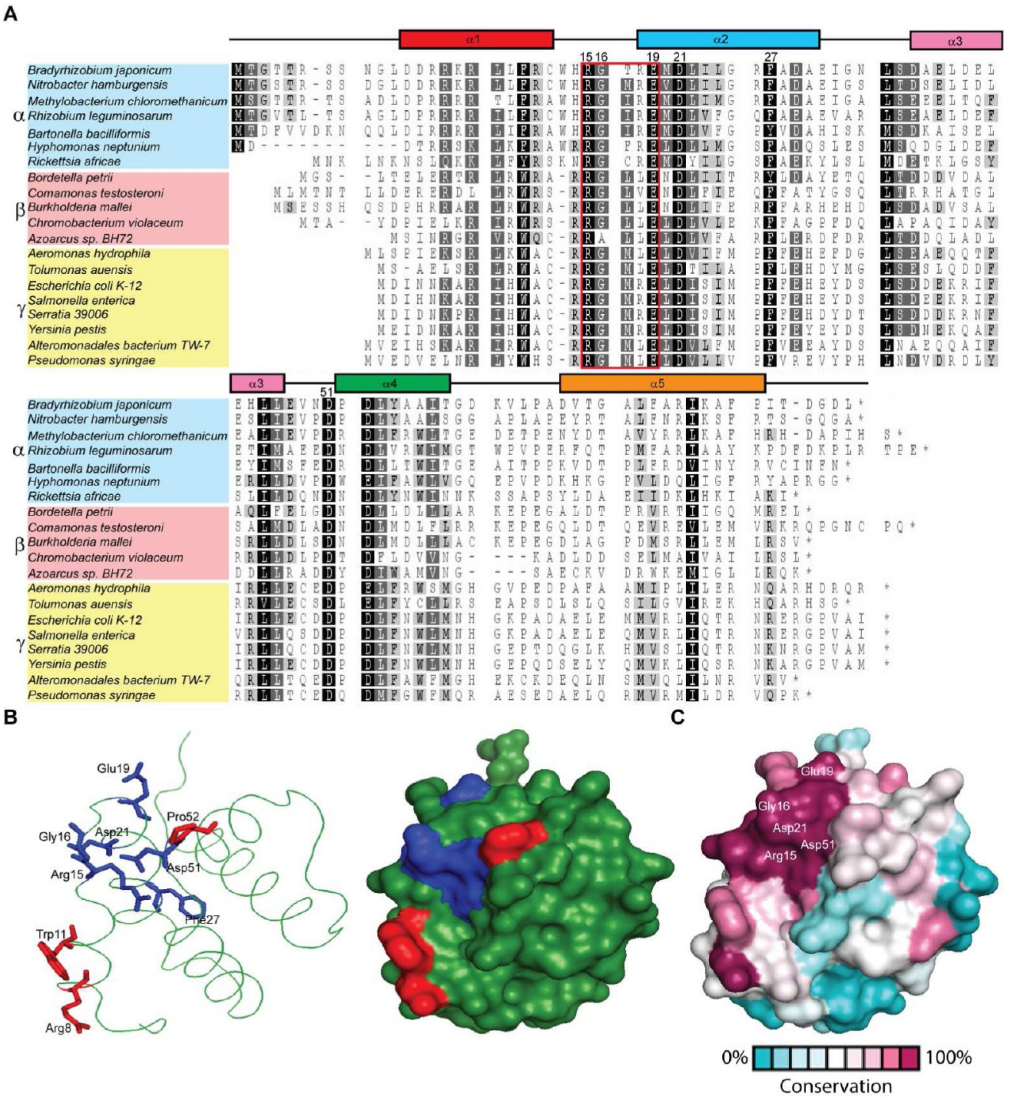

Figure 4. Sequence alignment and secondary structure predictions of SdhE homologues. (A) Secondary structure predictions were made with the *Serratia* 39006 SdhE sequence using Jpred.[21] Sequence alignments of SdhE homologues were made and shaded in Geneious using a ClustalW alignment. A black background denotes a highly conserved residue, while a white background denotes a poorly conserved residue. Accession numbers for each homologue are listed in the Supporting Information. The numbers above the respective residues correspond to residues that were mutated. The RGxxE motif is boxed in red. (B) Crystal structure of *E. coli* SdhE (Protein Data Bank entry 1X6J) in a ribbon (left) and spaced-filled model (right) highlighting the location of the residues mutated in this study (blue). The residues corresponding to those mutated by Eletsky and colleagues in Sdh5 (i.e. Arg8, Trp11 and Pro52) are coloured red.[17] (C) Conserved residues of SdhE homologues as generated using the Consurf server.[43] Structural images in panels B and C were generated using PYMOL.

The Conserved RGxxE Motif Is Required for SdhE Function. In phenotype rescue assays, D21A, F27A, and D51A complemented the growth of Δ*sdhE* in minimal succinate at levels similar to that of WT SdhE (Figure 5A). R15A partially complemented Δ*sdhE*, while G16R and E19A were nonfunctional (Figure 5A). Interestingly, although G16R produced a nonfunctional protein, the G16A substitution had no effect on function (Figure 5A). R15A, G16A, D21A, F27A, and D51A variants of SdhE complemented Δ*sdhE* when grown in LB, while G16R and E19A variants remained nonfunctional (Figure 5B). The inability of SdhE(G16R) and SdhE(E19A) to complement growth correlated with an inability to restore SDH activity in Δ*sdhE* membranes (Figure 5C). SDH activity detected for SdhE(R15A) was slightly lower than that upon complementation with SdhE(WT) (Figure 5C). Western blotting of C-terminally FLAG-tagged constructs demonstrated that G16R-FLAG and E19A-FLAG were expressed at levels similar to that of the WT version (Figure 5D). R15A-FLAG showed a reduced level of expression (Figure 5D), suggesting that partial functionality of R15A may arise from reduced protein stability. In conclusion, the highly conserved RGxxE motif is required for SdhE function, while alanine substitutions in other highly conserved residues are tolerated.

The Conserved RGxxE Motif Influences the Flavinylation of SDH. We predicted that the nonfunctional mutations G16R and E19A impaired the complete flavinylation of SdhA. His-SdhA was purified from Δ*sdhE* co-expressing C-terminally FLAG-tagged WT, R15A, G16R, or E19A SdhE. SdhE(WT)-FLAG and SdhE(R15A)-FLAG restored flavinylation of His-SdhA to levels similar to those observed in the WT background (Figure 6A). Consistent with a loss of function, SdhE(G16R)-FLAG failed to flavinylate His-SdhA (Figure 6A). SdhE(E19A)-

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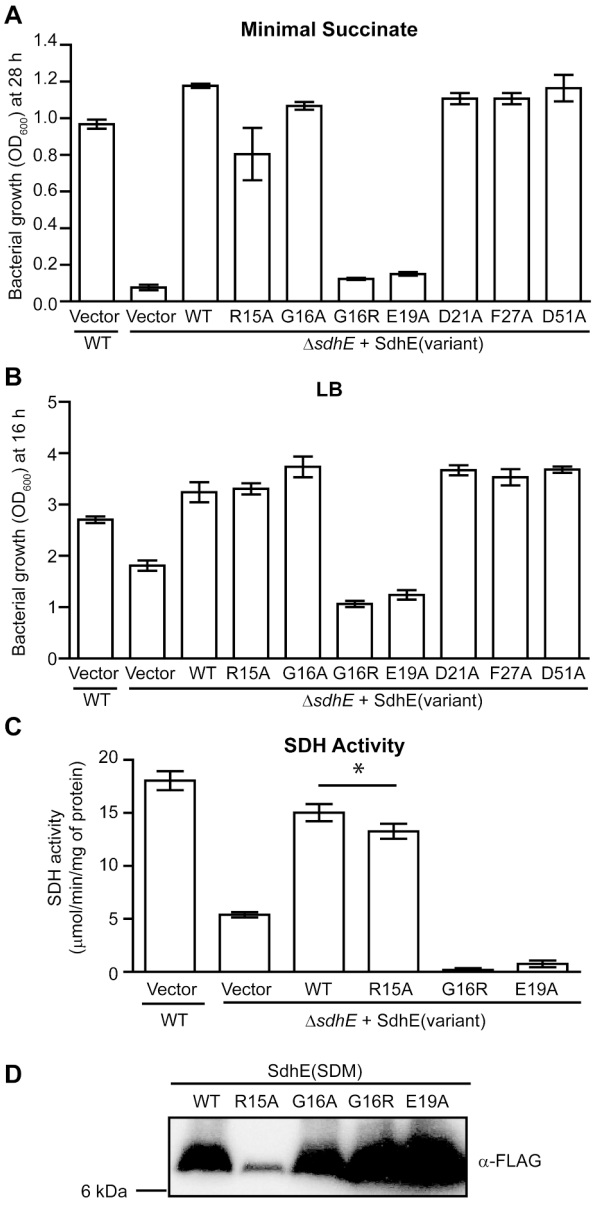

Figure 5. The RGxxE motif of SdhE is important for function. Phenotype rescue assays of SdhE site-directed variants (pMAT47−53) expressed in Δ*sdhE Serratia* 39006 grown in (A) minimal medium with succinate as the sole carbon source and (B) LB. (C) SDH assays were performed on membranes of WT and Δ*sdhE* strains expressing a vector control, untagged SdhE(WT), or SdhE RGxxE variants. *$p < 0.05$ as determined using a Student's *t* test. (D) C-Terminal FLAG-tagged versions of SdhE(SDM) expressed in WT *Serratia* 39006 grown in LB and detected by Western blotting.

FLAG flavinylated His-SdhA (Figure 6A), although the level of FAD incorporation was greatly reduced. The reduced level of covalent attachment of FAD to His-SdhA by SdhE(E19A)-FLAG was validated by FAD spectral analysis following acid precipitation of purified His-SdhA (Figure 6B). Despite the partial SdhA flavinyaltion by SdhE(E19A)-FLAG, this double-plasmid strain was unable to grow with succinate as the sole carbon source (Figure 6C). The low level of growth observed for SdhE(G16R)-FLAG and SdhE(E19A)-FLAG is due to the addition of arabinose required to induce expression of the P_{araBAD} promoter in pBAD30 (Figure 6C). Because arabinose is a carbon source, when it is supplied at 0.02% (w/v), Δ*sdhE* *Serratia* 39006 with SdhE(G16R) or SdhE(E19A) is able to grow to a very low OD_{600}, despite being unable to utilize the succinate that is present. In summary, Gly16 and Glu19 of the RGxxE motif are required for complete SdhA flavinylation and activation of SDH.

G16R and E19A Variants of the RGxxE Motif Do Not Affect the Secondary Structure, Interactions with SdhA, or *in Vitro* FAD Binding. On the basis of structural data, the RGxxE motif has been proposed to function in either protein folding or interactions with SdhA.[3,4,17] To investigate if RGxxE variants were correctly folded, His-SdhE variants were purified and analyzed by CD in the far-UV range. Consistent with structural data, His-SdhE(WT) produced a CD spectrum indicative of a largely α-helical protein, and His-SdhE(G16R) and His-SdhE(E19A) were indistinguishable from WT His-SdhE (Figure 6D). His-SdhE(R15A) could not be purified, consistent with SdhE(R15A) being an unstable variant. In conclusion, the G16R and E19A mutations did not cause major changes in the protein secondary structure, suggesting that SdhE variants are correctly folded.

To investigate the interaction between SdhA and RGxxE, we performed copurification assays on an anti-FLAG agarose using SdhE(variants)-FLAG proteins as bait and His-SdhA as prey. All experiments were performed in a Δ*sdhE* background to avoid any interference from WT SdhE.[4] His-SdhA copurified with SdhE(WT)-FLAG but did not purify with the anti-FLAG agarose in the absence of the bait protein, demonstrating that the copurification of His-SdhA is dependent on the interaction with SdhE-FLAG (Figure 6E). His-SdhA copurified with SdhE(R15A)-FLAG and interestingly copurified with SdhE-(G16R)-FLAG and SdhE(E19A)-FLAG (Figure 6E). This is consistent with SdhE(E19A) being able to partially flavinylate His-SdhA but was not expected for SdhE(G16R), which does not mediate flavinylation (Figure 6A). These results were reproduced in a reciprocal interaction using a Ni-NTA resin with His-SdhA as a bait and FLAG-tagged SdhE variants as prey (Figure S1 of the Supporting Information). In conclusion, the nonfunctional SdhE RGxxE variants were not impaired for interactions with the flavoprotein His-SdhA.

SdhE binds FAD *in vitro*, so we hypothesized that the RGxxE motif might be involved in FAD binding. To examine this hypothesis, purified His-SdhE(WT), His-SdhE(G16R) and His-SdhE(E19A) were incubated with FAD. The binding of FAD to His-SdhE(G16R) and His-SdhE(E19A) was indistinguishable from that to His-SdhE(WT) (Figure S2A,B of the Supporting Information). In conclusion, the SdhE mutations G16R and E19A do not detectably impair the ability of SdhE to bind FAD in these *in vitro* assays.

SdhE(G16R) and SdhE(E19A) Impair SDH Activation. The Δ*sdhE* strains expressing SdhE(G16R) or SdhE(E19A) had SDH activities lower than that of Δ*sdhE* with a vector control (Figure 5C). We hypothesized that G16R and E19A variants impaired the flavinylation and activation of SDH. In support of this concept, the expression of SdhE(G16R) or SdhE(E19A) in WT *Serratia* 39006 impaired growth in minimal medium with succinate as the sole carbon source (i.e., conditions at which SDH is required for growth[4]) (Figure 7A). SdhE(E19A) caused greater growth impairment than SdhE(G16R), which was eventually overcome, possibly by the chromosomal *sdhE* allele (Figure 7A). Consistent with growth analyses, SDH activity assays demonstrated that SdhE(G16R) and SdhE(E19A) reduced SDH activity by ∼65 and ∼97%, respectively, compared to that of the WT with a vector (Figure

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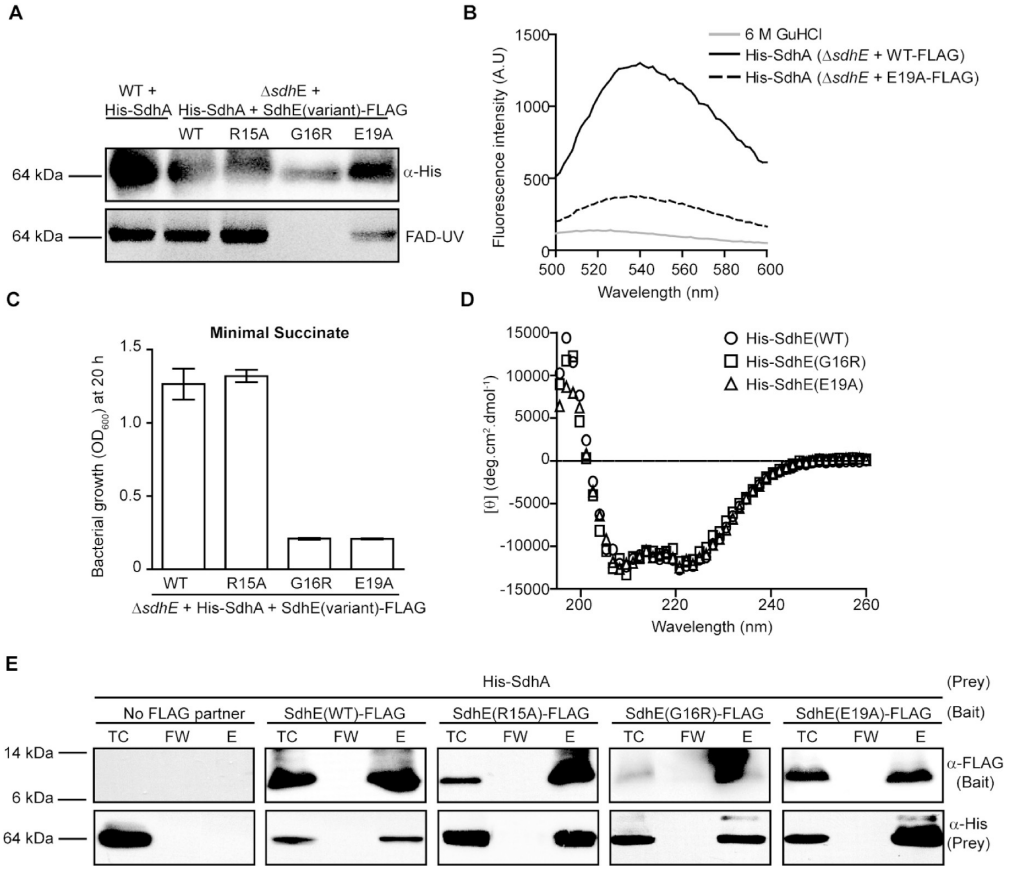

Figure 6. The RGxxE motif influences flavinylation of SDH. (A) His-SdhA (pMAT34) was purified from WT or Δ*sdhE* strains co-expressing SdhE(WT)-FLAG (pMAT7), SdhE(R15A)-FLAG (pMAT57), SdhE(G16R)-FLAG (pMAT59), or SdhE(E19A)-FLAG (pMAT60). Plasmids were induced with 1 mM IPTG and 0.2% arabinose at an OD_{600} of ~0.4. Purified His-SdhA was assessed for FAD incorporation by FAD-UV (bottom) normalized to total protein (1.3 μg per lane). Western blotting against the His tag (top). (B) Purified His-SdhA (100 μg) from panel A was precipitated with trichloroacetic acid, and the level of covalent FAD incorporation was determined using an excitation−emission scan of FAD. (C) Phenotype rescue assays of Δ*sdhE* co-expressing His-SdhA (pMAT34) and SdhE(WT)-FLAG, SdhE(R15A)-FLAG, SdhE(G16R)-FLAG, or SdhE(E19A)-FLAG grown in minimal medium with succinate as the sole carbon source. Plasmids were induced with 0.01 mM IPTG and 0.02% arabinose at time zero. (D) Far-UV spectra of His-SdhE(WT) (pMAT10), His-SdhE(G16R) (pMAT35), and His-SdhE(E19A) (pMAT56) purified from the WT were analyzed using CD. (E) Δ*sdhE* cells co-expressing His-SdhA (pMAT34) (prey) and SdhE(WT)-FLAG, SdhE(R15A)-FLAG, SdhE(G16R)-FLAG, or SdhE(E19A)-FLAG (bait) were grown, lysed, and purified on an anti-FLAG resin. TC denotes total cell expression prior to purification, FW the final wash fraction, and E the elution fraction.

7B). Interestingly, E19A expressed in a WT background reduced SDH activity to levels lower than that observed for a Δ*sdhE* + empty vector control (Figure 7B). The expression of SdhE(WT) in a WT background did not impair growth or SDH activity (Figure 7A,B). Furthermore, the overexpression of SdhE(G16R) and SdhE(E19A) in a WT background grown in minimal medium with glucose as the sole carbon source (i.e., conditions at which SDH activity is not required for growth) had no growth impairment compared to an empty vector control (data not shown). In summary, SdhE(G16R) and SdhE(E19A) impair the function of SDH.

To further investigate the functional importance of Glu19, we introduced an alternative conservative residue swap to aspartate (E19D). The SdhE(E19D) variant was able to complement Δ*sdhE* (Figure 7C) and did not impair the growth of the WT when it was grown in minimal medium with succinate as the sole carbon source (Figure 7D). In summary, a conservative residue E19D variant does not affect the function of SdhE under the conditions tested in this study.

DISCUSSION

SdhE belongs to a family of proteins required for the flavinylation and activation of SDH that are present in a wide range of bacteria and eukaryotes.[3,4,12,30] This study has provided insights into the function and potential mechanisms of the SdhE protein family.

We have shown that the bacterial flavoprotein subunit of SDH, SdhA, is flavinylated in an SdhE-dependent manner in the absence of the other SDH subunits (SdhB−D). These results suggest that SdhE interacts with and flavinylates SdhA prior to the assembly of the multiprotein complex in the bacterial membrane. The question of whether this occurs *in vivo* when SdhB−D are present requires further validation, but our results clearly demonstrate that other SDH components are not required for flavinylation. The *S. cerevisiae* homologue Sdh1 is also flavinylated in the absence of other SDH subunits,[16,26] highlighting the interkingdom conservation of SdhA flavinylation.

His-SdhA is folded into an α-helical protein irrespective of whether FAD is covalently attached. This suggests that SdhA is folded prior to flavinylation. This prefolding of His-SdhA is

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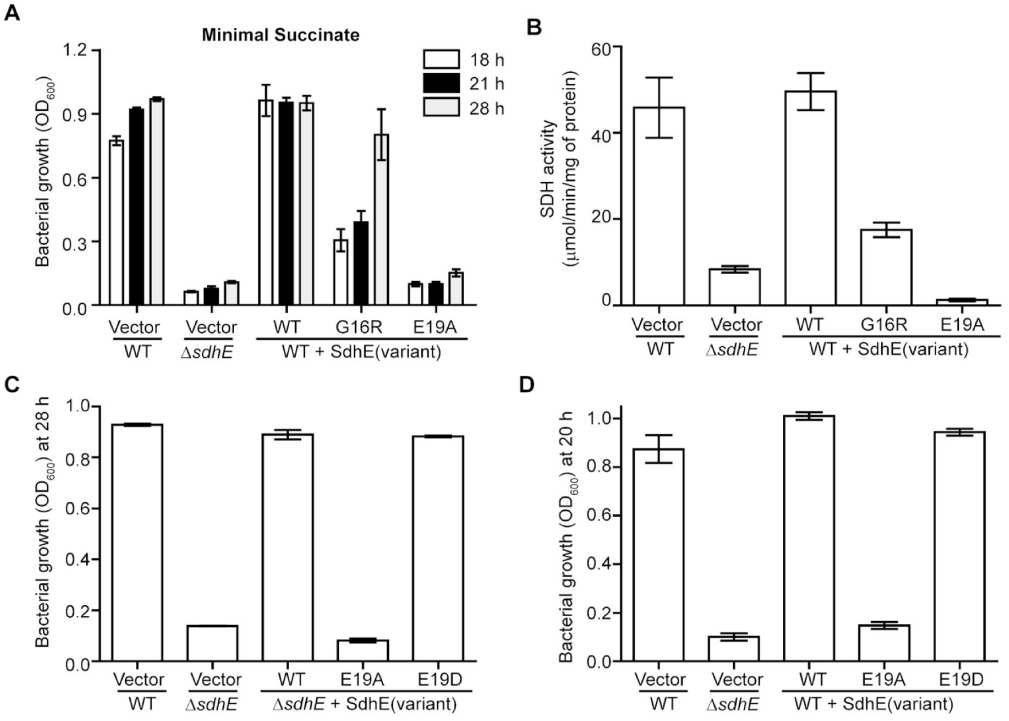

Figure 7. SdhE(G16R) and SdhE(E19A) impair SDH activity. (A) WT *Serratia* 39006 grown with succinate as the sole carbon source expressing untagged SdhE WT (pTA71), G16R (pMAT53) or E19A (pMAT49). Cultures were inoculated at a starting OD_{600} of 0.05 and induced with 1 mM IPTG at time zero. OD_{600} was measured at 18, 21, and 28 h. (B) SDH activity assays were performed on membranes of strains used in panel A. Plasmid expression was induced with 1 mM IPTG at 0 h. (C and D) Phenotype rescue assays of untagged SdhE E19A (pMAT49) and E19D (pMAT77) site directed-variants expressed in (C) Δ*sdhE* and (D) WT *Serratia* 39006 grown in minimal medium with succinate as the sole carbon source. Plasmids were induced with either (C) 0.1 or (D) 1 mM IPTG at time zero.

consistent with the observation that SDH is assembled into the membrane in the absence of FAD in both bacterial and eukaryotic species.[4,16] These results also support a model in which SdhA is folded prior to its interaction with SdhE. Interestingly, there were reproducible differences in protein spectra below 210 nm for His-SdhA purified from the WT and Δ*sdhE*. FAD contributes to protein spectra above 300 nm.[31−33] Therefore, the absence of FAD alone does not explain the observed divergence in protein spectra below 210 nm. Therefore, this divergence might represent small structural changes between His-SdhA with and without covalently bound FAD. However, this requires further investigation. In summary, His-SdhA is folded into an apo form prior to interactions with and flavinylation by SdhE.

SDH had a low basal activity in the absence of SdhE because of the flavinylation of a small proportion of SdhA proteins, possibly via an inefficient autocatalytic mechanism. On this basis, we propose that SdhE functions to increase the rate of an inefficient autocatalytic FAD attachment, thereby increasing the proportion of flavinylated SdhA proteins and SDH activity required for growth. Bacteria that do not belong to the α-, β-, or γ-proteobacteria contain SDH yet do not encode an identifiable SdhE homologue. Previously, we suggested that alternative FAD assembly factors might exist in these species.[4,15] However, it is possible that the rate of SdhA autocatalytic flavinylation is sufficient for SDH activity in these non-SdhE-containing species.

The G78R mutation in SdhAF2, the human SdhE homologue, is associated with cancer because of an inability to flavinylate SDH.[3,14] An equivalent variant, SdhE(G16R), was unable to flavinylate SdhA. Interestingly, a G16A variant did not affect SdhE function, consistent with an alanine at this position in the β-proteobacterium *Azoarcus* sp. BH72 (Figure 4A). It was previously hypothesized that Sdh5(G78R) was incorrectly folded and impaired for interactions with Sdh1.[3] However, in the bacterial system tested in this study, we demonstrate that SdhE(G16R) was not detectably impaired for protein folding or interactions with SdhA. The SdhE(G16R) impairment of SDH activity supports the interaction between SdhE(G16R) and SdhA. These differences between Sdh5(G78R) and SdhE(G16R) might highlight differences between eukaryotic and prokaryotic mechanisms of SDH flavinylation. Possible differences are likely to be due to the presence of 50 additional N-terminal residues in eukaryotic Sdh5 compared with bacterial SdhE, which is only 88 amino acids. Despite the ability to correctly fold and interact with SdhA, SdhE(G16R) was unable to flavinylate SDH. Gly is a small neutrally charged residue that commonly provides flexibility rather than directly contributing to catalysis.[34,35] The location of Gly16 in the loop between α-helices 1 and 2 (Figure 4B) suggests that Gly16 might provide flexibility to the SdhE protein backbone. Consequently, mutation to a larger positively charged arginine reduces flexibility and causes function to be lost, while mutation to a neutral alanine preserves this flexibility and function. Alternatively, it is possible that the positively charged arginine substitution may influence the dynamics of, but not prevent, the interaction between SdhE and SdhA.

SdhE(E19A) folded and interacted with SdhA yet failed to activate SDH despite partially flavinylating SdhA. Therefore, Glu19 is a critical residue required for SDH function but not for the interaction with SdhA. Interestingly, despite Glu19 being completely conserved across all SdhE homologues,[15,17,18,36] a

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conservative substitution to aspartate did not affect the activation of SDH in growth assays. Glutamate and aspartate are structurally similar residues (i.e., negatively charged and hydrophilic), suggesting that either the change in charge or hydrophobicity associated with E19A is the likely reason for the loss of function. These results suggest that a negatively charged residue is required at this position. Given the complete conservation of Glu19 within SdhE homologues, it is apparent that a glutamate at this position provides an evolutionary advantage over aspartate. However, this could not be detected under the experimental conditions of this study. Further investigations are required to determine the role of Glu19 in the covalent attachment of FAD to SdhA.

Eukaryotic Sdh5 R68E, Y71D, and W113A variants were impaired for SDH flavinylation.[17] These residues are present in surface-exposed regions adjacent to RGxxE[15,17] (Figure 4B). Recent structural analysis led to the hypothesis that this region was the site for interaction with Sdh1.[17] The study presented here demonstrates that mutations in the RGxxE region did not affect interactions with His-SdhA. It is further hypothesized that Arg15 is a structurally important residue not directly involved in the flavinylation and activation of SDH. This study demonstrates that the RGxxE motif was not required for FAD binding. Interestingly, recent NMR experiments suggest that eukaryotic Sdh5 does not interact with FAD,[17] suggesting that there may be further differences in the bacterial and eukaryotic mechanisms of SDH flavinylation. Further investigations are required to determine the mechanism and physiological relevance of the SdhE−FAD interaction.

Both G16R and E19A impaired the activation of SDH in the presence of WT SdhE. It is proposed that following the formation of an SdhA−SdhE(G16R) or -(E19A) complex, these variants are unable to perform subsequent flavinylation steps, which are currently uncharacterized. On the basis of our results, we propose two scenarios to guide future investigations: (1) SdhE induces small conformational changes in SdhA from an apo to holo form, or (2) SdhE catalyzes the attachment of FAD to SdhA. Neither of these scenarios is mutually exclusive, and both may occur. First, SdhE may induce small conformational changes in SdhA. Consequently, conformational changes occur at a slow rate in the absence of SdhE, and FAD is attached only through an inefficient autocatalytic mechanism. Second, SdhE might participate directly in the chemistry of the covalent attachment of FAD to SdhA. The exact catalytic mechanism is unknown but might involve Glu19, which is conserved across all homologues of SdhE, Sdh5, and SdhAF2. Determining the protein interfaces involved in the SdhE−SdhA interaction will provide significant insights into the mechanism of SdhE-mediated flavinylation. Eukaryotic Sdh5 binds to two Arg residues in the C-terminal tail of Sdh1.[16] This site is distal to the location of FAD in Sdh1, which would support a mechanism involving the induction of long-range conformational changes in Sdh1 induced by Sdh5.

Structures of multiple SdhE homologues have been determined and emphasize the structural similarity between homologues from different kingdoms.[17,28,29] Although models have been proposed, experimental evidence from examination of the molecular mechanism of SdhE/Sdh5-mediated flavinylation is scarce. The study presented here demonstrates that SdhE can interact with and flavinylate a folded form of SdhA in the absence of SDH assembly and that the highly conserved RGxxE motif of SdhE is required for flavinylation. Furthermore, SdhA can covalently attach FAD through an inefficient autocatalytic mechanism in the absence of SdhE. The conservation of SdhE homologues across kingdoms and the important role of SDH in bacterial pathogenesis and human pathologies underscore the value in continued genetic and biochemical analysis of this protein family and the process of complex II flavinylation.

■ ASSOCIATED CONTENT

Supporting Information

Supporting methods, including descriptions of the construction of chromosomal mutants and plasmids expressing tagged and untagged truncation and alanine swap variants; accession numbers for sequences used in sequence alignments; oligonucleotide sequences (Table S1); copurification of His-SdhA and WT and variant FLAG-tagged SdhE on a Ni-NTA resin (Figure S1); and FAD binding by SdhE RGxxE variants (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SDH, succinate dehydrogenase; FAD, flavin adenine dinucleotide; Pig, prodigiosin; WT, wild type; CD, circular dichroism.

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