The succinate dehydrogenase assembly factor, SdhE, is required for the flavinylation and activation of fumarate reductase in bacteria

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ABSTRACT

The activity of the respiratory enzyme fumarate reductase (FRD) is dependent on the covalent attachment of the redox cofactor flavin adenine dinucleotide (FAD). We demonstrate that the FAD assembly factor SdhE, which flavinylates and activates the respiratory enzyme succinate dehydrogenase (SDH), is also required for the complete activation and flavinylation of FRD. SdhE interacted with, and flavinylated, the flavoprotein subunit FrdA, whilst mutations in a conserved RGxxE motif impaired the complete flavinylation and activation of FRD. These results are of widespread relevance because SDH and FRD play an important role in cellular energetics and are required for virulence in many important bacterial pathogens.

Structured summary of protein interactions:

FrdA physically interacts with SdhE by anti tag coimmunoprecipitation (View interaction)

Abbreviations: FRD, fumarate reductase; SDH, succinate dehydrogenase; FAD, flavin adenine dinucleotide

1. Introduction

Oxidative phosphorylation, the dominant means of energy generation in the majority of eukaryotes and bacteria, is driven by two interlinked processes, termed the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) [1,2]. Succinate dehydrogenase (SDH), also known as complex II or succinate-ubiquinone oxidoreductase, oxidizes succinate to fumarate as part of the TCA cycle. This is coupled to the reduction of the mobile electron carrier ubiquinone, thereby donating electrons to the ETC. SDH consists of four protein subunits including a catalytic flavoprotein (SdhA), an iron–sulfur cluster protein (SdhB) and typically two hydrophobic membrane anchor subunits (SdhC and SdhD), which share a ubiquinone binding site [3]. Essential to SDH function is the covalent attachment of the redox cofactor flavin adenine dinucleotide (FAD) to the flavoprotein subunit SdhA [4]. The majority of proteins that bind FAD utilize a non-covalent linkage, with only 10% forming a covalent–FAD linkage. Many of the proteins that covalently bind FAD use an auto-catalytic mechanism that is independent of any accessory proteins or assembly factors [5–10]. However, recent studies identified a protein present in the α, β and γ proteobacteria, termed SdhE, that assists in the covalent attachment of FAD to SdhA [11,12]. Homologues of SdhE are present in yeast, humans and plants, where they also mediate the covalent attachment of FAD to SDH [13–15]. Biochemical characterization of SdhE identified a functionally important RGxxE motif that is required for the flavinylation and activation of SDH [16]. Interestingly, this motif is conserved across bacterial and eukaryotic homologues, suggesting that the mechanism of SdhE-mediated flavinylation of SdhA is conserved across kingdoms [12,16].

Fumarate reductase (FRD), also known as quinol-fumarate reductase, is a key component of anaerobic respiration in many bacterial species. FRD catalyzes the reduction of fumarate to succinate, allowing fumarate to act as a terminal electron acceptor in the absence of oxygen [4,17]. Like SDH, FRD is a four subunit enzyme typically consisting of two membrane-bound anchors (FrdC and FrdD), an iron–sulfur cluster protein (FrdB) and a catalytic flavoprotein subunit (FrdA) that contains a covalently bound FAD cofactor essential for FRD function [18]. To limit ROS production [3,19] and to optimize energy production, bacteria such as Escherichia coli utilize transcriptional regulators so that in the absence of oxygen the genes encoding FRD (frdABCD) are transcribed and those for SDH (sdhCDAB) are repressed, whilst in the presence of oxygen sdhCDAB is transcribed and frdABCD is repressed [20–22].
Given the structural and functional similarity between SDH and FRD, the aim of the current study was to determine if the covalent attachment of FAD to FRD was dependent on the FAD assembly factor, SdhE [12]. In this communication, we report that the bicistronic sdhEygX operon is expressed under conditions when a functional FRD is required, and SdhE is needed for FRD activity in Serratia species and E. coli. SdhE interacted with FrdA and was required for the complete flavinylation and activation of FRD. These data expand the repertoire of proteins that are dependent on SdhE for flavinylation.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Serratia sp. strain ATCC 39006 (Serratia 39006) and E. coli strains were grown at 30 and 37 °C, respectively. Aerobic cultures were grown in Luria Broth (LB: 5 g l⁻¹ yeast extract, 10 g l⁻¹ bacto tryptone and 5 g l⁻¹ NaCl), minimal media (0.1% w⁻¹ (NH₄)₂SO₄, 0.41 mM MgSO₄, 0.25% w⁻¹ glucose or 0.4% w⁻¹ sucinate, 40 mM K₂HPO₄, 14.7 mM KH₂PO₄, pH 6.9–7.1) at 180 rpm, or on LB agar supplemented with 1.5% (w⁻¹) agar (LBA) [23]. Minimal medium with 0.4% w⁻¹ sucinate was buffered with addition of 75 mM HEPES (pH 7.0). All aerobic cultures were grown in 10 ml of medium in 100 ml flasks. Anaerobic cultures were grown in 10 ml serum vials containing 80 ml glycerol-fumarate (GF) medium with 80 mM glycerol and 80 mM fumarate as previously described [24] with low shaking at 50 rpm. The medium was flushed with Nitrogen gas and sealed with a rubber stopper and metal lid prior to sterilisation. Growth (OD₆₀₀) and absorbance were measured in a Jenway 6300 spectrophotometer. When required, media were supplemented with antibiotics as follows: kanamycin 50 µg ml⁻¹ (Km), ampicillin 100 µg ml⁻¹ (Ap) and chloramphenicol 25 µg ml⁻¹ (Cm).

2.2. Strain and plasmid construction

Molecular biology techniques, unless stated otherwise, were performed using standard techniques. Oligonucleotides are shown in Table 1. Strain CC118 was a gift from Min Wang. Other strains and plasmids used in this study are shown in Table 3. The Serratia 39006 AfrdABCD::Cm deletion single and double mutants were constructed using an allelic exchange strategy similar to that described previously [25,26]. Details are provided in the Supplementary material and methods. Construction details for plasmids over expressing N-terminally His-tagged FrdA are also provided in the Supplementary material and methods.

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/phenotype</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Escherichia coli</td>
<td>araD, Δ(ara, leu), ΔlacZ4787, phoA20, galK, thi-1, rpsE, rpmR, argE, recA1, λpir</td>
<td>[33]</td>
</tr>
<tr>
<td>CC118</td>
<td>araBAD, recA1, lacZYA–argF1169, endA1, recA1, hsdR17 (λc mλc), deoR, thi-1, supE44, λ, gyrA96, relA</td>
<td>Gibco/BRL</td>
</tr>
<tr>
<td>DH5α</td>
<td>recA, pro, hsdR, recA::RPl-2-Tc::Mu, λpir, TrpR, SpR, SmR</td>
<td>[35]</td>
</tr>
<tr>
<td>S17-1</td>
<td>thi-1, thr, leu, tnaA, lacY, supE, recA::RPl-2-Tc::Mu, λpir, KmR</td>
<td>[35]</td>
</tr>
<tr>
<td>H26</td>
<td>Marker exchange mobilization strain for conjugal transfer</td>
<td>[34]</td>
</tr>
<tr>
<td>BW25113</td>
<td>ΔsdhE::Cm, ΔsdhF::AprdABC::Cm</td>
<td>[11]</td>
</tr>
<tr>
<td>JW2665</td>
<td>ΔsdhE::Cm, ΔsdhF::AprdABC::Cm</td>
<td>[11]</td>
</tr>
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<td>JW0713</td>
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<td>[11]</td>
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<tr>
<td>JW4114</td>
<td>ΔsdhE::Cm, ΔsdhF::AprdABC::Cm</td>
<td>[11]</td>
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2.3. β-Galactosidase assays

β-Galactosidase assays on the samples obtained from sdhEygX::lacZ were performed as described previously and expressed as Miller units (MU) [27].

2.4. His-tagged protein purification

All His-tagged proteins were purified using Ni–NTA agarose as previously described [11]. Details are provided in the Supplementary materials and methods.

2.5. Co-immunoprecipitation

Co-immunoprecipitation experiments were performed using anti-FLAG agarose as previously described [11,27]. Details are provided in the Supplementary materials and methods. Proteins were visualized by Western blotting.

2.6. Western blotting

Western blotting of SDS–PAGE gels was carried out using a Tris/ glycine buffer system following standard protocols. 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 and were visualized in an Odyssey FC Gel Doc.

2.7. Stability of SdhE variants

The stability of N-terminal His-tagged SdhE variants expressed in E. coli strain BW25113 was assessed by Western blotting. Cultures were grown anaerobically in GF medium from a starting ODb₀₀ of 0.06. Cultures were induced at 0 h with 0.01 mM IPTG and samples were collected after 72 h of growth. Twenty-five µl of ODb₀₀ adjusted culture was loaded per lane and analyzed by Western blotting.

2.8. FAD-UV and excitation/emission spectra of FAD

FAD binding assays and FAD-UV gel assays were performed as previously described [11]. Aliquots of each sample were separated on two gels for visualization using Coomassie Blue staining and FAD-UV. For excitation–emission analysis, 100 µl of FAD was loaded per lane and analyzed by centrifugation.
samples were resuspended in 50 μl of 6 M Guanidine HCl (dissolved in 0.1 M NaPO₄ pH 7). Resuspended protein samples were aliquoted into a 96 well microtitre plate, excited at a wavelength of 450 nm and the resulting emission between 500 and 600 nm was measured [28]. FAD produces a peak at approximately 530 nm. Measurements were performed in a Varioskan Flash Multimode Reader (Thermo Scientific) plate reader.

2.9. Fumarate reductase assays

Inverted membrane vesicles for FRD assays were prepared from microaerophilic cultures as described in the Supplementary materials and methods. FRD activity was measured by following the decrease in absorbance of benzyl viologen at 602 nm at 37 °C previously described [29]. Details are provided in the Supplementary materials and methods and FRD activity is expressed as μmol fumarate reduced min⁻¹ mg⁻¹.

3. Results

3.1. sdhE is expressed under aerobic and anaerobic conditions

It was hypothesized that if SdhE flavinylated FRD the bicistronic operon sdhEygfX, which includes the membrane protein YgfX [30], would be transcribed under both aerobic (SDH active) and anaerobic (FRD active) conditions. To test this hypothesis, a Serratia 39006 strain with a sdhEygfX::lacZ transcriptional fusion was grown under anaerobic conditions in GF medium (sdhCDAB is repressed),
and aerobic conditions with glucose (sdhCDAB is repressed) or succinate (sdhCDAB is transcribed) as a sole carbon source [20–22]. The sdhEygfX::lacZ operon was expressed to similar levels under aerobic conditions when either glucose or succinate was the sole carbon source, with only a 1.2-fold increase noted with succinate (Table 4). Similar sdhEygfX::lacZ expression was also observed with glycerol as the sole carbon source under anaerobic and aerobic conditions with only a 1.2-fold increase observed under anaerobic conditions (Table 4). In conclusion, sdhEygfX is transcribed under conditions that require either a functional SDH or FRD.

3.2. The deletion of sdhE in Serratia 39006 impairs anaerobic growth

To investigate if SdhE is required for FRD activity, wild-type (WT) and ΔsdhE mutant Serratia 39006 strains were grown in the

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Terminal electron acceptor</th>
<th>Oxygen</th>
<th>Fumarate</th>
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<tbody>
<tr>
<td>Succinateb</td>
<td></td>
<td>394 ± 24</td>
<td>ND</td>
</tr>
<tr>
<td>Glucoseb</td>
<td></td>
<td>307 ± 9</td>
<td>ND</td>
</tr>
<tr>
<td>Glycerolc</td>
<td></td>
<td>563 ± 33</td>
<td>672 ± 73</td>
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</table>

ND = Not determined.

a Samples were taken from cultures in early stationary phase and assessed for β-galactosidase activity (Miller units).

b Cultures were grown in minimal media containing glucose or succinate as a sole carbon source.

c Cultures were grown in glycerol fumarate medium, with fumarate omitted when oxygen was an electron acceptor.
absence of oxygen in GF medium. WT *Serratia* 39006 grew when both glycerol and fumarate were supplied together, but not when glycerol was supplied alone (Fig. 1A). A small amount of growth on fumarate was observed with WT *Serratia* (Fig. 1A). Deletion of *sdhE* (Δ*sdhE*) resulted in a partial growth defect under anaerobic growth in GF medium (Fig. 1B). The loss of SDH (Δ*sdhCDAB*::Kan) had no effect on growth compared with the WT, whilst the double Δ*sdhCDAB*::Kan, Δ*sdhE* mutant had a growth defect similar to Δ*sdhE* (Fig. 1B). Deletion of FRD (Δ*frdABCD*::Cm) resulted in a significant growth impairment similar to the growth of WT *Serratia* with fumarate (Fig. 1C). The double Δ*frdABCD*::Cm, Δ*sdhE* mutant had a growth impairment similar to Δ*frdABCD*::Cm (Fig. 1C). In conclusion, a functional FRD is required for optimal growth of *Serratia* 39006 under anaerobic conditions in GF medium. Furthermore, deletion of *sdhE* affects the growth of *Serratia* 39006 under these conditions, independent of SDH activity.

### 3.3. Deletion of *sdhE* in *E. coli* impairs anaerobic growth

The anaerobic growth of *Serratia* 39006 with fumarate alone complicated the analysis of whether SdhE was required for FRD activity. SdhE is functionally conserved between *Serratia* 39006 and *E. coli*, where it is also required for SDH activation [11,16]. To further investigate if SdhE was required for FRD activity, *E. coli* was utilised. Consistent with published data, WT *E. coli* could not grow anaerobically with glycerol or fumarate individually, but grew on glycerol and fumarate together (Fig. 1D) [24]. The deletion of *sdhA* did not impair anaerobic growth on glycerol and fumarate (Fig. 1E). Deletion of *frdA* resulted in a complete growth impairment, demonstrating that a functional FRD is essential for anaerobic growth in GF medium (Fig. 1E). Deletion of *sdhE* resulted in a less marked growth impairment (Fig. 1E). The reduced growth of Δ*sdhE*::Kan in GF medium was complemented by in trans expression of SdhE from both *E. coli* and *Serratia* 39006 (Fig. 1F). In conclusion, *sdhE* affects anaerobic growth of *E. coli* on glycerol and fumarate, independent of SDH activity.

### 3.4. *sdhE* is required for fumarate reductase activity

We predicted that the decreased anaerobic growth of *sdhE* mutants in GF medium was due to reduced FRD activity. To investigate this, the FRD activity of WT *E. coli*, Δ*sdhA*::Kan, Δ*frdA*::Kan and Δ*sdhE*::Kan deletion mutants was determined using membrane vesicles. Deletion of *sdhE* resulted in a 70% reduction in fumarate reductase activity compared with the WT (Fig. 2A). The absence of *frdA* completely abolished fumarate reductase activity, whilst the *sdhA* deletion mutant retained most of the activity compared with WT. These results correspond well with the growth defects observed in these *E. coli* mutants (Fig. 1E). In summary, SdhE is required for the complete activation of FRD under anaerobic conditions.

### 3.5. *sdhE* is required for complete flavinylation of FrdA

To investigate if the reduced FRD activity in *sdhE* mutants was due to the absence of covalently bound FAD, the N-terminally His-tagged flavoprotein subunit FrdA (His-FrdA) was purified from WT and Δ*sdhE* *Serratia* 39006. Covalently attached FAD will co-migrate with purified proteins when separated by SDS–PAGE whilst non-covalently bound FAD is dissociated [11]. Visualization of SDS gels under UV-light (i.e. FAD-UV) produces a fluorescent signal at the same molecular weight as the protein-FAD complex of...
interest [11]. His-FrdA purified from WT *Serratia* 39006 contained covalently bound FAD when analysed by FAD-UV (Fig. 2B). His-FrdA purified from ΔsdhE *Serratia* 39006 demonstrated a reduced FAD signal (Fig. 2B). This is consistent with the reduced FAD content in His-SdhA when purified from ΔsdhE (Fig. 2B) [11]. FAD emission/excitation analyses following acid precipitation validated these results, with His-FrdA from WT *Serratia* producing a spectral curve consistent with FAD, whilst His-FrdA from ΔsdhE produced a FAD spectral curve at a reduced intensity relative to the WT sample (Fig. 2C). In conclusion, SdhE is required for the complete flavinylation of His-FrdA.

### 3.6. SdhE interacts with FrdA

Essential to SdhE flavinylation of SdhA, is the interaction between SdhE and SdhA [11,12,16]. To investigate if SdhE interacted with the flavoprotein FrdA, co-immunoprecipitation experiments were performed using SdhE-FLAG (Bait) and His-FrdA (Prey) on an anti-FLAG resin. These results demonstrate that His-FrdA co-purified with SdhE-FLAG on an anti-FLAG agarose (Fig. 2D). His-FrdA could not be purified on an anti-FLAG agarose in the absence of SdhE-FLAG (Fig. 2D). In conclusion, SdhE interacts with the flavoprotein subunit FrdA.

### 3.7. The RGxxE motif of SdhE is required for FRD function

SdhE variants of the RGxxE motif are impaired for flavinylation and activation of SDH [16]. To investigate if SdhE flavinylated FRD with a similar mechanism, seven *Serratia* 39006 SdhE site-directed variants were assessed in phenotypic rescue assays of *E. coli* ΔsdhE::Kan mutants. Consistent with a recent study [16], the SdhE(G16R) and (G19A) variants failed to rescue aerobic growth in minimal succinate medium, whilst the SdhE variants R15A, G16A, D21A, F27A and D51A were functional (Fig. 3A and data not shown). The SdhE variants G16A, D21A, F27A and D51A rescued anaerobic growth in GF medium to levels similar to WT SdhE (data not shown). The SdhE(G16R) variant failed to rescue *E. coli* ΔsdhE::Kan grown anaerobically in GF medium (Fig. 3B). The SdhE(E19A) variant that does not activate SDH (Fig. 3A) rescued anaerobic growth of ΔsdhE::Kan (Fig. 3B), whilst the SdhE(R15A) variant that activates SDH, failed to rescue anaerobic growth of ΔsdhE::Kan (Fig. 3B). Furthermore, His-SdhE variants (R15A, G16R and E19A) are stably expressed in WT *E. coli* grown anaerobically in GF media (Fig. 3C). In conclusion, the RGxExE motif of SdhE is required for the activation of FRD, but in a different manner to the activation of SDH.

### 3.8. Mutations in the SdhE RGxxE motif affect flavinylation but not interactions with FrdA

To investigate if SdhE(R15A) and (G16R) were impaired for the flavinylation of FrdA, His-FrdA was purified from *Serratia* 39006 ΔsdhE co-expressing C-terminal FLAG-tagged SdhE(WT, R15A, G15R or E19A). Both WT and SdhE(E19A) restored the covalent attachment of FAD to His-FrdA (Fig. 4A and B). The non-functional SdhE(G16R) variant failed to flavinylate His-FrdA to levels greater than the ΔsdhE mutant (Fig. 4A and B). In conclusion, the Gly16, but not Arg15 or Glu19, of the RGxxE motif is required for the covalent flavinylation of His-FrdA.

The G16R and E19A SdhE variants are unable to fully flavinylate or activate SDH, yet still interact with SdhA [16]. To investigate if the SdhE variants (R15A) and (G16R) were impaired for interactions with FrdA, co-immunoprecipitation experiments were performed using His-FrdA (Bait) and SdhE(WT, R15A, G16R)-FLAG (Prey) on an anti-FLAG resin. These results demonstrate that His-FrdA co-purified with the SdhE variants (R15A) and (G16R) on an anti-FLAG agarose (Fig. 4C). In conclusion, the non-functional SdhE(R15A) and (G16R) still interact with the flavoprotein target, FrdA.

### 4. Discussion

The current study has shown that SdhE is required for the complete flavinylation and activation of FRD under anaerobic conditions in *Serratia* 39006 and *E. coli*. Consistent with the SdhE-dependent activation of SDH, FRD showed a reduced level of covalent FAD incorporation in the absence of SdhE. A low, but detectable, level of covalently bound FAD in the flavoprotein subunit FrdA was found in the absence of SdhE. It is likely that this background flavinylation is responsible for the partial FRD activity and growth under anaerobic conditions observed in *E. coli* and *Serratia* 39006 *sdhE* mutants. These results also suggest that FrdA
covalently attaches FAD through an inefficient auto-catalytic mechanism in the absence of SdhE. This observation supports our previous report demonstrating that SdhA can incorporate FAD through an inefficient auto-catalytic mechanism in the absence of SdhE [16]. Combined, these observations suggest that SdhE increases the rate of an auto-catalytic covalent FAD attachment to complex II enzymes (i.e. SDH and FRD) required to support cellular growth in the α, β and γ-proteobacteria.

It is predicted that the interaction between SdhE and SdhA is an important step in the flavinylation process. Here we have demonstrated that SdhE also interacts with the flavoprotein subunit FrdA, suggesting that SdhE may utilize a similar mechanism to flavinylate both SDH and FRD. The analysis of SdhE variants demonstrated that different mutations in the RGxxE motif have different effects on the activation of FRD and SDH. SdhE(R15A) flavinylated and activated SDH, but did not activate FRD. Despite the decreased activation of FRD by SdhE(R15A), it still interacted with and flavinylated FrdA. These results suggest that Arg15 of the RGxxE motif is not required for SDH activation but participates in the activation of FRD. Conversely, the SdhE(E19A) variant, that is impaired for the complete flavinylation and activation of SDH [16], flavinylated and activated FRD. These results suggest that Glu19 is important for SDH, but not FRD, activation. The SdhE(G16R) variant cannot flavinylate or activate SDH [16] or FRD, yet was not impaired for interaction with FrdA. Combined, these results suggest that the RGxxE motif of SdhE homologues is important for the flavinylation of both SDH and FRD. Furthermore, these results suggest that there are similarities, yet subtle differences in the mechanism of SdhE-mediated flavinylation of SdhA and FrdA.

On the basis of the results presented here and previous studies [11,16,20–22], the following model is proposed. Following the transition from aerobic to anaerobic conditions, and vice versa, transcriptional regulators ensure that the appropriate complex II respiratory enzyme is transcribed (i.e. FRD under anaerobic and SDH under aerobic conditions), whilst the transcription of sdhE remains constant. By targeting and interacting with a conserved, yet unidentified, motif present in both FrdA and SdhA, SdhE can facilitate the covalent attachment of FAD prior to the assembly of the appropriate respiratory enzyme complex [16]. Consequently, it is hypothesized that SdhE does not differentiate between the flavinylation targets, instead the decision of what enzyme is flavinylated is made by the cell at a transcriptional level in response to environmental stimuli.

This current study has further extended the repertoire of proteins that are dependent on SdhE for the covalent attachment of FAD. The conservation of SdhE homologues across kingdoms, the important role of both SDH and FRD in bacterial metabolism and pathogenesis, and the role of the eukaryotic homologue Sdh5 in

![Fig. 4. RGxxE variants of SdhE interact with FrdA: (A) Four μg of His-FrdA purified from ΔsdhE Serratia 39006 co-expressing SdhE(WT)-FLAG (pMAT7), (R15A)-FLAG (pMAT57), (G16R)-FLAG (pMAT59) or (E19A)-FLAG (pMAT60). Purified proteins were separated on 12% SDS–PAGE gels. Proteins were detected by Coomassie staining (top panel) and FAD content was detected using FAD-UV assays (bottom panel). (B) Excitation/emission analysis of 100 μg His-FrdA purified from (A). (C) Co-immunoprecipitation of His-FrdA, used as a bait protein, and SdhE(WT/R15A/G16R)-FLAG, used as the prey, performed on anti-FLAG agarose. Input: Total expression of protein prior to lysis; FW: Final wash fraction; Elute: Elution fraction.]

cancer progression [13,31,32], underscore the value in continued analysis of this protein family to decipher the molecular mechanism of complex II flavinylation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.12.019.

References