Biochimica et Biophysica Acta 1827 (2013) 637–647

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

Prokaryotic assembly factors for the attachment of flavin to complex II ☆

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ARTICLE INFO

Article history:
Received 30 July 2012
Received in revised form 5 September 2012
Accepted 7 September 2012
Available online 14 September 2012

Keywords:
Flavin adenine dinucleotide
Flavoprotein
Succinate dehydrogenase
sdhA
sdhE
sdh5

ABSTRACT

Complex II (also known as Succinate dehydrogenase or Succinate–ubiquinone oxidoreductase) is an important respiratory enzyme that participates in both the tricarboxylic acid cycle and electron transport chain. Complex II consists of four subunits including a catalytic flavoprotein (SdhA), an iron–sulphur subunit (SdhB) and two hydrophobic membrane anchors (SdhC and SdhD). Complex II also contains a number of redox cofactors including haem, Fe–S clusters and FAD, which mediate electron transfer from succinate oxidation to the reduction of the mobile electron carrier ubiquinone. The flavin cofactor FAD is an important redox cofactor found in many proteins that participate in oxidation/reduction reactions. FAD is predominantly bound non-covalently to flavoproteins, with only a small percentage of flavoproteins, such as complex II, binding FAD covalently. Aside from a few examples, the mechanisms of flavin attachment have been a relatively unexplored area. This review will discuss the FAD cofactor and the mechanisms used by flavoproteins to covalently bind FAD. Particular focus is placed on the attachment of FAD to complex II with an emphasis on SdhE (a DUF339/SDH5 protein previously termed YgfY), the first protein identified as an assembly factor for FAD attachment to flavoproteins in prokaryotes. The molecular details of SdhE-dependent flavinylation of complex II are discussed and comparisons are made to known cofactor chaperones. Furthermore, an evolutionary hypothesis is proposed to explain the distribution of SdhE homologues in bacterial and eukaryotic species. Mechanisms for regulating SdhE function and how this may be linked to complex II function in different bacterial species are also discussed. This article is part of a Special Issue entitled: Respiratory complex II: Role in cellular physiology and disease.

1. Introduction

Many protein complexes require one or more specific cofactors for function. Cofactors are typically recruited to the active sites of proteins and influence the chemistry that is performed [1–5]. Cofactors can be bound to target proteins either non-covalently or covalently, with different proteins able to bind the same cofactor with identical chemistry [1–5]. Alternatively, different proteins can bind various parts of the same cofactor using either non-covalent or covalent linkages. Consequently, there is a large diversity in protein–cofactor interactions. Furthermore, cofactors can be incorporated into proteins through either autocatalytic mechanisms or mechanisms that require accessory proteins or enzymes.

Oxidative phosphorylation is driven by the electron transport chain (ETC) and the tricarboxylic acid (TCA) cycle, which couple the oxidation of respiratory substrates to the generation of a proton gradient to drive the generation of cellular energy in the form of ATP [6–8]. Complex II (also known as succinate:quinone oxidoreductase or succinate dehydrogenase [SDH]) is the only membrane bound respiratory complex that participates in both the ETC and the TCA cycle. Furthermore, complex II is found in the majority of bacterial, archaeal and eukaryotic species, making it an integral component of oxidative phosphorylation amongst all organisms [9,10]. Complex II functions by coupling the oxidation of succinate to fumarate with the translocation of electrons to ultimately reduce the electron carrier ubiquinone to ubiquinol, and thereby donating electrons to the ETC [9–12]. Electron translocation to the ubiquinone binding site proceeds through the redox cofactors flavin adenine dinucleotide (FAD) and three Fe–S clusters ([2Fe–2S], [4Fe–4S], and [3Fe–4S]) that reside in flavoprotein and iron–sulphur subunits respectively [9,13]. Unlike complexes I and III of the ET, the electron translocation mediated by complex II does not contribute to the proton motive force that drives ATP synthesis [9,13]. Fumarate reductase (FRD) is an enzyme paralogue of complex II, which has a similar structural arrangement of four subunits, including a flavoprotein and iron–sulphur subunit, which performs the reverse reaction to complex II, i.e. reduction of fumarate to succinate, as part of anaerobic respiration [12,14–16]. Such is the sequence, structural and functional similarity between complex II and FRD that FRD is able to partially compensate for the loss of complex II and vice versa [17], leading to the hypothesis that complex II and FRD have evolved from a common ancestor [12,14].

The focus of this review is the mechanism by which FAD is attached to prokaryotic (specifically bacterial) complex II. Specific attention will

Abbreviations: FAD, Flavin adenine dinucleotide; TCA, Tricarboxylic acid; ETC, Electron transport chain

☆ This article is part of a Special Issue entitled: Respiratory complex II: Role in cellular physiology and disease.

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0005-2728/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbabio.2012.09.003

be paid to SdhE, the only identified FAD cofactor chaperone that was recently identified and the role that it has in activating complex II.

2. The redox cofactor FAD

Proteins that bind FAD cofactors, termed flavoproteins, play an important role in a diverse array of biological reactions by catalysing one and two electron oxidation/reduction reactions [18]. Mansoorabadi et al. [18] provides an excellent review that covers the biological diversity of flavoproteins. Structurally, the redox cofactor FAD is formed from an isoalloxazine ring linked to a ribitol group through a carbon-nitrogen bond, forming a riboflavin moiety, which is subsequently bound to an ADP molecule (Fig. 1A) [1,19,20]. The biosynthesis of the riboflavin moiety in prokaryotes involves a series of reactions that starts from one molecule of GTP and two molecules of ribulose 5-phosphate [21,22]. Riboflavin kinase converts riboflavin into the flavin cofactor flavin mononucleotide (FMN), with FAD synthetase catalysing the adenylylation of FMN to form FAD [21,22]. In many prokaryotic species the activities of riboflavin kinase and FAD synthetase are performed by a single bifunctional enzyme [23–28]. FAD switches between oxidised (FAD) and reduced ($FADH_2$) forms and facilitates the transfer of electrons between different chemical species (Fig. 1A) [1,20]. The complete reduction of FAD requires two electrons, so a semi-quinone flavin (FADH) is formed as an intermediate following one electron reduction reactions [20]. The isoalloxazine ring is the core component of flavin cofactors that is responsible for accepting and donating electrons. Furthermore, the isoalloxazine ring, when in an oxidised form, is responsible for the characteristic yellow colour of FAD. For a more detailed description of FAD chemistry and catalysis there are a number of excellent reviews [18–20,29].

2.1. FAD-flavoprotein linkages

Whilst the majority of flavoproteins bind FAD non-covalently, approximately 10% of flavoproteins covalently bind FAD [1,30]. The covalent bonds are characterised by the interaction between the 8α-methyl position of the isoalloxazine ring of FAD and either a Cys, His or Tyr residue of the flavoprotein. This results in either an 8α-*N*3-Histidyl, 8α-*N*1-Histidyl, 8α-*O*-Tyrosyl or 8α-*S*-Cysteinyl FAD covalent linkage (Fig. 1B). Interestingly, several bacterial flavoproteins are able to bind a single FAD molecule via bonds mediated by two amino acids, such as the 8α-*N*1-Histidyl-6-*S*-Cysteinyl bicovalent bond of aclacinomycin oxidoreductase (AknOx) [31–33]. AknOX participates in aclacinomycin biosynthesis, catalysing two consecutive FAD-dependent reactions [31]. It is hypothesised that by containing bicovalently-bound FAD, AknOx is able to use the same active site by making use of two distinct sets of catalytic residues in the two reactions [31]. Several flavoenzymes are also able to covalently bind the alternative flavin redox cofactor FMN. Like FAD, FMN can be covalently attached to His residues via 8α-*N*1-Histidyl and 8α-*N*3-Histidyl linkages or through the 8α-*N*1-Histidyl-6-*S*-Cysteinyl bicovalent bond [30,34–36]. Furthermore, FMN can be covalently attached to flavoenzymes through the FMN-specific 6-*S*-cysteinyl and phosphoester-threonyl covalent bonds [1,30,37–39]. The maintenance of covalently-bound FAD throughout evolution has raised interesting questions regarding the biological relevance of covalent FAD attachments. In recent years it has become apparent that covalently-bound FAD increases the redox potential of flavoproteins relative to non-covalently bound FAD [30] and is required for the structural integrity of some protein complexes [30]. It has also been hypothesised that covalent attachment may prevent the loss of FAD from membrane proteins that reside in cellular environments where FAD levels are low and difficult to replace if lost [1].

2.2. Prokaryotic mechanisms of FAD incorporation

Cofactor incorporation into target proteins can proceed through either an autocatalytic mechanism or by mechanisms dependent on accessory proteins often termed maturation factors, assembly factors or cofactor chaperones [5,40–48]. A number of different cofactor chaperones have been identified in various prokaryotic species, with examples listed in Table 1. The mechanisms of cofactor chaperones are diverse, including folding the target protein into a conformation that favours the binding of the cofactor, delivering the cofactor to the target protein or by assisting in the reaction of cofactor attachment [2,3,5,40,43–45,47,49–53]. However, these mechanisms are

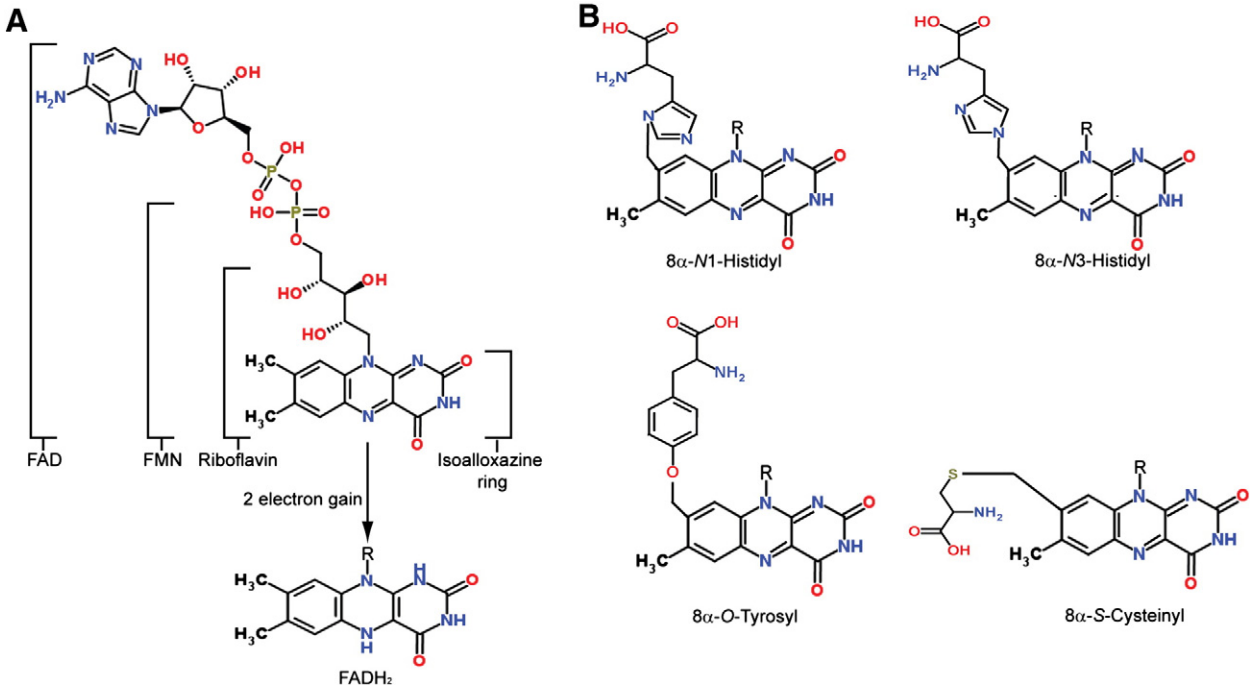

Fig 1. Flavin adenine dinucleotide and the covalent attachment of FAD to flavoproteins. (A) Chemical structure of the various components of FAD and FMN and the reduced form $FADH_2$. (B) Schematic representation of the different covalent linkages formed between the 8α methyl group of the isoalloxazine ring of FAD and either His, Tyr and Cys residues.

Table 1
Examples of prokaryotic cofactor chaperones.

Cofactor chaperone	Co-factor	Target protein	Function of target protein	Species	Reference
CopZ	Copper	CopA	Copper resistance	*B. subtilis*	[45,117–120]
CopZ	Copper	CopY	Copper homeostasis	*Enterococcus hirae*	[121–123]
CcmE	Haem	Cytochrome *c*	Electron carrier	*E. coli*	[47,104,105]
HypC	Ni–Fe	Ni–Fe hydrogenase (HypE)	Hydrogenase 3 (H_2 uptake)	*E. coli*	[46,124–125]
TorD	Molybdenum	TorA	Trimethylamine oxide reductase	*E. coli*	[40,51]
NarJ	Molybdenum	NarA	Nitrate reductase	*E. coli*	[44,126]
XdhC	Molybdenum	Xanthine dehydrogenase	Purine metabolism	*Rhodobacter capsulatus*	[53,127–129]

not necessarily mutually exclusive and one or all might be performed by a cofactor chaperone.

The identification of prokaryotic flavoproteins that covalently bind to FAD, apparently in the absence of accessory proteins, has led to the conclusion that covalent FAD incorporation into flavoproteins proceeds through an autocatalytic mechanism. The autocatalytic incorporation of FAD has been demonstrated by purifying the flavoprotein of interest in an apo-form (i.e. lacking FAD) and determining whether the apoprotein is able to incorporate FAD as detected using various *in vitro* biochemical techniques. The incubation of excess FAD with apo-monomeric sarcosine oxidase (MSOX), purified from a riboflavin-deficient mutant, resulted in the covalent attachment of FAD to MSOX via an 8α-*S*-Cysteinyl linkage and the restoration of catalytic activity in both a time- and concentration-dependent manner [54–56]. Similar to MSOX, apo-vanillyl-alcohol oxidase (VAO) was purified from a riboflavin-deficient mutant and incubated with excess FAD, which resulted in the autocatalytic covalent attachment of FAD via an 8α-*N*3-Histidyl bond [57,58]. FAD binding was detected by SDS-PAGE, tryptophan fluorescence and restoration of enzymatic activity [57,58]. The autocatalytic incorporation of FAD also promoted VAO multimerisation in the form of dimers and octamers [57,58]. Purification of apo-6-hydroxy-D-nicotine oxidase (6-HDNO) and incubation with FAD resulted in autocatalytic flavinylation and formation of the holoenzyme when assessed by SDS-PAGE and increases in enzyme activity [59]. The crystal structure of 6-HDNO demonstrated that FAD is attached via an 8α-*N*1-Histidyl linkage [60]. Furthermore, the presence of glycerol and sucrose improved the rate of 6-HDNO autoflavinylation, possibly by stabilising the apoenzyme and promoting conformational changes required for holoenzyme formation [59]. Finally, bacterial *p*-cresol methylhydroxylase (PCMH) exists as a $\alpha_2\beta_2$ tetramer with both α flavoprotein subunits (PchF) covalently binding FAD via an 8α-*O*-tyrosyl linkage [61,62]. By separately purifying the PchF and β cytochrome (PchC) subunits it was demonstrated that PchF autocatalytically incorporates FAD non-covalently [61]. Subsequent interactions with the PchC subunits induce conformational changes in PchF that allow for covalent binding to FAD [61,62].

With the identification of flavoproteins that bind FAD, with some using an autocatalytic mechanism of incorporation (e.g. MSOX, VAO, 6-HDNO and PCMH), structural and sequence alignments have identified a number of FAD attachment motifs [1,30,63]. However, there is no universally-conserved covalent FAD-binding motif [1,30,63]. Although a general reaction scheme for the covalent binding of FAD has been proposed, i.e. the quinone methide mechanism [19,30,64], the absence of a conserved covalent FAD-binding motif makes it difficult to draw conclusions regarding any general mechanism of FAD incorporation in other flavoproteins when characterised motifs are absent.

2.3. Complex II and FAD

The isolation of complex II in the 1950s provided the first experimental evidence for a covalent linkage between FAD and a flavoprotein [65–67]. The complex II–FAD attachment was later defined as 8α-*N*3-Histidyl [68]. Biochemical analysis of bovine heart mitochondrial complex II defined the FAD attachment site at His90 in the flavoprotein subunit Sdh1 [69]. Mutagenesis of the FAD attachment site (His90) to serine in *S. cerevisiae* prevented flavinylation [70], and, on the basis of sequence similarity, it was predicted that His44 was the FAD binding site in *E. coli* complex II [14]. Further biochemical and structural characterisation of *E. coli* complex II has confirmed that FAD is linked to the flavoprotein (SdhA) via an 8α-*N*3-Histidyl bond to His44 [13] (Fig. 2A). Sequence analysis of complex II from both prokaryotic and eukaryotic genomes has demonstrated that the FAD-binding His residue is completely conserved (Fig. 2B), demonstrating the importance of covalently-bound FAD for complex II function [9,14]. Although the attachment of FAD to prokaryotic complex II is thought to be a complex process, requiring the correct folding of the flavoprotein, it has been widely assumed that this process is autocatalytic, not requiring any accessory proteins [9,12].

Complex II has not only an important role in bacterial physiology and metabolism, but also plays an important role in bacterial pathogenesis. In uropathogenic *E. coli* (UPEC) the deletion of *sdhB* (iron sulphur subunit of complex II) resulted in a reduced colonisation of the bladder and kidneys in a murine model of urinary tract infections [71]. In *Salmonella enterica* serovar Typhimurium an *sdhCDA* deletion mutant had attenuated virulence in a murine model [72], whilst a double mutant of fumarate reductase (*frdACBD*) and *sdhCDA* was completely avirulent [73]. Interestingly, the *S.* Typhimurium *frdABCD*, *sdhCDA* double mutant was fully protective against subsequent challenges from WT *S.* Typhimurium, raising the possibility that a *frdABCD sdhCDA* double mutant may be used as a suitable live vaccine [73]. Alternatively, *sdhCDAB* mutant strains of *S.* Typhimurium have an improved survival in resting and active macrophages, suggesting that prokaryotic complex II is targeted by eukaryotic antimicrobial defence mechanisms [74]. Given the importance of complex II in bacterial virulence understanding the mechanism of complex II flavinylation would provide valuable insights into both bacterial metabolism and the establishment of bacterial infections.

In an attempt to understand the attachment of FAD to complex II, a number of interesting observations have been made. Firstly, biochemical analysis of the mutant forms of the flavoprotein subunit (SdhA) from *Bacillus subtilis* demonstrated that covalently-bound FAD was not required for the assembly or stability of complex II in membranes [75]. Secondly, the flavinylation of the SdhA flavoprotein subunit from *E. coli* is stimulated in the presence of succinate and fumarate, suggesting that target substrates might act as allosteric activators [76]. Studies on the eukaryotic flavoprotein subunit (Sdh1) in *Saccharomyces cerevisiae* have suggested that an additional protein component might be required for the flavinylation of Sdh1 [77]. The existence of an additional protein component was shown through the addition of unflavinylated Sdh1 to a mitochondrial matrix, which restored the flavinylation of Sdh1 [77]. However, treating the mitochondrial matrix with protease prior to the addition of unflavinylated Sdh1 prevented FAD attachment [77]. In spite of all of these studies, the mechanism of FAD incorporation into prokaryotic complex II had remained elusive until recently.

3. The identification of SdhE, an FAD attachment factor

Investigations into the functions of proteins that regulate the biosynthesis of the tripyrrole red pigment prodigiosin in the Gram-negative

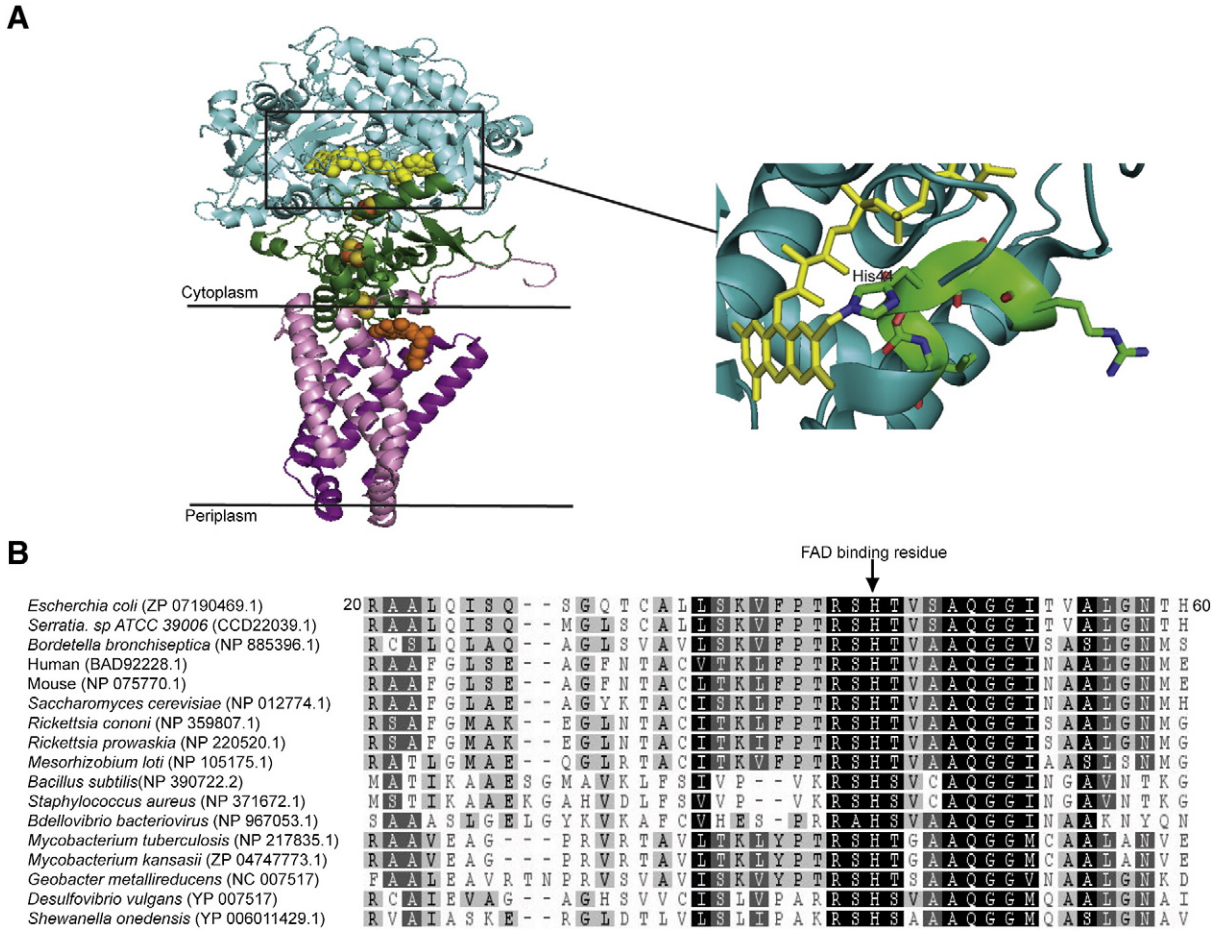

Fig. 2. Prokaryotic complex II and the covalently-bound FAD. (A) The crystal structure of *E. coli* complex II (PDB ID: 1NEK) with SdhA (pale blue), SdhB (green), SdhC (purple) and SdhD (pale pink) colour coded using PyMOL. The yellow cofactor in SdhA is FAD, three Fe–S clusters (pale yellow and red) are present in SdhB and the ubiquinone binding site (orange) is present in SdhC. The zoomed inset highlights the 8α-*N*3-Histidyl linkage between SdhA (His44) and FAD. (B) ClustalW partial sequence alignment of the SdhA flavoprotein subunits from various bacterial and eukaryotic species. This section of sequence highlights the conservation of the His residue that covalently binds FAD (as indicated by an arrow). Shaded residues are those that are conserved between different sequences, with highly conserved residues shaded in black and poorly conserved residues not shaded.

bacterium *Serratia* sp. ATCC 39006 (*Serratia* 39006) have led to the identification of many novel biochemical processes and transcriptional regulators [78–86]. A recent investigation into a putative regulator of prodigiosin serendipitously led to the identification of SdhE (succinate dehydrogenase protein E), previously termed YgfY [80,87]. SdhE is a member of the DUF339 (domain of unknown function/recently renamed SDH5) and COG2938 family of proteins that was listed as a conserved hypothetical protein, the characterization of which was considered to be of high priority [87–89]. SdhE homologues are widespread in both prokaryotic and eukaryotic species [49,87]. Bioinformatic analyses using the STRING database, which identifies putative functional linkages between proteins on the basis of genetic neighbourhood, co-occurrence, co-expression, experimental data and other parameters [90], suggested that SdhE might have a role in succinate metabolism via interaction or association with subunits of complex II [87]. Consistent with reported complex II mutants [11,91–93], an *sdhE* chromosomal deletion mutant (Δ*sdhE*) was unable to grow with succinate as a sole carbon source [87]. Deletion of *sdhE* impaired succinate metabolism due to a non-functional complex II, as determined by complex II activity assays [87]. Interestingly, the individual subunits of complex II correctly localised to the membrane and assembled into a trimeric complex II structure, as determined by denaturing and native gels respectively, suggesting that the loss of *sdhE* specifically impaired the activity but not the formation or stability of complex II [87]. The cytoplasmic localisation of SdhE led to the hypothesis that it might affect complex II function by interaction and activation of the cytoplasmic flavoprotein subunit, SdhA (Fig. 2A). As described above, the prokaryotic and eukaryotic flavoprotein subunit, SdhA, requires a covalently-bound FAD for complex II activity [75,94]. SdhA purified from WT *Serratia* contained covalently-bound FAD cofactor when analysed on denaturing gels, but when SdhA was purified from a Δ*sdhE* mutant FAD was absent [34]. These results demonstrated that *sdhE* was required for the attachment of the redox cofactor FAD to the flavoprotein subunit of complex II [87]. These findings also presented the first identification of an accessory protein that is required for the flavinylation of a flavoprotein in any bacteria.

3.1. SdhE: a FAD cofactor chaperone

The requirement of complex II for SdhE to mediate the attachment of FAD, and subsequent enzymatic activation, encouraged further investigations. Consistent with some previously reported cofactor chaperones, SdhE interacted, albeit weakly, with the target protein SdhA [87]. SdhA that was over-expressed in cells lacking SdhE (Δ*sdhE* + SdhA) was not flavinylated and the addition of exogenous FAD to these Δ*sdhE* + SdhA cell lysates did not restore the binding of FAD to SdhA [87]. However, the addition of purified His-SdhE and exogenous FAD to cell lysates of Δ*sdhE* + SdhA restored the incorporation of FAD into SdhA [87]. These results highlighted that SdhE facilitates the incorporation of FAD into SdhA and that lack of flavinylation of SdhA in the absence of SdhE is not due to defective FAD production [87].

In common with some previously reported cofactor chaperones, SdhE was able to interact with the target cofactor, FAD. His-SdhE purified from WT *Serratia* 39006 did not contain a bound FAD cofactor. However, multiple independent experiments (SDS-PAGE, UV-visible spectra, intact protein mass spectrometry, acid precipitation and TEV-cleavage of the $His_{\times 6}$ tag) demonstrated that SdhE binds FAD and forms an SdhE–FAD complex. Retention of protein–FAD complexes following SDS-PAGE, intact protein mass spectrometry and acid precipitation is commonly used to demonstrate a covalent linkage between flavoproteins and flavin cofactors [1,19,30,54–56,58,59,61,66,68,95,96]. As such, the retention of the SdhE–FAD complex following SDS-PAGE, intact protein mass spectrometry and acid precipitation was consistent with FAD covalently-bound to SdhE [87]. However, because the type and site of FAD linkage have not been characterised, a strong non-covalent interaction between SdhE and FAD cannot yet be unequivocally ruled out.

3.2. The SdhE–FAD complex

The covalent binding of FAD to proteins has been reported for numerous proteins including complex II. The data examining the interaction between SdhE and FAD was consistent with covalent FAD attachment. However, such binding would be highly unusual for a number of reasons. Firstly, FAD covalently binds to highly conserved Cys, His or Tyr residues [1,30]. However, bacterial homologues of SdhE lack any highly conserved Cys, His or Tyr residues (Fig. 3), making it difficult to predict where FAD could covalently bind. Secondly, previous structural characterisation of flavoproteins, including complex II, has shown that covalently attached FAD is present within flavoproteins in characteristic FAD binding folds, e.g. the Rossmann fold [63,97]. Structural genomics initiatives have led to structural determination of SdhE homologues by X-ray crystallography [98] and NMR [99] and demonstrated that these proteins consist of five α helices and lack characteristic FAD binding motifs such as the Rossmann fold (Fig. 4A) [98,99]. Furthermore, the residues that are highly conserved amongst SdhE homologues (e.g. R15, G16, E19, D21 and D51) (Fig. 3), are mainly surface exposed (Fig. 4A) and present in negatively or neutrally charged regions (Fig. 4B). However, these conserved residues represent the major region of the SdhE structure that is conserved amongst bacterial homologues (Fig. 4C), suggesting that this surface exposed region is likely to be of functional importance, possibly via protein–protein interactions or the binding of FAD. Conserved Arg, Asp, Glu and Gly residues often play an important role in the non-covalent attachment of FAD to flavoproteins, as thoroughly discussed by Dym and Eisenberg [63]. Examples include Asp and Glu residues of glutathione reductase that form hydrogen bonds with the adenosine moiety [63,100] and RebC where Arg, Gly and Glu residues establish non-covalent contacts with FAD [101,102]. Interestingly, an SdhE mutation, G16R, reduces the FAD binding affinity, suggesting that this region might be involved FAD attachment [87]. If these highly conserved residues of SdhE are involved in FAD binding, and if the SdhE–FAD linkage is covalent, then it might suggest a novel mechanism of covalent FAD attachment. Moreover, cofactor chaperones that bind target cofactors typically bind reversibly via non-covalent interactions to facilitate cofactor transfer to the target protein. Additionally, dialysis of the SdhE–FAD complex reduced the amount of FAD bound to SdhE and only a subpopulation of SdhE bound FAD (i.e. 0.1 mol of FAD/mol of His-SdhE and ~10% of the total SdhE protein population in intact protein mass spectrometry) [87], which is inconsistent with covalent binding. Furthermore, although the mechanism of FAD transfer remains uncharacterised a high level of energy would be required to

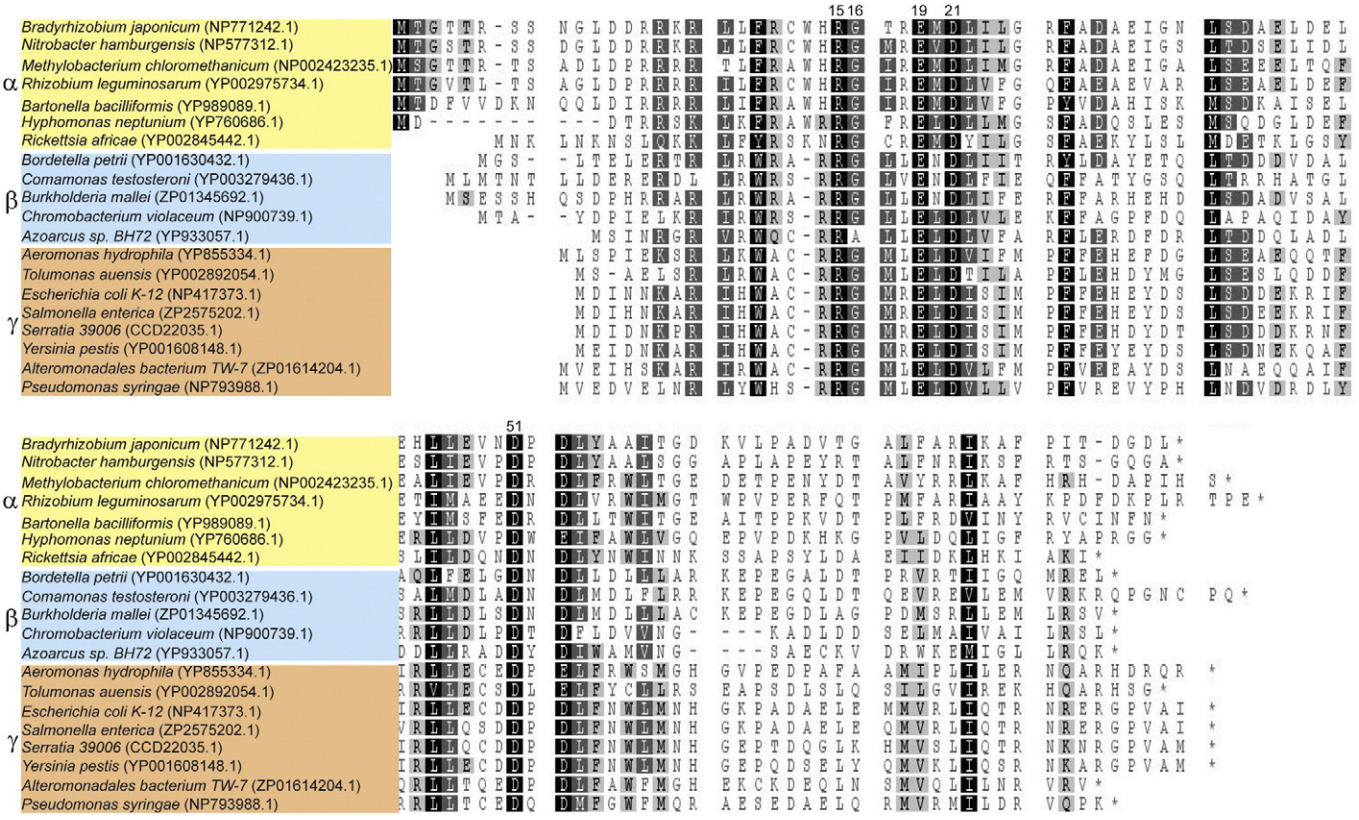

Fig. 3. Sequence analysis of the bacterial flavin attachment factor SdhE. Sequence alignment of SdhE homologues from various bacterial species. The proteobacterial families (α, β and γ) that the sequences are derived from are indicated. The most highly-conserved residues are indicated above the consensus sequence, with the numbers being relevant to their position in the *E. coli* sequence. Shaded residues are those that are conserved between different species, with highly conserved residues shaded in black and unconserved residues unshaded.

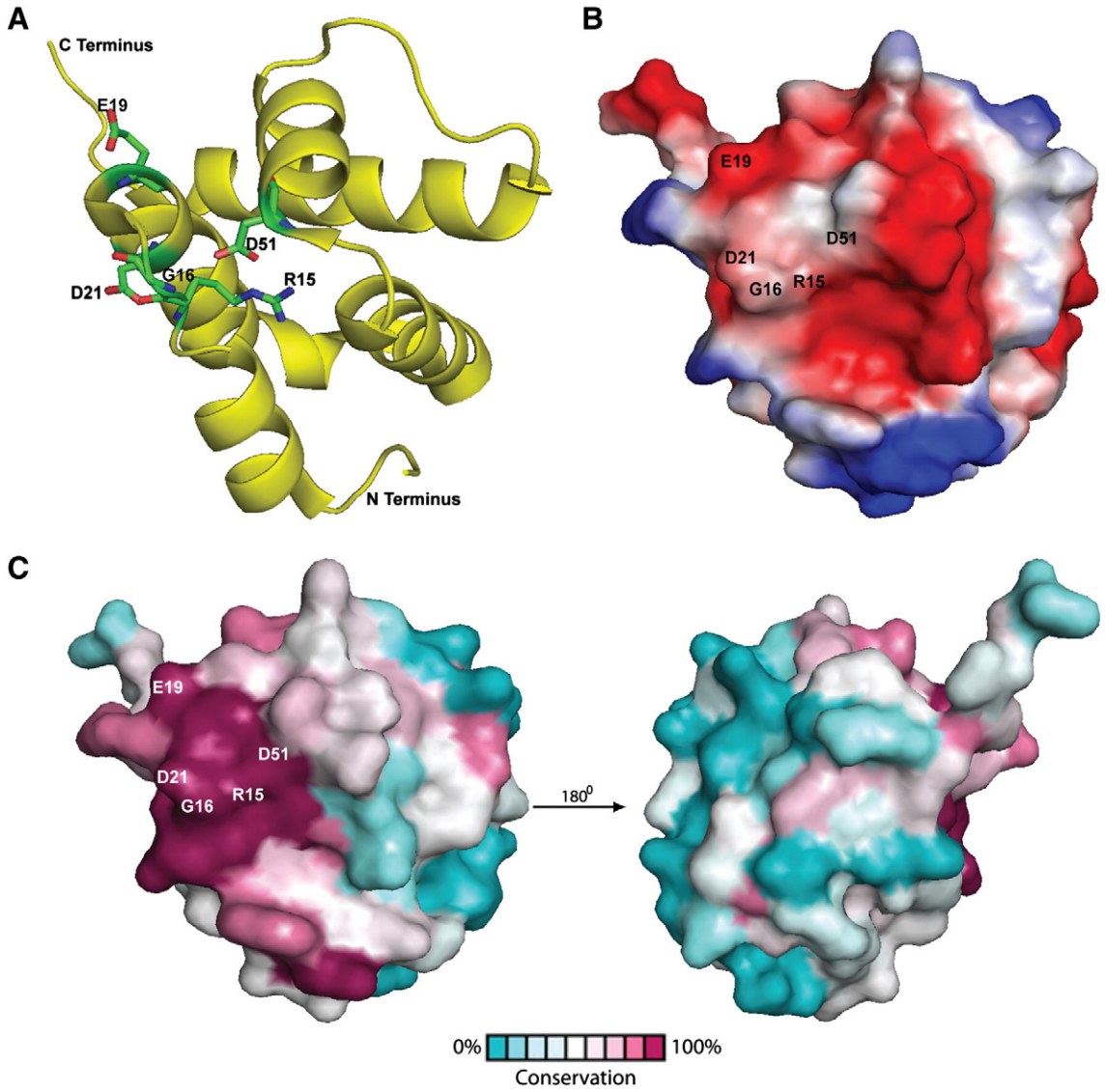

Fig. 4. Structural analysis of SdhE. (A) The crystal structure of *E. coli* SdhE (PDB ID: 1X6J) was visualised using PyMOL. The conserved, surface exposed, residues (R15, G16, E19, D21 and D51) are represented as sticks. (B) Crystal structure of *E. coli* SdhE with protein surface charges visualised using vacuum electrostatics in PyMOL. Blue and red colouring represent positive and negatively charged surfaces, respectively. (C) Surface conserved regions of SdhE homologues. Conserved residues were visualised in PyMOL using the Consurf server [116].

break any covalently-bound FAD to allow FAD transfer from SdhE to SdhA, which would be bioenergetically unfavourable.

However, there is a precedent for cofactor chaperones that covalently bind target cofactors. One example is the cytochrome *c* haem maturation factor (CcmE) from *E. coli*, which covalently binds haem before transferring the haem to apocytochrome *c* [47,48,103,104]. CcmE covalently binds haem via a single His residue that is present in a strictly conserved and unique haem binding motif [47,104]. Mutation of the His residue that binds haem prevents CcmE from covalently binding haem and cytochrome *c* maturation [47,104]. Upon binding haem, CcmE is able to form a CcmE–haem–apocytochrome *c* complex where CcmE and the apocytochrome *c* are linked via the haem binding His residue of CcmE and the haem binding Cys residue of apocytochrome *c* [105]. Although aspects of haem attachment, such as the mechanism of transfer of haem from CcmE to cytochrome *c*, require further characterisation this example provides evidence that some cofactor chaperones bind their cofactors covalently. In the case of SdhE and FAD, only by precisely identifying how SdhE binds FAD can the type of interaction be resolved.

3.3. SdhE: a 'bind and deliver' cofactor chaperone

Using the data presented in McNeil et al. 2012, the following working model of SdhE-dependent flavinylation of complex II was proposed (Fig. 5). Based on the observation that SdhE is purified without FAD bound, we hypothesised that SdhE exists predominately in a form without FAD bound (Fig. 5, step 1). In response to cellular conditions that require an active complex II, it is speculated that SdhE binds FAD to form an SdhE–FAD complex (Fig. 5 step 2), which then interacts with SdhA (Fig. 5, step 3). Next, through an unknown mechanism, FAD is transferred from SdhE to SdhA (Fig. 5, step 4). It is not known if SdhE can only interact with SdhA as an SdhE–FAD complex; consequently following FAD transfer to SdhA, SdhE might be unable to interact with the flavoprotein subunit (Fig. 5, step 4). Once released from activated complex II, SdhE might be able to participate in subsequent complex II flavinylation events.

Further investigations are required to decipher the details of SdhE-dependent flavinylation. Determining how SdhE binds FAD is of fundamental importance for understanding this process. Understanding how

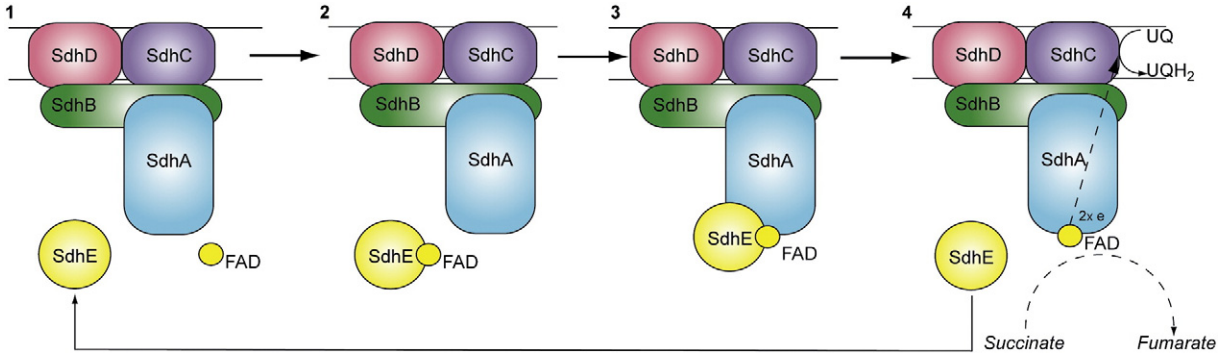

Fig. 5. Working model for SdhE-dependent flavinylation of complex II. The following model is proposed to describe the attachment of FAD to complex II. Step 1, SdhE exist predominately in a non-FAD bound state. Step 2, under metabolic conditions that require complex II activity, SdhE binds FAD. Step 3, SdhE–FAD interacts with the flavoprotein subunit SdhA and then FAD is transferred from SdhE to SdhA. Step 4, the transfer of FAD to SdhA activates complex II enabling activity, which results in the reduction of ubiquinone (UQ) to ubiquinol (UQH_2). The loss of FAD from SdhE might result in the destabilisation of the SdhE–SdhA interaction, allowing SdhE to be released. Free SdhE is able to flavinylate additional complex II flavoproteins.

SdhE binds SdhA is also a crucial component of this pathway. Does SdhE only interact with unflavinylated SdhA, is this interaction via direct protein–protein interactions or is it analogous to the interaction between CcmE and cytochrome *c* — for example, do SdhE and SdhA only interact via binding to independent regions of FAD? Furthermore, does SdhE interact with SdhA prior to, or following assembly of complex II? We demonstrated that complex II does not require SdhE for assembly into bacterial membranes, suggesting that SdhE might interact with SdhA once the complex has been assembled. However, this has yet to be tested directly. Furthermore, does SdhE cause conformational changes in SdhA to allow for flavinylation? Finally, deciphering the mechanism of FAD transfer from SdhE to SdhA is important. Is transfer based on SdhA having a higher affinity for FAD than SdhE, does SdhE activate FAD in some unknown manner or is SdhE required for catalysing the SdhA–FAD covalent linkage? Clearly, these many questions need addressing to further our understanding of this 'missing link' in complex II activation.

4. Evolution of the SdhE protein family

SdhE is a member of the DUF339/COG2938 protein family. Members of this family are widespread and are present in a number of bacterial and eukaryotic species. Bioformatic analyses of this distribution demonstrated that SdhE is found specifically amongst the alpha-, beta- and gamma-proteobacteria [87], whilst in eukaryotes COG2938 is a nuclear-encoded mitochondrial protein termed Sdh5 [106]. Recent work on the eukaryotic homologue has demonstrated that Sdh5 is also required for the flavinylation of complex II [49]. Subsequent phylogenetic analysis supported the hypothesis that the COG2938 protein family evolved only once in an ancestral α-proteobacteria prior to the evolution of the mitochondria and has remained in subsequent bacterial (α-, β- and γ-proteobacteria) and eukaryotic descendants (Fig. 6A) [87]. The function of the COG2938 protein family appears to be conserved, with both eukaryotic and bacterial homologues required for complex II activity and flavinylation [49,87]. Interestingly, in *S. cerevisiae* Sdh5 was required for complex II stability and assembly [49], whilst in *Serratia* 39006 SdhE was not required for complex II stability or assembly [87]. These observations may reflect differences in the influence that FAD has on the structural integrity of complex II between bacterial and eukaryotic species [49,87]. Alternatively, it may be indicative of different modes of SdhE/Sdh5 function. For example, in bacteria, SdhE may flavinylate SdhA once complex II has assembled and FAD might not be a prerequisite for assembly or stability, whilst in eukaryotes Sdh5 may flavinylate Sdh1 (the SdhA flavoprotein subunit homologue) prior to complex II assembly with an absence of flavinylation preventing assembly [49,87]. It is worth noting that eukaryotic Sdh5 also has a longer N-terminal domain (by approximately 50 residues) than most prokaryotic homologues [49], which might influence the properties of Sdh5 and SdhE and lead to differences in the mechanism of complex II flavinylation. Furthermore, as *Serratia* 39006 and *E. coli* are members of the γ-proteobacteria, and that proteobacteria have evolved linearly from α- to β- and finally γ-proteobacteria [107] (Fig. 6A) it is speculated that SdhE homologues in members of the α- and β-proteobacterial species would be required for complex II activation and flavinylation. However, further validation of these hypotheses is required.

5. Regulation of the SdhE protein family in bacteria

Metabolic enzymes, such as complex II, are generally subject to a tight transcriptional regulation so that they function only when required. Such is the case for complex II in *E. coli*, with the complex II biosynthetic operon (*sdhCDAB*) being negatively regulated by ArcAB and FNR in response to oxygen conditions (i.e. anaerobic) and positively regulated by CRP in the absence of glucose [108–110]. The identification of SdhE as an important component of complex II function suggests that additional regulatory pathways might exist to control the activity of complex II via flavinylation. In the majority of β-proteobacterial species, *sdhE* homologues are predicted to be in an operon with the genes encoding complex II (Fig. 6B). In these species it is hypothesized that *sdhE* is transcriptionally regulated with *sdhCDAB* so that SdhE is only produced when complex II activity is required. Given this transcriptional link with complex II it is surprising that the function of SdhE was not discovered earlier in a β-proteobacterial species. However, any transcriptional co-regulation between *sdhE* and *sdhCDAB* is not obvious in the α- and γ-proteobacterial species. Amongst the majority of the α-proteobacteria, *sdhE* homologues are in a putative operon with the transcription repair factor *mfd* (Fig. 6B) [87,111]. Such a putative transcriptional coupling suggests that SdhE may link cellular metabolism with DNA repair in some α-proteobacterial species. Amongst the majority of the γ-proteobacteria, *sdhE* homologues are in operons with a conserved hypothetical protein that is commonly the membrane protein YgfX (DUF1434) (Fig. 6B) [87]. The co-transcription of the *sdhEygfX* operon has been confirmed in *Serratia* 39006 and we demonstrated that the *sdhEygfX* operon is present in almost all sequenced Enterobacterial species [87]. Amongst many other γ-proteobacteria, *sdhE* is operonic with other putative membrane bound conserved hypotheticals that are likely to represent poorly annotated DUF1434 proteins. Transcriptional analysis of the *sdhEygfX* operon in *Serratia* 39006 demonstrated that it is constitutively expressed in LB [80]. Furthermore, the *sdhEygfX* operon is expressed to similar levels in conditions that repress complex

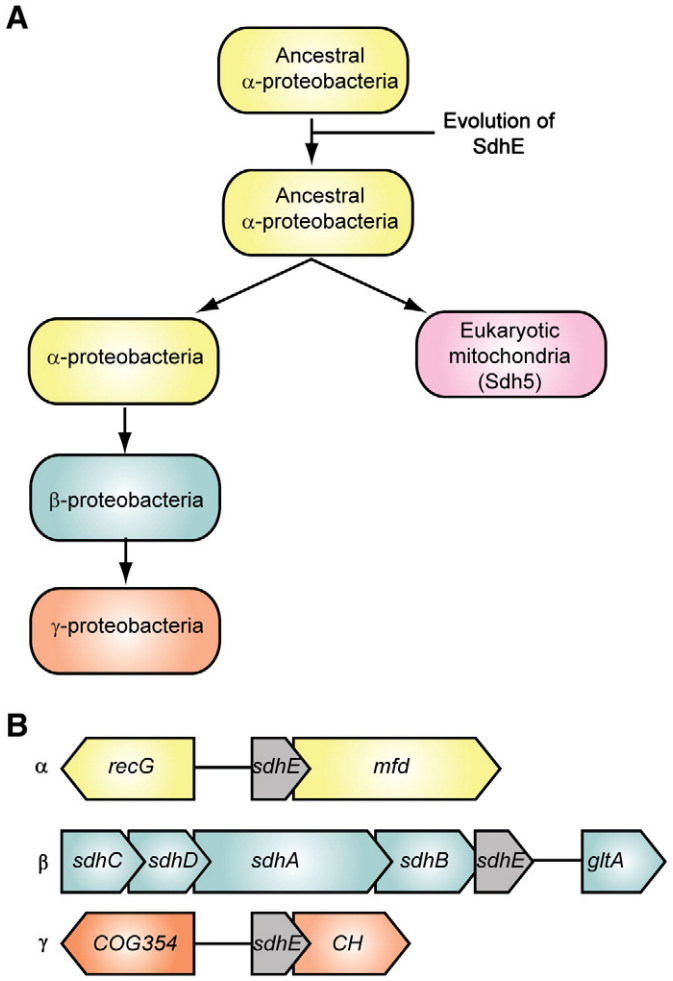

Fig. 6. Evolutionary hypothesis and genomic organisation of bacterial SdhE. (A) Evolutionary model for the evolution of SdhE (COG2938) suggests that it evolved once in an ancestral α-proteobacteria and remained in both bacterial and eukaryotic decendants. (B) The dominant (i.e. >50%) genomic organisation of SdhE homologues found in the α-, β- and γ-proteobacteria [87]. CH; conserved hypothetical.

II activity (i.e. anaerobic or aerobic with glucose as a sole carbon source) compared with conditions that require an active complex II (i.e. aerobic with succinate as a sole carbon source) [41]. Therefore, *sdhE* does not appear to be subject to transcriptional regulation via ArcAB, FNR or CRP when it is co-transcribed with YgfX in the Enterobacteriaceae. However, in *Serratia*, the *sdhEygfX* operon is repressed by the DNA-binding master pleiotropic regulator PigP, but the significance of this is unclear [80]. Whether YgfX and SdhE are functionally link remains to be determined. It has also been suggested that YgfX and SdhE form a toxin–antitoxin system in *E. coli* by preventing the polymerization of cell division proteins FtsZ and MreB [112]. Alternatively, SdhE may still be transcribed when complex II activity is not required as it might be required for the flavinylation of additional flavoproteins in the Enterobacteriaceae.

6. SdhE: evidence for a promiscuous flavinylation accessory protein

As previously mentioned, flavoproteins have traditionally been viewed as incorporating FAD through autocatalytic mechanisms. Consequently, SdhE is the first accessory protein shown to be required for FAD incorporation in bacteria. An obvious question regarding this process is whether additional FAD cofactor chaperones are required for the flavinylation of other flavoproteins? Furthermore, cofactor chaperones (including metallochaperones) are generally regarded as being specific for a single target cofactor and target protein [5,42,113]. Interestingly, the Δ*sdhE Serratia* 39006 mutant was highly pleiotropic with reduced growth in aerobic culture when grown in LB media, reduced swimming motility and reduced production of quorum sensing molecules (*N*-acyl homoserine lactones), prodigiosin, carbapenam and the plant cell wall degrading enzyme activities cellulase and pectate lyase, which culminated in reduced virulence in a potato tuber model [87]. This Δ*sdhE* pleiotropy might be caused by a reduced metabolic capacity, resulting from a non-functional complex II. Alternatively, SdhE might be required for the flavinylation of additional flavoproteins, with the loss of SdhE affecting multiple biological pathways. Investigation of the complex II literature supports this promiscuous flavinylation model because, although complex II is required by a number of Enterobacterial pathogens for the establishment of infections [71–74], there are no previous reports (to the best of our knowledge) of complex II being required for motility in Enterobacterial species. Interestingly, FRD, the enzymatic paralogue of complex II, is required for flagella-mediated swimming motility under aerobic conditions in *E. coli* [51]. Like complex II, FRD has four subunits that are arranged in a similar manner, with the cytoplasmic FrdA flavoprotein subunit containing a covalently bound FAD molecule [12,114,115]. If SdhE acts as an accessory protein for the flavinylation of other flavoproteins, the similarity between complex II and FRD makes FRD a likely flavinylation target. Whether SdhE flavinylates FRD or other proteins requires further investigation.

7. Concluding remarks

Complex II is an important respiratory complex that contains multiple cofactors, including FAD that is covalently linked to the flavoprotein subunit. Through a combination of genetic and biochemical approaches the conserved hypothetical protein, SdhE, was identified as being required for the flavinylation of complex II. The current working model is that SdhE functions as a 'bind and deliver' cofactor chaperone that directly binds FAD and delivers it to the SdhA flavoprotein subunit of complex II. It is important to investigate the remaining questions surrounding the SdhE protein family and their mode of action given the metabolic importance of complex II, the requirement for an active complex II for virulence in many pathogens and the widespread conservation of these FAD cofactor chaperones. Additionally, the presence of complex II in many bacterial species that lack SdhE homologues raises the intriguing question of how complex II is flavinylated in these species. In these other bacteria is flavinylation autocatalytic, or do additional complex II FAD attachment accessory protein(s) await discovery?

Acknowledgements

The authors wish to thank Stan Brouns and Gregory Cook for critically reading the manuscript. Members of the Fineran and Cook groups for are also thanked for useful discussions. This work was supported by the University of Otago and the Marsden Fund, Royal Society of New Zealand. MBM was funded by Bright Futures Top Achiever Doctoral Scholarship from the Tertiary Education Commission, NZ, a University of Otago Postgraduate Publishing Bursary (PhD) and a Division of Health Sciences Career Development Postdoctoral Fellowship.

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