Flavinylation and Assembly of Succinate Dehydrogenase Are Dependent on the C-terminal Tail of the Flavoprotein Subunit*

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Background: Succinate dehydrogenase (SDH) requires a covalent addition of FAD for catalytic function.

Results: Mutational analyses of Sdh1 implicate C-terminal region Arg residues involvement in covalent flavinylation and SDH assembly.

Conclusion: SDH assembly is dependent on FAD binding to Sdh1 but not covalent binding.

Significance: These results document the basis for the SDH deficiency and pathology seen with mutations in human Sdh1.

The enzymatic function of succinate dehydrogenase (SDH) is dependent on covalent attachment of FAD on the ~70-kDa flavoprotein subunit Sdh1. We show presently that flavinylation of the Sdh1 subunit of succinate dehydrogenase is dependent on a set of two spatially close C-terminal arginine residues that are distant from the FAD binding site. Mutation of Arg582 in yeast Sdh1 precludes flavinylation as well as assembly of the tetrameric enzyme complex. Mutation of Arg638 compromises SDH function only when present in combination with a Cys630 substitution. Mutations of either Arg582 or Arg638/Cys630 do not markedly destabilize the Sdh1 polypeptide; however, the steady-state level of Sdh5 is markedly attenuated in the Sdh1 mutant cells. With each mutant Sdh1, second-site Sdh1 suppressor mutations were recovered in Sdh1 permitting flavinylation, stabilization of Sdh5 and SDH tetramer assembly. SDH assembly appears to require FAD binding but not necessarily covalent FAD attachment. The Arg residues may be important not only for Sdh5 association but also in the recruitment and/or guidance of FAD and or succinate to the substrate site for the flavinylation reaction. The impaired assembly of SDH with the C-terminal Sdh1 mutants suggests that FAD binding is important to stabilize the Sdh1 conformation enabling association with Sdh2 and the membrane anchor subunits.

Succinate dehydrogenase (SDH),4 also known as succinate:quinone oxidoreductase, is a citric acid cycle enzyme that links directly to the aerobic respiratory chain. The enzyme catalyzes the FAD-dependent oxidation of succinate to fumarate coupled with the reduction of ubiquinone to ubiquinol. SDH is a heterotetrameric integral membrane protein complex. The eukaryotic enzyme is embedded within the mitochondrial inner membrane by a hydrophobic module associated with the catalytic module protruding into the mitochondrial matrix (1). The hydrophilic catalytic module consists of Sdh1 and Sdh2 subunits and the electron transfer cofactors. Succinate oxidation occurs in the FAD-containing Sdh1 with the abstracted electrons from the reaction shuttled via three iron-sulfur centers in Sdh2 to the ubiquinone reduction site (QP−proximal) at the interface of Sdh2 and the membrane anchor (1–4). The Sdh3/Sdh4 membrane domain contains a bound heme b moiety at the subunit interface of Sdh3 and Sdh4 with each providing one of the two axial His ligands, although the role of the heme in eukaryotic SDH is unresolved (5).

The FAD of Sdh1 is covalently attached at an active site His residue (2). This covalent bond increases the FAD redox potential by ~60 mV to permit succinate oxidation (6). SDH is the major mitochondrial protein containing a covalent bound flavin (7). Sdh1 containing a H90S substitution is enzymatically inactive in succinate oxidation but assembles into a tetrameric complex that exhibits fumarate reductase activity (8). Fumarate reductase activity in SDH does not require covalent flavinylation. SDH is related to the bacterial fumarate reductase and both enzymes can catalyze succinate oxidation and fumarate reduction with different efficiencies (9). Flavinylation of Sdh1 was found to occur after import into the matrix and to be influenced by the presence of the iron/sulfur cluster subunit Sdh2 but largely independent of the membrane anchor (10). The presence of citric acid intermediates stimulated the flavinylation process (10).

The covalent addition of FAD was proposed to be autocatalytic (7), but recently, a dedicated assembly factor Sdh5 was identified that is required for covalent flavinylation (11). The role of Sdh5 in Sdh1 flavinylation was discovered by the interaction of the two proteins and the demonstration that sdh5Δ yeasts were devoid of SDH activity and lacked flavinylated Sdh1. The direct role of Sdh5 in Sdh1 flavinylation was shown by the enhanced covalent addition of FAD to recombinant Sdh1 expressed in bacteria in the presence of Sdh5. However, the

4 The abbreviations used are: SDH, succinate dehydrogenase; BN-PAGE, blue native PAGE; FAD, flavin adenine dinucleotide; DDM, n-dodecyl-β-d-maltoside.

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mechanism by which the 22-kDa Sdh5 protein mediates flavinylation of Sdh1 remains unresolved.

The human Sdh5 ortholog, SDHAF2, was shown to have a corresponding role in covalent flavinylation in human cells (11). Mutations in SDHAF2 are associated with a neuroendocrine tumor designated as paraganglioma. Mutations in the SDH structural genes are also detected in a range of tumors, including paraganglioma, pheochromocytomas, gastrointestinal stromal tumors, and renal cell carcinoma (12). Recently, the bacterial ortholog of Sdh5 was identified in the bacterium *Serratia*. The factor designated SdhE was shown to interact with the flavoprotein (SdhA) and independently associate with FAD (13). This is the first suggestion that the assembly factor may mediate Sdh1 flavinylation by presenting bound FAD. Whereas *sdh5Δ* cells exhibited a specific defect in SDH, the bacterial *sdhE* mutant showed a more pleiotropic defect, suggesting SdhE may flavinylate other flavoproteins (13).

SDH is one of many flavoproteins with a covalently bound cofactor. SDH has an 8α-N3-histidyl-FAD linkage, but other flavoproteins use cysteinyl-FAD or tyrosyl-FAD linkages (14). Two large families of covalent flavoproteins are the glucose oxidase/methanol oxidase family and the vanillyl-alcohol oxidase family. Within the vanillyl-alcohol oxidase family, FAD oxidase/methanol oxidase family and the vanillyl-alcohol oxidase family, FAD linkages to histidine, cysteine, and tyrosine are known, although no ancillary enzyme is known to catalyze the covalent addition. Covalent flavinylation with FAD is known to proceed nonenzymatically, albeit slowly.

In the present study, we identify essential arginines in the C-terminal tail in yeast Sdh1 that are required for flavinylation and SDH1 assembly. The impaired assembly of the tetrameric enzyme with these Sdh1 mutants correlates with the lack of FAD binding.

**MATERIALS AND METHODS**

**Yeast Strains and Vectors**—All *Saccharomyces cerevisiae* strains used in this study were derivatives of Trp0 (Mata *ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112*). Deletion strains *sdh1Δ*, *sdh2Δ*, *sdh3Δ*, and *sdh4Δ*, in addition to plasmid pRS414 *Sdh2-His6Myc2*, were kindly provided by Dr. Jared Rutter at the University of Utah. Additional deletion strains (*fkhΔ, sdxΔ, sdx3Δsdx4Δ* double) were constructed by homologous recombination using either the *KanMX4* or the *HIS3MX6* disruption cassettes (15). The C-terminal mutants of *SDH1* along with *SDH1* WT under the control of its own promoter and *CYC1* terminator were expressed in *sdh1Δ* strain or subcloned into integrating pRS405 vectors for chromosomal expression. The integrating constructs were further digested with AflII and integrated at the *leu2-3,112* locus of *sdh1Δ* to be able to express wild type and mutants *SDH1* chromosomally. All integrated strains were confirmed by PCR analysis of the locus. The C-terminal point mutations were introduced by QuikChange mutagenesis PCR system (Agilent Technology). All mutations were confirmed by DNA sequencing. Yeast strains were transformed using lithium acetate. Strains were grown in synthetic complete medium lacking the amino acid(s) to maintain plasmid selection with either 2% galactose or 2% glycerol/lactate as the carbon source.

For carbon swap cultures overnight, 50-ml glucose-grown cultures were used to inoculate 1 liter of medium containing 2% galactose as the carbon source. Cells were grown to an A600nm ~ 0.4 to 0.5 and harvested in a sterile manner. The cell pellet was resuspended in fresh medium containing 2% glycerol/lactate as the carbon source and grown for ~10 h (~3–4 doublings) before harvesting.

**Mitochondria Isolation**—Intact mitochondria were isolated using previously described methods of Glick and Pon (16) and Diekert et al. (17). For HPLC experiments, isolated mitochondria were further purified using ultracentrifugation through a Histodenz (Sigma Aldrich) step gradient (14 and 22%). Total mitochondrial protein was quantified using either the Bradford (18) or the bicinchoninic acid assays (19).

**Immunoblotting and Blue-native PAGE**—Steady-state levels of mitochondrial proteins were analyzed using the NuPAGE Bis-Tris gel system (Invitrogen) using MES as the buffer system. Proteins were subsequently transferred to nitrocellulose membrane and probed using the indicated primary antibodies and visualized using enhanced chemiluminescence (ECL) reagents with horseradish peroxidase-conjugated secondary antibodies. Primary antibodies were obtained from the following: anti-Sdh1, Sdh2, Sdh3, and Sdh5 were generated in this study (21st Century Biochemicals). Anti-HA, anti-Myc, and anti-porin were purchased from Rockland, Roche Applied Science, and Molecular Probes, respectively. Anti-F1 ATP synthase was a generous gift from Alex Tzagoloff.

Analysis of yeast mitochondrial native membrane complexes was performed using the native PAGE gel system (Invitrogen) that is based on the blue-native polyacrylamide gel electrophoresis (BN-PAGE) technique developed by Schägger and von Jagow (20). Solubilized mitochondria (1% digitonin for 20–40 μg of mitochondrial protein) were separated on either 3–12% (18) or the bicinchoninic acid assays (19).

**Hemin-agarose Pulldown Assays and Immunoprecipitation**—Purified mitochondria (0.75 to 1 mg of total protein) were solubilized using either 0.1% DDM or 1% digitonin in PBS buffer and clarified at 20,000 × g for 5 min. For hemin-agarose pull-down assay, the clarified lysates were incubated with 20 to 40 μl of hemin-agarose beads (Sigma), which had been preincubated using DTT. Binding was performed for 2 h or overnight at 4 °C. Beads were subsequently washed (15 min × 4) with PBS buffer, 0.1% DDM or 1% digitonin, ± DTT, and eluted with 2× SDS-PAGE loading buffer. For immunoprecipitation, the clarified lysates were incubated with anti-HA or anti-Myc beads for 2 h or overnight at 4 °C, washed four times with PBS buffer + 0.1% DDM or 1% digitonin, and eluted with 2× SDS-PAGE loading buffer. The clarified lysate, final wash, and eluate fraction were analyzed by SDS-PAGE and immunoblotting. Bands from COomasie-stained SDS-PAGE gel from hemin-agarose pulldown assay were also excised for identification using MS analysis of the tryptic digests.

**SDH Activity Assay**—SDH activities in isolated yeast mitochondria were measured spectrophotometrically at 22 °C following the succinate-dependent, phenazine methosulfate-mediated reduction of either 2,6-dichlorophenolindophenol or
cytochrome c as the terminal electron acceptor described for intact mitochondria (21).

Analysis of Sdh1-bound FAD and Total Mitochondrial FAD Levels—Levels of covalently attached FAD to Sdh1 were analyzed as described previously (11, 22). Mitochondrial proteins were resolved on SDS-PAGE, and the gel was placed in a 10% acetic acid solution for 20 min to oxidize flavin. The FAD band was visualized upon exposure to UV light using a Bio-Rad Gel Doc transilluminator.

Freely soluble mitochondrial FAD levels were analyzed as described previously (23). Purified mitochondria (500 μg) were pelleted at 20,000 x g for 10 min and resuspended in 500 μl of deionized water and boiled at 105 °C for 10–15 min to precipitate proteins. The resulting yellowish supernatant was clarified at 20,000 x g for 5 min and analyzed on a Waters Sunfire C18 HPLC column (5 μm 4.6 x 150 mm) equilibrated with 15% MeOH in 10 mM ammonium acetate, pH 6.4. Flavin was eluted using a linear MeOH gradient to 75% in 10 mM ammonium acetate, pH 6.4, for 25 min and monitored at 450 nm. Elution times were compared with FAD and FMN flavin mononucleotide standards (Sigma). The concentration of FAD for the peak area was determined using an extinction coefficient of 11,300 M^-1 cm^-1 at 450 nm (9).

RESULTS

Adsorption of Sdh1 on Heme Agarose—The focus on SDH flavinylation initiated from a goal to isolate heme-binding proteins within the mitochondria using affinity purification with a heme-agarose matrix. Detergent-solubilized mitochondrial lysates were chromatographed on heme-agarose affinity beads either in the oxidized (Fe³⁺; hemin) or reduced (Fe²⁺; heme) state (Fig. 1A). Proteins adsorbed on the heme matrix were eluted by SDS treatment and analyzed by SDS-PAGE. With reduced heme-agarose, a single major band was apparent with Coomassie staining that was identified as Sdh1 by mass spectrometry. The 67-kDa band was further verified to be Sdh1 by

\[ \text{FIGURE 1. Subunit 1 of SDH (Sdh1) binds hemin in vitro, and truncation of its last 13 residues abrogates this heme binding and renders the cells respiratory defective. A, Coomassie-stained SDS-PAGE gel of hemin-agarose purified Sdh1 from DDM-solubilized mitochondria ± DTT (lane 4 versus 5) and in the presence of increasing concentrations of free hemin as a competitor to the hemin-agarose beads (lanes 6–8). Activated agarose beads conjugated to a six-carbon linker (minus hemin) failed to capture Sdh1 (lane 9) std, protein standards; wash, final wash of the resin concentrated 10-fold. B, the last 13 residues of Sdh1 is a featureless strand (highlighted in red) located on the surface of the protein and away from the buried FAD cofactor (colored sticks). Sdh1 is shown in light beige, Sdh2 is shown in light gray, and its iron-sulfur clusters are shown as dark gray spheres. PyMOL was used to generate the figure from Protein Data Bank code 2H88 (avian). C, WT and sdh1Δ strains transformed with vectors (Vec) encoding full-length and truncated Sdh1 were spotted on fermentable (glucose) and non-fermentable (glycerol/lactate) medium with serial dilution and incubated at 30 °C. Cells with truncation of the last 13 residues in Sdh1 (Clip K629) failed to grow on respiratory medium. D, SDS-PAGE and immunoblot analysis of mitochondria isolated from strains in B. Covalent flavinylation of Sdh1 is lost in cells with truncation of the last 13 residues in Sdh1 (Clip K629) as analyzed by UV illumination of SDS-PAGE gel. Immunoblots show that steady-state levels of Sdh2 and Sdh3 are also affected by C-terminal truncation. E, immunoblot analysis of heme-agarose purification of Sdh1 using mitochondria isolated from strains indicated in B shows that heme binding is lost in the Sdh1 C-terminal truncate (Clip K629). Flavo, flavoprotein. KanMX is the deletion cassette.} \]
immunoblotting using Sdh1 antisera. Subunits Sdh2 and Sdh3 were also detected with their respective antisera but in less abundance compared with Sdh1 (data not shown). The adsorption of Sdh1 on heme-agarose was competed by preincubation of the lysate with free heme, and no Sdh1 was retained on activated agarose beads lacking the heme moiety.

The retention of Sdh1 on heme-agarose suggested Sdh1 may possess a heme-binding motif. Inspection of the yeast Sdh1 protein sequence showed a single CP motif in the C-terminal tail found in a subset of heme-binding proteins (Fig. 1B). This C-terminal tail strand lies on the surface of Sdh1 and is located far from the buried FAD cofactor. Deletion of a C-terminal 13-residue segment in Sdh1 generated a truncate (designated ClipK628) that was stably expressed, although cells harboring this truncate failed to propagate on non-fermentable carbon sources (Fig. 1C). The presence of the Sdh1 truncate abrogated assembly of the tetrameric SDH enzyme and steady-state levels of Sdh2 and Sdh3 were markedly attenuated (Fig. 1D). The truncated Sdh1 was not flavinylated as shown by the lack of a FAD fluorescence band after SDS-PAGE (10). Chromatography of detergent lysates on the mutant cells on heme-agarose did not result in any retention of the Sdh1 truncate on the column (Fig. 1E).

**C-terminal Segment of Sdh1 Is Critical for Flavinylation**—Site-directed mutagenesis was carried out to map the residues responsible for flavinylation and heme-agarose binding. C630A, V633S, or a double P634A,P635A mutation in Sdh1 had no effect of glycerol/lactate growth (Fig. 2A). Furthermore, the C630A mutant in the CP motif did not attenuate SDH activity or compromise steady-state protein levels (Fig. 2B). Heme-agarose binding was also unaffected (Fig. 2C). However, cells harboring a mutant allele of Sdh1 with a R638A substitution were compromised in glycerol/lactate growth (Fig. 2A) and SDH activity (Fig. 2D). Flavinylation of the mutant Sdh1 was attenuated by 60% relative to the WT subunit. Assembly of the mutant Sdh1 into the tetrameric complex was not impaired as seen by BN-PAGE (Fig. 2A) and SDH activity (Fig. 2D). Flavinylation of the mutant Sdh1 was attenuated by ~60% relative to the WT subunit. Assembly of the mutant Sdh1 into the tetrameric complex was not impaired as seen by BN-PAGE (Fig. 2A). The R638A mutant Sdh1 retained the ability to associate with the heme-agarose matrix (Fig. 2C).

A double C630A,R638A mutant revealed a more impaired phenotype than the single R638A mutant. The double mutant in Sdh1 failed to support SDH assembly of the tetrameric complex (Fig. 2E), resulting in a marked drop in steady-state levels of Sdh2 and Sdh3 (Fig. 2D). The mutant Sdh1 exhibited no flavinylation, although the mutant Sdh1 was stably expressed. Furthermore, the double mutant failed to associate with the heme-agarose matrix (Fig. 2F).
**SDH Flavinylation**

**FIGURE 3.** The steady-state level of Sdh5 is diminished in the C630A,R638A double mutant. A, steady-state immunoblot analysis of endogenous Sdh5 from isolated mitochondria from strains expressing chromosomally integrated SDH1 C-terminal mutants. B, immunoprecipitation of HA-tagged Sdh5. Isolated mitochondria from cells expressing exogenous HA-tagged Sdh5 on top of the strains in A were solubilized in digitonin and subjected to IP. The Sdh1-Sdh5 interaction was analyzed by SDS-PAGE immunoblot analysis of the HA-agarose purification eluate and probed with antibodies to HA (SDH5) and Sdh1.

**Isolation of Intragenic SDH1 Suppressors**—In the absence of a more marked rescue of the respiratory defect of R638A Sdh1 mutant cells by overexpression of SDH5, we isolated genetic suppressors in the mutant cells to gain possible insight into the mechanism underlying the point mutants. In addition to focusing on the R638A mutant, we analyzed a Sdh1 mutant in a second conserved arginine residue (Arg582) that is spatially adjacent to Arg638. The corresponding residue in human SDHA is Arg589 and a R589W mutation was reported in a patient afflicted with paraganglioma (24). We generated R582W and R582A mutations in yeast Sdh1 and observed that both substitutions resulted in lack respiratory growth (Fig. 5A).

Plating sdh1Δ cells expressing either R638A, R589A, or R589W Sdh1 from high-copy plasmids at high density on glycerol/lactate medium resulted in the appearance of papillae that contained mutants proficient in respiratory growth. The suppressors failed to respire in each case when the plasmid expressing the Sdh1 point mutation was shed, suggesting intragenic suppression. Rescue of the plasmid in *Escherichia coli* and retransformation in parent sdh1Δ cells resulted in glycerol/lactate growth.

DNA sequencing of the R638A mutant SDH1 gene revealed a second-site mutation resulting in a G70V substitution. The respiratory competency of the G70V,R638A Sdh1 mutant is shown in Fig. 5A. Gly70 is located 35 Å from Arg638 but is close to the FAD. It is also on the opposite face from the His90 that forms the covalent bond (Fig. 5B). SDH activity was restored in these second-site mutant cells (Fig. 6A).

The cultures used for the studies in Fig. 6 were propagated using a carbon swap protocol in which cultures initially grown in glucose to an A_600 nm ~ 0.5 were switched to glycerol/lactate for the last 10 h of the experiment. Under these conditions, even the sdh1Δ cells maintained viability during the last phase of growth. Propagation of sdh1Δ cells with the R638A Sdh1 mutant on galactose medium rather than the carbon swap protocol resulted in markedly reduced SDH activity as seen in Figs. 2D and 4B; however, the presence of the G70V,R638A Sdh1 suppressor mutant did not exhibit enhanced SDH activity. The abundance of wild-type and mutant SDH complexes is markedly enhanced in glycerol/lactate medium relative to galactose medium (data not shown).

The genetic suppressor screen carried out with the R582A Sdh1 mutant cells resulted in the recovery of a second-site suppressor with a M599R Sdh1 substitution in addition to the original R582A mutation (Fig. 5A). Met599 is spatially close to Arg582 and Arg638 (Fig. 5, C and D). The double R582A,M599R Sdh1 mutant was catalytically active unlike the R582A single mutant (Fig. 6A). Steady-state levels of Sdh1, Sdh2, and Sdh3 were restored. The suppressor was able to partially assemble, unlike the R582A single mutant, into a tetrameric SDH complex that could be visualized upon extended exposure of the blue-native immunoblot (Fig. 6B). In addition, the double mutant was competent to bind the heme-agarose matrix (Fig. 6D). The suppressor mutation resulted in a stabilized Sdh5 polypeptide.

A second site suppressor was also found for the non-functional R582W Sdh1 mutant (Fig. 5A). The suppressor mutant consisted of a conversion of the Trp to a Cys residue (Fig. 5A).
FIGURE 4. Overexpression of Sdh5 partially restores growth of the single R638A mutant; the double C630A,R638A is unaffected. Similar effects are observed for steady-state covalent flavinylation and protein levels. A, growth test of strains with chromosomally integrated SDH1 C-terminal point mutants minus (top panel) or plus (bottom panel) low-copy vector (vec) expressing Sdh5. Cells were grown on glucose (fermentable) and glycerol/lactate (non-fermentable) media at 30 °C. B, upper panel: SDH activities of isolated mitochondria from strains in A expressed as a percentage of WT (n = 3 ± S.D.). Lower panels: corresponding immunoblot analysis of mitochondria isolated from above strains showing steady-state levels of covalent flavinylation levels (UV illumination) and SDH subunit. The flavoprotein (Flavo) Δ% is a relative band density (quantified using ImageJ software) comparison of the Sdh5 overexpressing strain to that of the non-overexpressing strain.

FIGURE 5. Spontaneous intragenic second-site suppressors of the Sdh1 flavinylation defect shows restoration of growth. A, growth test of intragenic second-site spontaneous suppressors to Sdh1 Arg mutants. The plasmid-borne SDH1 second site mutations were integrated chromosomally into sdh1Δ strains at LEU2 locus along with the original Arg mutation and tested for growth on fermentable (glucose) and non-fermentable (glycerol/lactate) medium at 30 °C. B, PyMOL-generated representation of avian SDH (Protein Data Bank code 2H88) showing the two critical Arg residues that are located on (Arg638) or near (Arg582) the C terminus (red strand) that when mutated causes a covalent flavinylation defect. C, the second-site mutation G70V that restores the growth defect of the R638A mutation lies on a helix that makes contact with the phosphate group of FAD but lies 35 Å away from the Arg638 and 14 Å away from FAD. PyMOL generated representation of avian SDH (Protein Data Bank code 2H88). D, the second-site mutation M599R is spatially close to the C-terminal tail (~6 Å) of Sdh1 and to the original R582A (~3.5 Å) mutation. In eukaryotes, the residue corresponding to Met599 in yeast has already been replaced to Arg. PyMOL generated figure using avian SDH (Protein Data Bank code 2H88). vec, vector.
The R582C Sdh1 mutant is active and assembles into the tetrameric complex (Fig. 6C) and is capable of adsorption on heme-agarose beads (Fig. 6D). Thus, Sdh1 with either an Arg or Cys at sequence position 582 is functional.

**Role of FAD in SDH Complex Assembly**—The lack of assembly in the R582A and the double C630A,R638A mutant Sdh1 and the lack of Sdh1 flavinylation in those mutants raised the question of whether SDH assembly is dependent on flavinylation. Yeast lacking Sdh5 were reported to be impaired in SDH assembly (11), but cells containing a mutant H90S Sdh1 mutant with a replacement of the histidyl residue forming the covalent FAD adduct was able to assemble into the tetrameric complex (8). We confirmed the latter result; the H90S Sdh1 yeast lacked any observable covalent FAD (Fig. 7A), yet it exhibited a tetrameric complex on BN-PAGE (Fig. 7B). Although no covalent FAD was seen, Robinson et al. (8) reported that FAD was still associated non-covalently with the enzyme. Sdh5 levels were normal in cells containing the H90S mutant Sdh1 (Fig. 7A).

In the case of *sdh5Δ* cells, despite no covalent flavinylation (Fig. 7A), we observed varying degrees of SDH assembly in multiple independent experiments with three different *sdh5Δ* strains (Fig. 7C). This variability occurs within a given deletion strain. The variable assembly states seen by BN-PAGE may imply that the SDH complex is unstable and dissociates under the Coomassie gel conditions. In these mitochondrial lysates, we quantified steady-state Sdh2 and Sdh3 levels to assess the dependence of Sdh1 flavinylation on the presence of other SDH subunits. Robinson and Lemire (10) reported that Sdh1 flavinylation is independent of the membrane anchor subunits Sdh3 and Sdh4 (Fig. 8A). Cells lacking Sdh4, or Sdh3 and Sdh4 double, possess a similar phenotype as cells lacking Sdh3 only (data not shown). Sdh2 steady-state levels were near normal in cells lacking Sdh3, but Sdh2 levels were absent in cells lacking Sdh1. Sdh1 flavinylation was partially depressed in cells lacking Sdh3, but Sdh2 levels were near normal in cells lacking Sdh2. We extended their observations and found that steady-state Sdh1 levels were normal in cells lacking Sdh2 and the membrane anchor Sdh3 (Fig. 8A). Cells lacking Sdh4, or Sdh3 and Sdh4 double, possess a similar phenotype as cells lacking Sdh3 only. Flavinylation of Sdh1 was shown to occur upon mitochondrial import and proteolytic processing of Sdh1. In a pulse-chase study, the flavinylation of Sdh1 was assessed (10) in strains lacking one of the other SDH subunits to assess the dependence of Sdh1 flavinylation on the presence of other subunits (10). Robinson and Lemire (10) reported that Sdh1 flavinylation is independent of the membrane anchor subunits Sdh3 and Sdh4 and partially attenuated in cells lacking Sdh2. We extended their observations and found that steady-state Sdh1 levels were normal in cells lacking Sdh2 and the membrane anchor Sdh3 (Fig. 8A). Cells lacking Sdh4, or Sdh3 and Sdh4 double, possess a similar phenotype as cells lacking Sdh3 only (data not shown). Sdh2 steady-state levels were near normal in cells lacking Sdh3, but Sdh2 levels were absent in cells lacking Sdh1. Sdh1 flavinylation was partially depressed in *sdh2Δ* cells. However, flavinylation of Sdh1 occurred normally in *sdh3Δ* cells. The Sdh1 and Sdh2 subunits persisting in double, possess a similar phenotype as cells lacking Sdh3 only (data not shown). Sdh2 steady-state levels were near normal in cells lacking Sdh3, but Sdh2 levels were absent in cells lacking Sdh1. Sdh1 flavinylation was partially depressed in *sdh2Δ* cells. However, flavinylation of Sdh1 occurred normally in *sdh3Δ* cells. The Sdh1 and Sdh2 subunits persisting in double, possess a similar phenotype as cells lacking Sdh3 only (data not shown). Sdh2 steady-state levels were near normal in cells lacking Sdh3, but Sdh2 levels were absent in cells lacking Sdh1. Sdh1 flavinylation was partially depressed in *sdh2Δ* cells.
important for efficient covalent FAD binding. Interestingly, the Sdh5 steady-state levels were elevated in sdh2/H9004 or sdh3/H9004 cells (Fig. 8A).

**DISCUSSION**

We show presently that flavinylation of the Sdh1 subunit of succinate dehydrogenase is dependent on a set of two spatially close Arg residues near the C terminus, which are distant (>20 Å) from the FAD binding site but are critical in flavinylation. These residues are also important for the assembly of Sdh1 into the tetrameric enzyme complex. Mutant Sdh1 proteins with either a R582A or double C630A,R638A substitution are neither flavinylated nor assembled into the SDH complex (Table 1). With each mutant Sdh1, second-site Sdh1 suppressor mutations were recovered in Sdh1 permitting both flavinylation and SDH assembly. In the case of the single R638A mutation, the second site suppressor was a G70V substitution, whereas the R582A second site suppressor was a M599R substitution. The presence of the Arg at residue 599 restores a positively charged residue in proximity to residue position 582. It is of interest that the corresponding residue to Met599 in humans and metazoans is Arg. In the human SDHA (Sdh1 equivalent), the Arg residue corresponding to yeast Arg589 is Arg582. Substitution of this Arg589 to a Trp (R589W) has been reported in a patient afflicted with paraganglioma (24). Yeast harboring a corresponding R582W mutant Sdh1 are compromised in SDH assembly and flavinylation, but a reversion mutant of R582C restores both Sdh1 flavinylation and SDH assembly.
One major question emerging from the present studies concerns the role of the C-terminal Arg residues in Sdh1 flavinylation. These mutations do not appear to destabilize the Sdh1 polypeptide; rather, they only impair Sdh1 maturation. Three candidate roles for the C-terminal Arg residues may be envisioned. One candidate link involves the binding of Sdh5 and its importance in Sdh1 flavinylation. Sdh5 levels are dependent on the presence of Sdh1. The reduced steady-state levels of Sdh5 in the Sdh1 mutants may reflect attenuated binding. However, cells harboring the mutant Sdh1 alleles are markedly impaired in SDH assembly, whereas sdh5Δ cells are only partially attenuated in SDH stability as seen by the variable levels of the assembled SDH complex in our series of isolates. Thus, the phenotypes observed with either the R582A or double C630A,R638A mutants appear distinct from that of sdh5Δ cells.

A second scenario is that the flavinylation may occur in a nascent conformation of Sdh1 that is somewhat distinct from the final mature conformation. In this scenario, the C-terminal Arg residues may be in juxtaposition for FAD flavinylation. The G70V second site suppressor mutation in the SDH-deficient R638A Sdh1 mutant is consistent with this postulate of a distinct nascent conformation in which these two residues are now in closer proximity. However, two observations argue against this model. The observation that citric acid cycle intermediates can stimulate the flavinylation process (10) suggests that a flavinylation-competent conformation may have a preformed native-like substrate binding site. The dependence of Sdh2 on efficient Sdh1 flavinylation suggests that the flavinylation-competent conformation of Sdh1 must be quite similar to the final mature fold enabling Sdh2 association. In the flavoprotein vanillyl-alcohol oxidase containing a histidyl-linked FAD, structural similarity between the holo- and the apo-forms indicates that FAD and substrate bind to a folded, highly preorganized cofactor/active site cavity, followed by autocatalytic covalent flavinylation (27).

A third candidate role for the C-terminal Arg residues may relate to FAD binding. Although the C-terminal Arg residues are distant removed from the FAD or substrate site in the mature Sdh1 structure, the Arg residues may be important in recruitment and/or guidance of FAD and or succinate to the substrate site. As mentioned, flavinylation of Sdh1 is dependent on succinate (10).

Another conserved Arg residue (human Arg408) stabilizes succinate or inhibitor binding through two hydrogen bonds. Mutations in the human gene yielding a R408C substitution was reported in a patient with a late onset neurodegenerative disease (28). Engineering the corresponding mutation in the E. coli enzyme resulted in impaired covalent flavinylation and the absence of membrane-associated enzyme (28).

The impaired assembly of SDH with the C-terminal Sdh1 mutants suggests that FAD binding is important to stabilize the Sdh1 conformation enabling association with Sdh2 and the membrane anchor subunits. To address the role of FAD binding in SDH assembly, we utilized a yeast flx1Δ deletion strain. This mutant was reported to have attenuated levels of matrix FAD levels (23), and we confirmed this observation. Cells lacking Flx1 are known to be deficient in two FAD-containing enzymes SDH and lipoamide dehydrogenase (22). We show the mutant cells are also impaired in SDH assembly and stability of Sdh2. The impaired SDH assembly in the FAD-deficient flx1Δ cells suggests that FAD binding is important for Sdh1 maturation enabling assembly of the tetrameric enzyme. Cells containing the H90S Sdh1 mutant that precludes covalent flavinylation assembly into the SDH complex consistent with a non-covalent association of FAD as was reported previously (8).

The covalent addition of FAD to Sdh1 likely occurs in a specific folded conformation of Sdh1 that brings a set of amino acids in juxtaposition for the autocatalytic addition. Sdh5 as well as succinate as a substrate are proposed to stabilize the flavinylation-competent conformation of Sdh1 for the reaction. The conserved C-terminal Arg residues (Arg582 and Arg638) could contribute to FAD recruitment and or its binding prior to formation of the covalent attachment. The G70V second site suppressor mutation in the SDH-deficient R638A Sdh1 mutant may merely partially deform the Sdh1 conformation allowing FAD binding in the absence of Arg638. The C-terminal Arg residues may also have a secondary role in the binding of Sdh5.

The propensity of Sdh1 to adsorb onto heme-agarose beads may relate to either a hydrophobic pocket that fortuitously accommodates heme with no physiological consequence of this binding. Alternatively, the presence of the positively charged Arg residues in the C-terminal segment could interact electrostatically with the dianionic propionate groups of heme, facilitating the association of Sdh1 with heme-agarose. This interaction may be analogous to the possible dianionic succinate or phosphates of FAD. Thus, in this scenario, heme is acting merely as a dianionic mimic of FAD or succinate. This notion is supported by the fact that protoporphyrin IX (heme lacking the iron center, but still possessing the dianionic propionates) can compete for heme binding to Sdh1 (results not shown). This observation argues that the iron center is not important for binding to Sdh1. Thus, the increased affinity of Sdh1 to heme in the presence of a reductant may be more related to the reduced state of Sdh1 than the heme iron.
A third less likely scenario is that heme has an effect role in the flavinylation reaction. We have no evidence that SDH biogenesis requires heme for Sdh1 maturation. Because heme is essential for cell survival and important in yeast for Hap1-mediated gene expression of mitochondrial proteins, the investigation of a role of heme in Sdh1 maturation is challenging and will be the topic of future studies.

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