Identification of the Heme Axial Ligands in the Cytochrome b\textsubscript{562} of the Saccharomyces cerevisiae Succinate Dehydrogenase*

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Succinate dehydrogenase (SDH) plays a key role in energy generation by coupling the oxidation of succinate to the reduction of ubiquinone in the mitochondrial electron transport chain. The \textit{Saccharomyces cerevisiae} SDH is composed of a catalytic dimer of the Sdh1p and Sdh2p subunits containing flavin adenine dinucleotide (FAD) and iron-sulfur clusters and a heme b-containing membrane-anchoring domain comprised of the Sdh3p and Sdh4p subunits. We systematically mutated all the histidine and cysteine residues in Sdh3p and Sdh4p to identify the residues involved in axial heme ligation. The mutants were characterized for growth on a non-fermentable carbon source, for enzyme assembly, for succinate-dependent quinone reduction, for heme b content, and for heme spectral properties. Mutation of Sdh3p His-106 or His-113 leads to a marked reduction in the catalytic efficiency of the enzyme for quinone reduction, suggesting that these residues form part of a quinone-binding site. We identified Sdh3p His-106 and Sdh4p Cys-78 as the most probable axial ligands for cytochrome \textit{b}\textsubscript{562}. Replacement of His-106 or Cys-78 with an alanine residue leads to a marked reduction in cytochrome \textit{b}\textsubscript{562} content and to altered heme spectral characteristics that are consistent with a direct perturbation of heme b environment. This is the first identification of a cysteine residue serving as an axial ligand for heme b in the SDH family of enzymes. Loss of cytochrome \textit{b}\textsubscript{562} has no effect on enzyme assembly and quinone reduction; the role of the heme in enzyme structure and function is discussed.

Energy generation in most eukaryotes depends on the activity of the mitochondrial respiratory chain (MRC).\textsuperscript{1} The MRC is composed of four multisubunit enzymes, complexes I-IV, that transfer electrons from NADH and succinate to molecular oxygen and capture the energy of substrate oxidation in the form of proton gradient, which is used for ATP synthesis by complex V. Succinate dehydrogenase (SDH), also known as complex II or the succinate-ubiquinone oxidoreductase, is a member of both the Krebs cycle and the MRC and oxidizes succinate to reduce ubiquinone (1–6). In addition to its role in energy generation, SDH functions as a tumor suppressor (7) and may be involved in regulating mitochondrial redox status by serving as an oxygen sensor (8, 9). Mutations of the human SDH are associated with a wide variety of clinical manifestations, including tumor formation, encephalopathy, optic atrophy, and myopathy (10–12). The involvement of SDH in these clinical conditions has generated significant interest in understanding the structure of mitochondrial members of the SDH family.

Membrane-bound fumarate reductases (FRDs), which are found in anaerobic organisms respiring with fumarate as terminal electron acceptor, are functionally and structurally related to SDH (2, 13–15). FRD catalyzes the reduction of fumarate to succinate, the reverse of the reaction catalyzed by SDH.

The crystal structures of the \textit{Escherichia coli} SDH (16), the \textit{E. coli} FRD (17), and the \textit{Wollinella succinogenes} FRD (18) have tremendously increased our understanding of the structures of these enzymes. SDH and FRD enzymes consist of a hydrophilic catalytic dimer that protrudes into the mitochondrial matrix or the bacterial cytoplasm and either one or two integral membrane subunits. The catalytic subunits exhibit a high degree of sequence conservation across species and, as expected, adopt very similar structures. The larger subunit carries a covalently bound flavin adenine dinucleotide (FAD), while the smaller subunit contains three iron-sulfur centers. In contrast, the SDH and FRD membrane subunits show considerable diversity in their primary structures and cofactor composition, and are believed to contribute to the distinct properties of the enzymes in different biological systems (4, 14, 19).

In the \textit{E. coli} SDH and FRD enzymes, two hydrophobic subunits are present (16, 17). The \textit{E. coli} FRD was crystalized without heme and with two bound menaquinone molecules, while the \textit{E. coli} SDH was crystalized with one heme and one ubiquinone. FRD from \textit{W. succinogenes} has a single membrane subunit, which contains two hemes, but the crystals did not contain any bound quinone (18).

All SDHs characterized to date are known to interact with one or two protoheme b molecules. The role of heme in electron transfer from succinate to quinone remains poorly understood despite the recent high resolution crystal structures. The \textit{Saccharomyces cerevisiae} SDH is a tetrameric (Sdh1p-Sdh4p), single heme-containing enzyme that has been extensively studied as a model mitochondrial enzyme (6, 20). Based on the kinetic and mutagenesis studies, it harbors two non-equivalent quinone-binding sites (21, 22). Although the \textit{S. cerevisiae} SDH contains heme b (20), the axial heme ligands have yet to be experimentally identified. Two histidine residues have been found to coordinate the hemes in all SDH and FRD enzymes investigated to date (4, 16, 18, 23, 24). Primary sequence analysis and homology modeling of the \textit{S. cerevisiae} SDH structure (PDB code 1PB4 and Ref. 44) have identified Sdh3p His-106 and Sdh4p Cys-78 as the most likely candidates for axial ligation of cytochrome \textit{b}\textsubscript{562}. In addition, molecular dynamics simulation studies suggest that the cysteine residue is used as the

\section*{References}

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\textsuperscript{3} The abbreviations used are: MRC, mitochondrial respiratory chain; SDH, succinate dehydrogenase; FRD, fumarate reductase; PMS, phenazine methosulfate; DB, 2,3-dimethoxy-5-methyl-6-decy1-1,4-benzoquinone; DCPIP, 2,6-dichlorophenolindophenol.
Yeast SDH Cytochrome b<sub>562</sub> Axial Ligands

Table I

| Gene   | Amino acid  | Oligonucleotide sequence 5′-3′                                      | Mutant residue |
|--------|-------------|--------------------------------------------------------------------|---------------|
| SDH3   | His-30      | CCAATTTTCTCCAGCCGTCGAGCACTTACC<sup>a</sup>                         | Alanine       |
|        |             | GTGAAGAATGGCATCGAGGCCGAAGAAAG<TAGG>                                |               |
| SDH3   | His-46      | CTCTTCTGTCCGGCTATTTCC                                              | Alanine       |
|        |             | TCTTGCATGGCCGTTGAGGAGCC                                         |               |
| SDH3   | His-46      | CCAAATACCGTTCAAGAGAAAGGACGG                                       | Aspartic acid |
|        |             | TGGCTCATTGCCGTAATTCG                                              |               |
| SDH3   | His-84      | AATTGGTACGCCGAGAGTATTTCC                                          | Alanine       |
|        |             | TAGGCAGTATTTCC                                                   |               |
| SDH3   | His-106     | TTTGGCAATTTCCGTATGTTGG                                            | Alanine       |
|        |             | GGCATTAGACCTGTTGAGG                                              |               |
| SDH3   | His-113     | CCAATACGCGGCTGATTGCC                                              | Histidine     |
|        |             | CCAATACGCGGCTGATTGCC                                              |               |
| SDH4   | Cys-78      | GTATGGTCATGATACATGCGAGTTAATTTCC                                    | Alanine       |
|        |             | CATGTGTTGCGGCCAAGTACCGGATATTTCC                                   |               |
| SDH4   | Cys-85      | GTTATTCCCACTGATGACATCCAAAAGG                                       | Alanine       |
|        |             | GGAATTTCCATGCATGACCATTCAAAAGG                                     |               |
| SDH4   | His-99      | TTGATGTTGCGGCCAAGTACCGGATATTTCC                                    | Alanine       |
|        |             | CATGGCCATCTTGGGCAACACACCA                                        |               |

<sup>a</sup> Letters in bold indicate the mutated nucleotides.

axial ligand because a more bulky histidine could not be accommodated without distorting the protein backbone conformation (44). In this report, we employ site-directed mutagenesis and spectroscopic and catalytic analysis to investigate these predictions. Our results strongly suggest that the yeast SDH differs from other family members by using a cysteine residue as an axial heme ligand.

EXPERIMENTAL PROCEDURES

Strains, Media, and Culture Conditions—The parental S. cerevisiae strain, MH125 (MATa, trp1, ura3-52, leu2-3, his3, his4, rme, p<sup>1</sup>), and the E. coli strain, DH5<sup>a</sup> have been described (22). The SDH3 and SDH4 knockout strains, sdh3<sup>W3</sup> (sdh3::TRP1) and sdh4<sup>W2</sup> (sdh4::TRP1), respectively, are isogenic derivatives of MH125 (21, 22). The yeast media (YPD, YPG, SD, SG, YPGal, and semisynthetic media) and culture conditions have been reported (22). Plasmid loss was routinely monitored by plating out aliquots of cultures grown under non-selective conditions onto YPD and selective medium (22). The proportion of <i>p</i> cells in a culture was determined by mating to the <i>p<sup>0</sup></i> strain, MS10 (matα, kar1-1, leu2-3, canR, <i>p<sup>0</sup></i>), and testing the respiratory competence of the diploid on YPG plates. Bacterial strains were routinely grown on LB medium at 37 °C using ampicillin as the selectable marker.

Construction of Mutants—Site-directed mutagenesis was performed by PCR as described (25, 26). The mutagenic oligonucleotides used are shown in Table I. A single mutagenic oligonucleotide was used for introducing alanine residues at the Sdh3p His-46, His-84, His-106, and His-113 codons (26), while two complementary mutagenic oligonucleotides in parenthesis were used for the other mutants (25). The flanking primers for the SDH3 mutagenesis are 5′-GTAAAGGATGTCTAGGTG-3′ (sense primer; nucleotides -307 to -326) and 5′-ATAGGTTCCTGTTAATCGGCC-3′ (antisense primer; nucleotides +719 to +738). For the SDH4 mutagenesis, the flanking primers are 5′-GTTAAAGGATGTCTAGGTG-3′ (sense primer; nucleotides -307 to -326) and 5′-ATAGGTTCCTGTTAATCGGCC-3′ (antisense primer; nucleotides +966 to +986). Letters in bold indicate non-template nucleotides introduced to create an XbaI restriction site (underlined). Each mutation was confirmed by sequencing the entire gene. Sequencing was performed by the Department of Biochemistry Core DNA Facilities, University of Alberta (Edmonton, Alberta).

Isolation of Mitochondria and Submitochondrial Membranes—Stationary phase cultures grown on YPGal were harvested by centrifugation, lysed in a French pressure cell, and submitochondrial preparations were extracted by differential centrifugation as described (21). For the preparation of mitochondria, cultures were grown in semisynthetic galactose to late logarithmic phase (OD<sub>600</sub> ~ 3), and lysed enzymatically (21).

Enzyme Assays—The succinate-dependent reduction of quinone was monitored spectrophotometrically at 22 °C as the malonate-sensitive, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DBQ)-mediated reduction of 2,6-dichlorophenolindophenol (DCPIP). The succinate-dependent, phenazine methosulfate (PMS)-mediated reduction of DCPIP is a measure of membrane-associated Sdh1p/Sdh2p dimer. Other enzyme assays (succinate-cytochrome <i>c</i> reductase, succinate oxida-
genes were cloned into a single copy shuttle vector YCp416 and introduced into the SDH3-knockout strain sdh3W3. Similarly, mutant SDH4 genes were cloned into the vector pRS416 and the resulting plasmids introduced into sdh4W2. The respiratory efficiencies of the mutant strains were determined on a minimal glycerol medium (Table II). All mutant strains were able to support respiratory growth, although to varying degrees. The growth of the sdh3W3/H30A and the sdh3W3/C78A strains were indistinguishable from that of the wild-type strain. sdh3W3/H46A and sdh3W3/H46D exhibited the slowest growth, indicating a respiratory defect. Similarly, all SDH4 mutant strains supported growth on the minimal glycerol medium, although the growth of the sdh4W2/C78H was reduced. To quantify respiratory growth, the growth yields on semisynthetic galactose media were measured. S. cerevisiae exhibits a diauxic shift when cultured in this medium; an initial fermentative growth is achieved, by fermentation alone, a growth yield of 11 ± 1% (Fig. 2A). The growth yields of MH125 and the sdh3W3 strain carrying a wild-type plasmid-borne SDH3 gene, sdh3W3/pYCSDH3 are similar. The growth yields of sdh3W3/H30A and sdh3W3/C78A strains are comparable to the wild type at 95 ± 3 and 90 ± 4%, respectively. The sdh3W3/H110A mutant retained a significant capacity for respiratory growth with a 75 ± 4% growth yield. In contrast, the H46A, the H46D, and the H113A mutants showed impaired respiratory growth with yields of 50 ± 2, 55 ± 3, and 65 ± 3%, respectively, consistent with their slower growths on a minimal glycerol medium. The Sdh4p mutations H37A and C78A have no significant effect on growth yields. The sdh4W2/C78H and sdh4W2/H99A achieved growth yields of 50 ± 2% and 75 ± 3%, respectively. These observations suggest that the Sdh3p H46A, H46D, and H113A mutations and the Sdh4p C78A substitution compromise respiratory growth in S. cerevisiae.

*Assembly of Mutant and Wild-type SDH Enzymes*—To investigate the effect of the mutations on enzyme assembly, we measured the levels of covalent FAD in mitochondrial membranes (Table II). SDH is the major flavoprotein in S. cerevisiae and the covalent flavin levels of mitochondrial membranes quantitatively reflect SDH assembly (22). The covalent FAD contents of all the SDH3 mutant strains, with the exception of the sdh3W3/H106A strain, were not significantly different from the wild type. Similarly, the covalent FAD contents of the sdh4W2/H30A and the sdh4W2/C78A strains were comparable to wild-type level. The covalent flavin level of the sdh4W2/C78H membrane was reduced by about 2-fold, while that of the sdh4W2/H99A was slightly, but significantly reduced. We conclude that enzyme assembly proceeds normally in most mutants but that the Sdh3p H106A, the Sdh4p C78H, and the Sdh4p H99A mutations mildly impair enzyme assembly or stability.

Membrane-associated succinate-dependent PMS reductase activity depends on the membrane anchoring functions of the hydrophobic subunits and not on their catalytic competence (Table II). To compare catalytic efficiencies, we determined turnover numbers based on the covalent FAD contents. There was marked reduction of activity in the sdh4W2/C78H mutant strain, indicating impairment of enzyme assembly or stability. Similarly, the succinate-PMS reductase activity of the sdh4W2/H99A enzyme was significantly reduced. In contrast, the activities of the remaining mutant enzymes were comparable to wild-type enzyme. These data confirm that enzyme assembly proceeds normally in these mutant strains and that the mutations do not compromise the anchoring roles of the Sdh3p and Sdh4p subunits.

*Quinone-dependent Enzymatic Activities of Mitochondrial Membranes*—To explore the effect of the mutations on electron transfer, we assayed mitochondrial membranes for malonate-sensitive succinate-dependent reduction of exogenous quinone (Table III). The succinate-DB reductase activities of sdh3W3/H46A, sdh3W3/H46D, and sdh3W3/H113A, and the sdh4W2/C78H were reduced to about half of wild-type activities, indicating that the mutations affected the interaction of the enzyme with quinone. Similarly a slight reduction in the activity of the sdh4W2/H99A enzyme was observed. In contrast, the activities of the remaining mutant enzymes were not significantly different from wild-type values. The succinate-DB reductase activities correlate well with the levels of respiratory growth seen on minimal glycerol and on semisynthetic galactose media.

We also measured the malonate-sensitive, succinate-dependent cytochrome c reductase and the succinate oxidase activi-
ties, which rely on the reduction of endogenous quinone and are physiologically relevant assays (Table III). The former assay depends on complexes II and III of the respiratory chain; the latter assay requires complexes II-IV. The activities of mutant and wild-type enzymes parallel the succinate DB reductase activities. Neither activity is detectable in sdh3W3 and the wild-type enzymes parallel the succinate DB reductase activities. The catalytic efficiencies of the sdh3W3/H46A, the sdh3W3/H46D, and the sdh4W2/C78H and the H99A exhibited decreased catalytic efficiencies. We conclude that the Sdh3p substitutions (H46A, H46D, and H113A) and the Sdh4p substitutions (C78H and H99A) mark-

### Table II

| Yeast strains | Growth on minimal glycerol | Covalent FAD<sup>a</sup> | Specific activity<sup>b</sup> | Turnover number<sup>c</sup> |
|---------------|----------------------------|--------------------------|-------------------------------|-----------------------------|
| MH125         | + + + +<sup>d</sup>        | 35 ± 3                   | 133 ± 3.5                     | 3800 ± 100                  |
| sdh3W3/pYC33SDH3 | + + + +           | 32 ± 4                   | 118 ± 3                       | 3680 ± 90                   |
| sdh3W3/H30A | + + + +                   | 31 ± 3                   | 114 ± 2                       | 3700 ± 50                   |
| sdh3W3/H46A | + + + +                   | 32 ± 2                   | 108 ± 4                       | 3600 ± 126                  |
| sdh3W3/H46D | + + + +                   | 33 ± 2                   | 119 ± 3                       | 3600 ± 100                  |
| sdh3W3/H84A | + + + +                   | 31 ± 1                   | 107 ± 3                       | 3680 ± 99                   |
| sdh3W3/H106A | + + + +                   | 20 ± 1                   | 60 ± 2                        | 3000 ± 70                   |
| sdh3W3/H113A | + + + +                   | 31 ± 2                   | 108 ± 3                       | 3500 ± 100                  |
| sdh3W3 | ND                        | ND                       | ND                            | —                           |
| sdh4W2/pSdh4-17 | + + + +                  | 34 ± 4                   | 129 ± 3                       | 3800 ± 100                  |
| sdh4W2/H57A | + + + +                   | 32 ± 3                   | 124 ± 2                       | 3900 ± 50                   |
| sdh4W2/C78A | + + + +                   | 30 ± 2                   | 108 ± 4                       | 3600 ± 126                  |
| sdh4W2/C78H | + + + +                   | 16 ± 1                   | 32 ± 3                       | 2000 ± 100                  |
| sdh4W2/C85A | + + + +                   | 32 ± 3                   | 115 ± 6                       | 3600 ± 200                  |
| sdh4W2/H99A | + + + +                   | 25 ± 2                   | 75 ± 3                        | 3000 ± 100                  |
| sdh4W2 | NG                        | ND                       | ND                            | ND                          |

<sup>a</sup> Covalent flavin contents are expressed as pmol of FAD mg of protein<sup>−1</sup>. Each value represents the mean of triplicate determinations ± S. E.

<sup>b</sup> Specific activities are expressed as nmol of PMS-mediated DCPIP reduced min<sup>−1</sup> mg of protein<sup>−1</sup>.

<sup>c</sup> Turnover numbers are expressed as pmol of PMS-mediated DCPIP reduced min<sup>−1</sup> µmol of FAD<sup>−1</sup>.

<sup>d</sup> + + + +, the growth phenotype is essentially the same as the wild type; + + + , + + , progressivel y less growth; ND, no growth.

<sup>e</sup> ND, not detectable; less than 5% of wild-type value.

<sup>f</sup> —, Not determined.

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### DISCUSSION

All SDHs characterized to date are believed to contain at least one 6-type heme (3, 6). The heme axial ligands in all SDH and FRD enzymes examined so far are two histidine residues (16, 18, 23). In this study, we present two lines of evidence that strongly suggest that the S. cerevisiae Sdh3p His-106 and the Sdh4p Cys-78 are the axial ligands of cytochrome b<sub>562</sub>. First, mutating these residues to alanines leads to sharp reductions in cytochrome b<sub>562</sub> contents. These observations strongly implicate Sdh3p His-106 and the Sdh4p Cys-78 as axial ligands for cytochrome b<sub>562</sub>. In addition, both mutations lead to spectral shifts of λ<sub>max</sub> from 562 to 560 nm and to reductions in the spectral bandwidth measured at half peak height (Fig. 3 and Table V). The cytochrome b<sub>562</sub> content of the H113A membranes is reduced by about half but the difference spectrum is similar to that of wild type (no change in λ<sub>max</sub> or the bandwidth). Interestingly, the cytochrome b<sub>562</sub> content of the Sdh4p C78H membrane was comparable to wild-type level, though the maximum absorption peak shifted to 558 nm. There were no significant differences in both the cytochrome b<sub>562</sub> contents and the spectra of the remaining mutant membranes compared with that of the wild type (data not shown), suggesting that these residues are not involved in axial coordination of heme.
circles spectrum, effectively ruling them out as heme ligands. The only produced a similar effect on heme content or on the heme this model. None of the other His mutations we introduced ate distances from the iron ion of the heme for axial ligation. Of Sdh4p Cys-78 are in the right orientations and at appropriate distances as the main heme ligand. The data presented in this report validate a major prediction of this model. None of the other His mutations we introduced produced a similar effect on heme content or on the heme spectrum, effectively ruling them out as heme ligands. The only exception is the Sdh3p H113A mutation, which led to a small reduction in cytochrome b₅₆₂ content (Table V). However, the spectral characteristics of this mutant are identical to that of the wild type (Fig. 3).

In our previous mutagenesis studies, we had suggested that Sdh3p His-106 was an axial heme ligand because its replacement with a Tyr residue led to a 3-fold reduction in heme content (21). However, the H106Y mutant cytochrome spectrum had spectral properties characteristic of the wild type, suggesting that there had not been a large change in the heme environment. Surprisingly, the H106A mutation appears to be a more severe change than the H106Y. The introduction of the smaller Ala residue instead of the Tyr may interfere with the packing of the transmembrane helices around the heme.

Homology modeling and molecular dynamics simulation studies (44) indicated that substitution of Sdh4p Cys-78 with histidine could not allow proper coordination of heme without distorting the protein backbone. The sdh4W2/C78H enzyme contains a wild-type level of heme b (Table V and Fig. 3), confirming that histidine at this position can serve as an axial ligand. However, the deleterious effects of the C78H substitution on enzyme assembly and quinone reduction (Tables II, III, and IV) strongly suggest that the mutation is very destabilizing. This conclusion is in excellent agreement with the prediction from our model.

The role of heme b in SDH has long eluded investigators (3, 6). Although heme can function as an electron carrier, it does not appear to do so in all cases. Bovine SDH cytochrome b₅₆₀ is not reducible by succinate (34), but the heme in the E. coli SDH (Eₚ₅₆₀ = +36 mV) is (35). In the B. subtilis SDH, the high potential heme (Eₚ₅₆₀ = +65 mV) is reducible by succinate, while the low potential heme (Eₚ₅₆₀ = −95 mV) is not (31). There has been speculation that di-heme SDH or FRD enzymes are involved in transmembrane electron transfer (36–38), and this has been confirmed in the case of the B. subtilis SDH (39) but is not supported by the experimental observations for the W. succinogenes FRD (40). In contrast, the E. coli FRD, which lacks heme, catalyzes with similar efficiency the same reactions as the E. coli SDH (17, 41), ruling out an obligatory role for heme in catalysis. Our data indicate that the heme in the yeast SDH is not essential for catalysis. Loss of heme has no commensurate effect on enzyme activities requiring quinone reduction. Taken together, these observations suggest that electron flux through heme b is not essential for quinone reduction in the single heme containing enzymes.

Another possible role of heme b is in the maintenance of the structural integrity of the membrane polypeptides. While such a role has been demonstrated for the B. subtilis SDH hemes (42), the situation is less clear for the E. coli SDH. When a hemA mutant was transformed with a plasmid carrying the sdh operon, membrane-associated SDH activity could only be detected when the cells were grown in the presence of 5-aminolevulinic acid, suggesting that heme is required for enzyme assembly (28). However, when the heme axial ligands (SdhC His-84 and SdhD His-71) were mutated, succinate-quinone reductase activity was retained in the mutant enzymes despite an apparent absence of heme (24). The same mutations were re-investigated and found to retain heme, although with altered redox potentials and spectroscopic properties (41). The mutant enzymes were also found to be less thermostable and more sensitive to aeration, which may account for the discrepancies between the two reports. The heme likely has a structural role in the E. coli SDH given its interactions with residues in the SdhB subunit and in the membrane subunits (16). However, our data suggest that catalytically functional enzyme can be assembled without heme. There is the formal possibility that
the heme ligand mutations lead to loss of heme from the mutant enzymes under the conditions used for the determination of heme contents, which involves detergent, but not under assay conditions. However when detergent-solubilized mitochondrial membranes were assayed for quinone-mediated activities, the turnover numbers obtained were not significantly different from those obtained from native membranes (data not shown).

While the current study does not provide evidence for a catalytic role for cytochrome b\(_{562}\), possible redox functions cannot be entirely ruled out. All known SDHs contain heme b, but this is not the case for the FRD enzymes. Since FRDs are functional in anaerobic environments, they may not have to evolve to minimize reactivity with oxygen. In fact, the \(E.\ coli\) FRD produces copious amounts of reactive oxygen species compared with the \(E.\ coli\) SDH (2, 43). Heme b in SDH may be an adaptation to aerobic environments that prevents oxygen toxicity, possibly by serving as electron sink to prevent electron leakage from the flavin (16).

Four of the mutations characterized in this study markedly affect quinone reductase activities. The most profound effects were observed when \(Sdh3p\) His-46 was replaced with Ala or Asp residues (Table III). This histidine residue is conserved in all known SDHs and is part of the quinone-binding site sequence motif, LXXXHXX (32, 33). In the \(E.\ coli\) SDH structure, the equivalent residue (\(SdhC\) His-30) is part of the quinone-binding site sequence motif, LXXXHXX (32, 33). In the \(E.\ coli\) SDH structure, the equivalent residue (\(SdhC\) His-30) is part of the quinone-binding site sequence motif, LXXXHXX (32, 33).

### TABLE III

| Strain                  | Succinate-DB reduction | Succinate-cytochrome c oxidase | Glycerol-1-P oxidase | Succinate-DB reductase | NADH oxidase |
|-------------------------|------------------------|-------------------------------|--------------------|-----------------------|--------------|
| MH125                   | 2900                   | 1890                          | 81                 | 1690                  | 132          |
| \(sdh3W3/YC33SDH3\)     | 2840                   | 1850                          | 81                 | 1620                  | 130          |
| \(sdh3W3/H90A\)         | 2850                   | 1850                          | 79                 | 1650                  | 125          |
| \(sdh3W3/H46A\)         | 1500                   | 950                           | 80                 | 840                   | 130          |
| \(sdh3W3/H46D\)         | 1650                   | 960                           | 81                 | 850                   | 129          |
| \(sdh3W3/H84A\)         | 2500                   | 1630                          | 77                 | 1680                  | 129          |
| \(sdh3W3/H106A\)        | 2400                   | 1565                          | 80                 | 1500                  | 129          |
| \(sdh3W3/H113A\)        | 2100                   | 1440                          | 75                 | 1200                  | 126          |
| \(sdh3W3\)              | ND                     | ND                            | 68                 | ND                    | 107          |
| \(sdh4W2/pSDH4–17\)     | 2900                   | 1850                          | 83                 | 1670                  | 130          |
| \(sdh4W2/H57A\)         | 2850                   | 1860                          | 81                 | 1650                  | 130          |
| \(sdh4W2/C78A\)         | 2700                   | 1800                          | 81                 | 1600                  | 130          |
| \(sdh4W2/C78H\)         | 1500                   | 1000                          | 77                 | 900                   | 126          |
| \(sdh4W2/C85A\)         | 2800                   | 1700                          | 74                 | 1500                  | 123          |
| \(sdh4W2/H99A\)         | 2100                   | 1390                          | 81                 | 1200                  | 129          |
| \(sdh4W2\)              | ND                     | ND                            | 60                 | ND                    | 100          |

### TABLE IV

The apparent kinetic parameters for the succinate-DB reductase activities of mutant and wild-type mitochondrial membranes

The initial velocities of succinate-DB reductase were measured by varying the concentrations of the soluble quinine analogue, dechylubiquinone (DB) at fixed saturating concentrations of succinate and DCPIP. \(K_m\) and \(V_{max}\) values were calculated from a nonlinear regression fit to the Michaelis-Menten equation, using initial estimates from the double-reciprocal plots. Each value represents the mean of triplicate determinations ± S.E.

| Strain                  | \(K_m^a\) | \(V_{max}^b\) | \(b_{cat}^c\) | \(k_{cat}/K_m^c\) |
|-------------------------|-----------|---------------|---------------|-------------------|
| MH125                   | 3.4 ± 0.1 | 126 ± 5       | 3800 ± 150    | 1.1 × 10^4        |
| \(sdh3W3/YC33SDH3\)     | 3.5 ± 0.2 | 112 ± 3       | 3500 ± 100    | 1.0 × 10^4        |
| \(sdh3W3/H90A\)         | 3.8 ± 0.2 | 109 ± 3       | 3500 ± 110    | 1.0 × 10^4        |
| \(sdh3W3/H46A\)         | 15 ± 0.5  | 54 ± 2        | 1700 ± 50     | 1.3 × 10^4        |
| \(sdh3W3/H46D\)         | 13 ± 0.3  | 59 ± 2        | 1800 ± 50     | 1.4 × 10^4        |
| \(sdh3W3/H84A\)         | 3.7 ± 0.2 | 109 ± 4       | 3500 ± 120    | 1.0 × 10^4        |
| \(sdh3W3/H106A\)        | 4.6 ± 0.2 | 70 ± 3        | 2800 ± 100    | 1.0 × 10^4        |
| \(sdh3W3/H113A\)        | 9.5 ± 0.4 | 68 ± 3        | 2200 ± 100    | 3.0 × 10^4        |
| \(sdh4W2/pSDH4–17\)     | 3.5 ± 0.2 | 119 ± 3       | 3500 ± 100    | 1.0 × 10^4        |
| \(sdh4W2/H57A\)         | 3.6 ± 0.2 | 115 ± 3       | 3800 ± 110    | 1.0 × 10^4        |
| \(sdh4W2/C78A\)         | 4 ± 0.5   | 102 ± 2       | 3400 ± 50     | 1.0 × 10^3        |
| \(sdh4W2/C78H\)         | 7 ± 0.2   | 30 ± 1        | 1900 ± 70     | 3 × 10^2          |
| \(sdh4W2/C85A\)         | 3.5 ± 0.1 | 109 ± 3       | 3400 ± 100    | 1.0 × 10^3        |
| \(sdh4W2/H99A\)         | 5 ± 0.3   | 68 ± 1        | 2700 ± 50     | 5 × 10^2          |

\(^a\) Values are expressed in \(\mu\)m.

\(^b\) Values are expressed as nmol of DB-mediated DCPIP reduced \(\mu\)mol of protein.

\(^c\) The \(K_m\) values are based on covalent FAD levels of mitochondrial membrane. Values are expressed as min.\(^{-1}\).
TABLE V

| Membrane sample | Cytochrome $b_{562}$ contents | $\lambda_{\text{max}}$ | Spectral bandwidth |
|-----------------|-------------------------------|--------------------|-------------------|
| MH125           | 1.11 ± 0.18                   | 562                | 50                |
| sdh3W3/pYC33SDH3| 1.08 ± 0.20                   | 562                | 50                |
| sdh3W3/H30A     | 1.10 ± 0.14                   | 562                | 50                |
| sdh3W3/H46A     | 0.99 ± 0.20                   | 562                | 50                |
| sdh3W3/H46D     | 1.00 ± 0.22                   | 562                | 50                |
| sdh3W3/H94A     | 1.06 ± 0.21                   | 562                | 50                |
| sdh3W3/H106A    | 0.23 ± 0.05                   | 560                | 16                |
| sdh3W3/H113A    | 0.65 ± 0.09                   | 562                | 50                |
| Sdh4W2/H37A     | 1.10 ± 0.10                   | 562                | 50                |
| Sdh4W2/C78A     | 0.20 ± 0.04                   | 560                | 16                |
| Sdh4W2/C78H     | 1.1 ± 0.10                    | 558                | 50                |
| Sdh4W2/C85A     | 1.04 ± 0.11                   | 562                | 50                |
| Sdh4W2/H99A     | 0.98 ± 0.20                   | 560                | 50                |

![Fig. 3. Absorption spectra of wild type and selected mutants of the S. cerevisiae SDH.](image)

results were obtained when His-113 was replaced with a glutamine residue by random mutagenesis (21). The molecular basis for the loss of activity is not immediately clear. In our model (PDB code 1PB4 and Ref. 44), His-113 is about 10 Å away from the proximal quinone-binding site, ruling it out as a direct ligand. We suggest that the His-113 mutations produce a structural perturbation that is propagated to the quinone-binding pocket, possibly by altering the packing of the Sdh3p second transmembrane helix. The properties of the Sdh4p H99A can also be interpreted in this manner.

We have presented data that strongly support the roles of the S. cerevisiae Sdh3p His-106 and the Sdh4p Cys-78 as axial ligands of cytochrome $b_{562}$. To our knowledge, this is the first time that a cysteine residue has been shown to be involved in axial ligation of heme $b$ in the SDH or FRD family of enzymes. Although our data do not establish a catalytic role for heme $b$ in the reduction of quinone, it does not rule out other redox or physiological functions that ensure its strict conservation among SDHs. Determining the role of heme $b$ may be critical to understanding how mammalian complex II participates in disease processes.

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