The Saccharomyces cerevisiae Succinate-ubiquinone Oxidoreductase

IDENTIFICATION OF SDH3P AMINO ACID RESIDUES INVOLVED IN UBIQUINONE BINDING*

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Succinate dehydrogenase (SDH) participates in the mitochondrial electron transport chain by oxidizing succinate to fumarate and transferring the electrons to ubiquinone. In yeast, it is composed of a catalytic dimer, comprising the Sdh1p and Sdh2p subunits, and a membrane domain, comprising two smaller hydrophobic subunits, Sdh3p and Sdh4p, which anchor the enzyme to the mitochondrial inner membrane. To investigate the role of the Sdh3p anchor polypeptide in enzyme assembly and catalysis, we isolated and characterized seven mutations in the SDH3 gene. Two mutations are premature truncations of Sdh3p with losses of one or three transmembrane segments. The remaining five are nonsense mutations that are clustered between amino acids 103 and 117, which are proposed to be located in transmembrane segment II or the matrix-localized loop connecting segments II and III. Three mutations, F103V, H113Q, and W116R, strongly but specifically impair quinone reductase activities but have only minor effects on enzyme assembly. The clustering of the mutations strongly suggests that a ubiquinone-binding site is associated with this region of Sdh3p. In addition, the biphasic inhibition of quinone reductase activity by a dinitrophenol inhibitor supports the hypothesis that two distinct quinone-binding sites are present in the yeast SDH.

The mitochondrial respiratory chain consists of four discrete prosthetic group-containing protein complexes. One of these, the succinate-ubiquinone oxidoreductase (complex II or succinate dehydrogenase (SDH)) donates electrons derived from its substrate, succinate, to the respiratory chain via the reduction of ubiquinone to ubiquinol. Generally, SDH is made up of two parts: a soluble catalytic dimer and a membrane domain (1–5). In the yeast Saccharomyces cerevisiae, the catalytic dimer is composed of the 67-kDa Sdh1p subunit, to which is attached a covalent FAD cofactor (6–9), and the 28-kDa Sdh2p subunit (10), in which are located three iron-sulfur clusters. The membrane-anchoring domain is composed of the two hydrophobic subunits, Sdh3p and Sdh4p, of 16.7 and 16.6 kDa, respectively (11–13). Each subunit is believed to have its amino terminus in the matrix and three transmembrane segments.

The catalytic dimer can catalyze electron transfer from succinate to artificial electron acceptors, such as phenazine methosulfate (PMS), but not to the physiological acceptor, ubiquinone. The presence of the membrane domain anchors the catalytic dimer subunits to the inner membrane, restores ubiquinone reductase activity, and renders the enzyme sensitive to quinone analog inhibitors (1, 3). Thus, the membrane domain is believed to contain at least one quinone-binding site.

In the bovine heart mitochondrial SDH, quinone binding has been assigned to the QPs1 and QPs3 membrane domain subunits by cross-linking with photoaffinity analogs of ubiquinone (14, 15). The quinone-binding site in the QPs1 subunit, which corresponds to Sdh3p, is localized in the matrix-facing loop connecting transmembrane segments II and III. The quinone-binding domain in QPs3, which corresponds to Sdh4p, is localized to the end of the first transmembrane segment toward the cytosolic side of the membrane (15, 16). These results place the two quinone-binding sites on opposite sides of the membrane, consistent with a model formulated from inhibitor binding studies (17).

Recently, photoaffinity labeling identified a ubiquinone-binding site in the Escherichia coli SdhC subunit, which corresponds to Sdh3p, near the cytoplasmic end of the first transmembrane segment (18). The site contains serine 27 and arginine 31, which are necessary for succinate-ubiquinone reductase activity. It remains to be determined whether a second ubiquinone-binding site is present.

The E. coli fumarate reductase, which uses electrons from menaquinol to reduce fumarate to succinate, is structurally and catalytically similar to the succinate-ubiquinone oxidoreductases (1–5, 19). Mutagenesis and inhibitor studies suggest that it contains two distinct quinone-binding sites, designated QA and QB (17, 20). The QA site harbors a nonexchangeable quinone that is the primary electron acceptor from the iron-sulfur clusters in the catalytic dimer; it cycles between the oxidized and the semiquinone states. The QA site successively passes two electrons to the QB site, which harbors an exchangeable quinone.

We have previously demonstrated the importance of residues 128–135 of the carboxyl terminus of the yeast Sdh4p for ubiquinone reduction (21). The positive charge provided by Lys-132 is crucial for this function (22). In addition, we have recently documented the need for the membrane domain in the assembly of the SDH-associated cytochrome b562, although the role of this heme in catalysis remains unclear (23, 24).

To further investigate the structure and function of the yeast SDH membrane domain, we isolated and characterized seven SDH3 mutants. The mutant enzymes were characterized for their ability to support respiratory growth, as well as for enzyme stability, quinone reduction, heme b assembly, and inhib-
itor sensitivity. From our analyses, we conclude that a ubiqui-
one-binding site is likely associated with the matrix-facing loop connecting Sdh3p transmembrane segments II and III. Thus, the yeast SDH is suggested to contain two ubiquinone-

binding sites that are topologically localized to different faces of the membrane.

EXPERIMENTAL PROCEDURES

Strains—The S. cerevisiae strains MH125 (MATα, trp1, ura3-52, leu2-3, leu2-112, his3, ade2, rie, p′) and sdh3W3 (MH125, sdh3::TRP1), and the E. coli strain DH5α were described earlier (21).

Media, Yeast Culture Conditions, and Isolation of Mitochondria—The yeast media used are SD (0.67% yeast nitrogen base, 2% glucose), SG (0.67% yeast nitrogen base, 3% glycerol), YPGal (1% yeast extract, 2% peptone, 2% galactose), YPDPG (1% yeast extract, 2% peptone, 0.1% glucose, 2% glycerol), and semisynthetic galactose (0.3% yeast extract, 0.1% KH2PO4, 0.1% NaCl, 0.06% MgSO4·7H2O, 0.05% CaCl2, 0.063% FeCl3, 2% galactose). Cultures were grown on SD for 2–3 days to select for plasmid retention, used to inoculate YPGal medium supplemented with 0.01% glucose to a starting A600 = 0.05, and grown aerobically at 30 °C to stationary phase. Cells were harvested and lysed in a French pressure cell for the preparation of submitochondrial particles (21). For the preparation of mitochondria, cultures were grown in semisynthetic galactose to late logarithmic phase (A600 about 3), harvested, and lysed enzymatically (21).

Random Mutagenesis—An EcoRI-BglII fragment containing the promoter and the entire coding region of the SDH3 gene was subcloned into the yeast E. coli shuttle vector, YCplac111, to yield the plasmid pYCSDH3 and transformed into the E. coli strain DH5α. Cells were UV-irradiated at 254 nm with a dose rate of 1.4 J m−2 s−1 on LB plates in the dark to achieve 5–10% survival. The plates were incubated overnight at 37 °C in the dark. Colonies were scraped from a total of 100 plates, and plasmids were isolated. For chemical mutagenesis, suspensions of the DH5α pYCSDH3 were treated with 3% ethyl methanesulfonate to give 5–10% survival. Mutagenized plasmids were isolated and introduced into sfd3W3. Transformants were replica-plated onto SG and YPDG media. Colonies that exhibited impaired growth were further analyzed. Plasmids were recovered and retransformed into sfd3W3 to ensure that any growth defects were plasmid-mediated. Mutations were identified by sequencing the entire SDH3 gene.

Enzyme Assays—Unless otherwise stated, 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-1,4-cis 2,6-dichlorophenol-indophenol coefficient is 16 mM−1 cm−1. For thermal stability measurements, membrane fractions (20 mg/ml) were incubated at temperatures ranging from 25 to 65 °C in 50 mM potassium phosphate, 50 mM succinate, and the E. coli strain DH5α were described earlier (21).

RESULTS

Mutagenesis of the SDH3 Gene—We mutagenized an E. coli strain carrying the plasmid-borne yeast SDH3 gene with ultraviolet light and reintroduced the mutated plasmids into the SDH3 knockout strain, sfd3W3. Mutagenesis in E. coli, rather than in yeast, greatly reduced the production of respiration-deficient E. coli strains and petite. About 5000 Leu+ sfd3W3 transformants were tested for respiratory growth on SG and YPDPG media. Ten strains were impaired for growth, indicating a respiration deficiency. The strains were further analyzed by determining their growth yields on semisynthetic medium containing 0.5% galactose as carbon source. We isolated and sequenced the SDH3 genes from six strains that displayed growth yields ranging from 10 to 50%. We have found that growth yields of less than 10% are often associated with null alleles that abolish enzyme assembly. Five SDH3 mutants contained single base alterations, resulting in the substitutions of Phe–103 with Val (F103V), His–106 with Tyr (H106Y), His–113 with Glu (H113Q), Trp–116 with Arg (W116R), and Leu–122 with a stop codon (L122stop). This latter lesion truncates Sdh3p by removing the third transmembrane segment. A sixth mutant contains a single base deletion in the codon for Leu–52 that causes a frameshift mutation (L52FrShift) and truncates Sdh3p after the addition of two new amino acid residues, removing all three transmembrane segments.

Ethyl methanesulfonate-treated plasmids were also isolated and tested for the presence of mutations by transformation into sfd3W3. Of the 1000 colonies screened, only one was incapable of respiratory growth. Sequencing revealed that the SDH3 gene contained two A to T transversions, replacing His–106 and His–113 with glutamine and histidine, respectively (H106Q). This latter lesion truncates Sdh3p with growth yields of 35 and 40%, respectively, in SG and YPDG media.

FIG. 1. Growth of yeast strains on galactose media. Yeast strains were grown at 30 °C on semisynthetic liquid medium containing 0.1, 0.2, 0.3, 0.4, and 0.5% galactose and the absorbance values at 600 nm were measured. Cultures were inoculated at a starting A600 of 0.1 and allowed to reach late stationary phase (approximately 100 h). The relative growth yields were calculated using the final absorbance values reached on 0.5% galactose. Open squares, MH125; crosses, sfd3W3; closed circles, W116R; closed squares, H106Y; closed triangles, H113Q; closed diamonds, F103V; and open circles, sfd3W3, L122stop, H106Q/L117V, and L52FrShift. The growth characteristics of these latter four mutants are indistinguishable, and for simplicity, only one symbol is used.

Fig. 1 shows the growth of the seven mutants on semisynthetic medium containing 0.1–0.5% galactose. The growth yield of sfd3W3 is 10% of its parent, MH125; this represents only fermentative and no respiratory growth. Sequencing revealed that the SDH3 gene contained two A to T transversions, replacing His–106 and Asp–117 with Leu and Val, respectively (H106Y). This latter lesion truncates Sdh3p by removing the third transmembrane segment. A sixth mutant contains a single base deletion in the codon for Leu–52 that causes a frameshift mutation (L52FrShift) and truncates Sdh3p after the addition of two new amino acid residues, removing all three transmembrane segments.
the importance of this segment for SDH function.

**Assembly of Mutant and Wild Type SDH Enzymes—** To determine whether the SDH3 mutations interfere with enzyme assembly, we measured the levels of covalent FAD in mitochondrial membranes from mutant and wild type strains (Table I). In *S. cerevisiae*, SDH is the major covalent flavoprotein and the covalent flavin levels of mitochondrial membranes reflect SDH assembly (27). The covalent FAD levels of the F103V, H113Q, and W116R strains were not significantly different from the wild type. We conclude that SDH assembly is not significantly impaired in these mutants. The covalent FAD levels of the H106Y, H106LD117V, and L122stop strains were reduced, indicating some impairment of enzyme assembly.

Also shown in Table I are the succinate-PMS reductase activities of the mutant and wild type enzymes. Membrane-associated succinate-dependent PMS reductase activity depends only on the membrane anchoring function of the membrane domain and not on its catalytic competence. To compare catalytic efficiencies, we determined turnover numbers using covalent FAD levels. The succinate-PMS reductase activities of the F103V, H113Q, and W116R mutant enzymes are similar to that of the wild type enzyme in MH125 or sdh3W3 pYCSDH3. These data confirm that the primary defects in these mutants are not due to impaired assembly. The L122stop mutant has a turnover number only slightly less than that of MH125, indicating that its catalytic dimer retains wild type activity, although the lower level of covalent FAD in this mutant may indicate that the enzyme is only weakly attached to the membrane. In contrast, the turnover number of the H106Y/D117V mutant enzyme is reduced, suggesting that an altered Sdh3p structure influences catalysis by the attached catalytic dimer.

**Steady State Sdh3p Levels—** The steady state levels of the wild type and mutant Sdh3p subunits were compared by Western blot analysis (Fig. 2) (23). The W116R, H106LD117V, H113Q, H106Y, and F103V Sdh3p levels (Fig. 2, lanes 4–8, respectively) were similar to those of the wild type strains (lanes 2 and 9). A truncated Sdh3p, expected to migrate at about 13.8 kDa, was not detectable in the L122stop mutant (lane 3). Its absence may be due to protein degradation, to enzyme instability, or to an altered behavior in the gel or blotting systems. However, the L122stop mutation did not eliminate enzyme assembly, as judged by the covalent FAD level and the succinate-PMS reductase activity of isolated membranes. As expected, Sdh3p was absent in sdh3W3 (Fig. 2, lane 1).

**Quinone Reductase Activities of SDH3 Mutants—** Mitochondrial membranes of mutant and wild type strains were assayed for the DB-mediated reduction of 2,6-dichlorophenol indophenol (Table II). The succinate-DB reductase activities of the L122stop, F103V, H106LD117V, and W116R mutants were sharply reduced (25, 24, 20, and 18% of the wild type turnover number, respectively). The H113Q mutant enzyme retained a low but significant activity (35% of wild type level), whereas the H106Y mutant was not greatly impaired (60% of wild type level). These values are consistent with the levels of respiratory growth seen on galactose medium (Fig. 1) and with their respective abilities to grow on minimal glycerol medium. As expected, sdh3W3 had no detectable activity.

We also measured the malonate-sensitive, succinate-cytochrome *c* reductase and the succinate-oxidase activities of the mutant enzymes (Table II). The first assay depends on complexes II and III of the respiratory chain, whereas the second depends on complexes II, III, and IV. Both assays rely on the reduction of endogenous ubiquinone. The succinate-cytochrome *c* reductase and the succinate oxidase activities paralleled the succinate-DB reductase activities. The membranes of the SDH3-deficient strain, sdh3W3, had undetectable levels of these enzymatic activities. The NADH oxidase and glycerol-1-phosphate-cytochrome *c* reductase activities were largely unaffected in all the mutants. Because these activities do not depend on succinate as the source of electrons, they demonstrate the integrity of the remainder of the respiratory chain.

From these data, we conclude that the effects that we have observed arise from defective SDH function and that the SDH3 mutations do not have pleiotropic effects on the other respiratory complexes present in the mitochondrial inner membrane.

**Kinetics of Exogenous Quinone Reduction—** The kinetic parameters, *K*~m~ and *V*~max~ for the DB-mediated reduction of DB at four fixed concentrations of other substrates. The results are summarized in Table III. The apparent *K*~m~ values of the W116R and the H113Q mutant enzymes were increased by 3–4-fold, whereas those of the F103V and L122stop mutants were increased 2-fold. To compare the catalytic efficiencies of the enzymes, we also expressed the apparent *V*~max~ as maximal turnover numbers (*k*~cat~) based on covalent

### Table I

| Yeast strain          | Covalent FAD | Specific activity | Turnover number | Cytochrome b~562~ |
|-----------------------|--------------|------------------|-----------------|-------------------|
| MH125                 | 37 ± 4       | 148 ± 5          | 4000 ± 135      | 1.14 ± 0.16       |
| sdh3W3/pYCSDH3        | 34 ± 2       | 127 ± 6          | 3700 ± 185      | 0.54 ± 0.05       |
| F103V                 | 33 ± 4       | 118 ± 3          | 3500 ± 126      | 0.76 ± 0.05       |
| W116R                 | 32 ± 3       | 110 ± 4          | 3500 ± 112      | 0.65 ± 0.11       |
| H106Y                 | 24 ± 3       | 77 ± 3           | 3000 ± 111      | 0.55 ± 0.05       |
| H113Q                 | 30 ± 3       | 103 ± 3          | 3000 ± 111      | 0.55 ± 0.05       |
| L122stop              | 19 ± 2       | 57 ± 2           | 1800 ± 65       | 0.43 ± 0.05       |
| H106LD117V            | 17 ± 3       | 31 ± 1           | —               | —                 |
| sdh3W3                | ND           | 6                | —               | —                 |

* All values represent the mean of triplicate determinations ± S.E. Covalent flavin contents are expressed as pmol of FAD mg of protein~1~.

* Specific activities are expressed as μmol of PMS-mediated DCPIP reduced min~1~ mg of protein~1~.

* Turnover numbers are expressed as μmol of PMS-mediated DCPIP reduced min~1~ μmol of covalent FAD~1~.

* Cytochrome levels are expressed as nmol of heme nmol~1~ covalent FAD.

* Not determined.

* Not detectable.

**Fig. 2.** Western blot analysis of mutant and wild type mitochondria. Mitochondria were isolated from galactose-grown cultures. 10 μg of protein per lane were electrophoresed on 1% Tricine denaturing gels for Western blot analysis using an anti-Sdh3p antibody. Lanes 1–9 are sdh3W3, MH125, L122stop, W116R, H106LD117L, H113Q, H106Y, F103V, and sdh3W3/pYCSDH3, respectively.

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**TABLE II**

**Measurements of SDH assembly in mitochondrial membranes**

| Yeast strain          | Covalent FAD | Specific activity | Turnover number | Cytochrome b~562~ |
|-----------------------|--------------|------------------|-----------------|-------------------|
| MH125                 | 37 ± 4       | 148 ± 5          | 4000 ± 135      | 1.14 ± 0.16       |
| sdh3W3/pYCSDH3        | 34 ± 2       | 127 ± 6          | 3700 ± 185      | 0.54 ± 0.05       |
| F103V                 | 33 ± 4       | 118 ± 3          | 3500 ± 126      | 0.76 ± 0.05       |
| W116R                 | 32 ± 3       | 110 ± 4          | 3500 ± 112      | 0.65 ± 0.11       |
| H106Y                 | 24 ± 3       | 77 ± 3           | 3000 ± 111      | 0.55 ± 0.05       |
| H113Q                 | 30 ± 3       | 103 ± 3          | 3000 ± 111      | 0.55 ± 0.05       |
| L122stop              | 19 ± 2       | 57 ± 2           | 1800 ± 65       | 0.43 ± 0.05       |
| H106LD117V            | 17 ± 3       | 31 ± 1           | —               | —                 |
| sdh3W3                | ND           | 6                | —               | —                 |

* All values represent the mean of triplicate determinations ± S.E. Covalent flavin contents are expressed as pmol of FAD mg of protein~1~.

* Specific activities are expressed as μmol of PMS-mediated DCPIP reduced min~1~ mg of protein~1~.

* Turnover numbers are expressed as μmol of PMS-mediated DCPIP reduced min~1~ μmol of covalent FAD~1~.

* Cytochrome levels are expressed as nmol of heme nmol~1~ covalent FAD.

* Not determined.

* Not detectable.
A Quinone-binding Site in the Yeast SDH

**TABLE II**

Quinone-mediated enzymatic activities of mitochondrial membranes

| Strain          | Succinate-DB reductase | Succinate-cytochrome c reductase | Succinate oxidase | NADH oxidase | Glycerol-3-P-cytochrome c reductase |
|----------------|------------------------|---------------------------------|------------------|--------------|-----------------------------------|
|                 | Activity (nmol/min/mg) | Activity (nmol/min/mg)          | Activity (nmol/min/mg) | Activity (nmol/min/mg) | Activity (nmol/min/mg)          |
| MH125           | 2.0 ± 0.2              | 1.0 ± 0.1                       | 0.5 ± 0.1        | 0.3 ± 0.1    | 0.1 ± 0.0                         |
| sdh3W3/pYCSDH3  | 0.9 ± 0.1              | 0.4 ± 0.1                       | 0.2 ± 0.1        | 0.1 ± 0.1    | 0.0 ± 0.0                         |
| F103V           | 1.0 ± 0.1              | 0.5 ± 0.1                       | 0.3 ± 0.1        | 0.2 ± 0.1    | 0.1 ± 0.0                         |
| H106Y           | 1.5 ± 0.3              | 0.8 ± 0.2                       | 0.4 ± 0.2        | 0.2 ± 0.2    | 0.1 ± 0.1                         |
| H106LD117V      | 1.2 ± 0.5              | 0.6 ± 0.3                       | 0.3 ± 0.3        | 0.2 ± 0.3    | 0.1 ± 0.1                         |
| H113Q           | 1.2 ± 0.5              | 0.6 ± 0.3                       | 0.3 ± 0.3        | 0.2 ± 0.3    | 0.1 ± 0.1                         |
| W116R           | 1.8 ± 0.1              | 0.9 ± 0.1                       | 0.4 ± 0.1        | 0.2 ± 0.1    | 0.1 ± 0.0                         |
| L122stop        | 1.1 ± 0.3              | 0.5 ± 0.2                       | 0.3 ± 0.2        | 0.2 ± 0.2    | 0.1 ± 0.1                         |

**TABLE III**

The apparent Michaelis constants for succinate-DB reductase activities

| Strain          | $K_m$ (μM) | $V_{max}$ (nmol/min/mg) | $k_{cat}$ (nmol/min/mg) |
|----------------|------------|-------------------------|-------------------------|
| MH125          | 4.8 ± 0.1  | 125.8 ± 2.8             | 3400 ± 75               |
| sdh3W3/pYCSDH3 | 5.5 ± 0.2  | 123.2 ± 3.4             | 3250 ± 100              |
| F103V          | 10.0 ± 0.3 | 32.9 ± 1.2              | 1000 ± 35               |
| H106Y          | 7.0 ± 0.2  | 53.7 ± 1.7              | 2200 ± 70               |
| H106LD117V     | 7.3 ± 0.1  | 11.7 ± 0.3              | 690 ± 15                |
| H113Q          | 15.0 ± 0.5 | 39.8 ± 1.5              | 1300 ± 50               |
| W116R          | 18.0 ± 1.0 | 19.3 ± 0.9              | 612 ± 30                |
| L122stop       | 11.0 ± 0.3 | 15.5 ± 0.4              | 820 ± 20                |

Values are expressed as μmol of DB-mediated DCPIP reduced min$^{-1}$ mg of protein$^{-1}$.

**Fig. 3.** Thermal stability profiles of succinate-DB reductase activities. DB reduction was monitored spectrophotometrically at the wavelength pair of 280 and 325 nm after incubating mitochondrial membranes at the indicated temperatures for 10 min. Activities are expressed as percentages of turnover numbers observed at 25 °C for each strain. Values are the means of triplicate determinations ± S.E.

**Sensitivities of SDH3 Mutants to a Quinone Analog Inhibitor**—We have shown that 2-sec-butyl-4,6-dinitrophenol (s-BDNP) inhibits the yeast SDH with nonlinear noncompetitive kinetics (21). We expect that a mutation that affects quinone-reductase activity by altering a quinone-binding site will also alter the sensitivity of that enzyme to a quinone analog inhibitor. We determined the inhibitor sensitivities of the H106Y, H113Q, and wild type enzymes. No reasonable estimates of the inhibition constants could be determined for the remaining mutants due to their low succinate-DB reductase activities. With both mutants, complete inhibition could be attained. As previously reported (21), s-BDNP inhibits the S. cerevisiae SDH in a noncompetitive manner (Fig. 4, inset). The inhibition patterns of the mutant enzymes are also noncompetitive (data not shown). Replots of the abscissa intercepts against the inhibitor concentrations are hyperbolic for the wild type and the mutant enzymes (Fig. 4). Nonlinearity of replots can result from a number of possibilities, including partial inhibition, allosteric effects, inhibition by an alternative product, or binding of the inhibitor to more than one site. In light of two-quinone binding site models for SDH and related enzymes, we consider the latter possibility most likely. Accordingly, the data...
were analyzed with an equation for noncompetitive inhibition having two nonequivalent \( K_s \) values (28),

\[
y = \frac{(1 + 1/K_{1a})}{(1 + 1/K_{2a})} \tag{Eq. 1}
\]

where \( y \) is the slope or intercept in the presence of a fixed concentration of the inhibitor \( I, a \) is the slope or intercept in the absence of inhibitor, and \( K_{1a} \) and \( K_{2a} \) are the high affinity and low affinity inhibition constants, respectively. There are good fits of the data to the equation (Fig. 4). The apparent \( K_s \) values for inhibitor binding at sites 1 and 2 (\( K_{1s} \) and \( K_{2s} \)) are presented in Table IV. Consistent with our earlier observations, there was a 10-fold difference between the affinities of the two sites for s-BDNP in the wild type enzyme. There were no significant apparent affinity differences at the mutant low affinity inhibitor sites (\( K_{2s} \)) compared with the wild type. In contrast, the \( K_{1s} \) values for the high affinity sites in the H106Y and the H113Q mutants were increased 2- and 3-fold, respectively. These results are consistent with a two quinone-binding site model in which the H106Y and the H113Q mutations only affect one of the sites.

**DISCUSSION**

In this study, we provide several lines of evidence that three amino acid residues (Phe-103, His-113, and Trp-116) in the S. cerevisiae Sdh3p subunit are important in the formation of a quinone-binding site in SDH. First, the three mutants have impaired but not abolished respiratory growth, indicating that the mutant SDH enzymes are assembled and partially functional *in vivo* (Fig. 1). Second, the three mutants contain near normal levels of membrane-associated covalent FAD, wild type turnover numbers with the succinate-PMS reductase assay (Table I), and wild type levels of Sdh3p polypeptide (Fig. 2). The mutant enzymes are thus assembled and inserted into the membrane in normal amounts. Third, these three mutants are significantly impaired in succinate-DB reductase, succinate-cytochrome \( c \) reductase, and succinate oxidase activities, assays that require the ability to reduce quinones (Table II). Fourth, the mutations lead to increases in the apparent \( K_m \) values, with the highest increase observed for the W116R enzyme (Table III). Fifth, two of the mutations, H113Q and W116R, do not affect the thermostabilities of the mutant enzymes, suggesting that the structural perturbations are minor. Sixth, for the H113Q mutation, the inhibitor sensitivity analysis reveals that only one of two inhibitor sites and by extension, one of the two quinone-binding sites is affected. Finally, the F103V, H113Q, and W116R residues are topologically clustered (Fig. 5), strongly arguing for their involvement in a common function, which we postulate is the formation of a quinone-binding site in or near the loop connecting transmembrane segments II and III. The specific roles of these and neighboring residues in the formation of this site can now be further explored by site-directed mutagenesis.

The L122stop mutation results in many of the same effects as the F103V, H113Q, and W116R mutations, such as a lowered succinate-DB reductase activity and an elevated \( K_m \) value. However, it also results in lower covalent FAD (Table I) and Sdh3p levels (Fig. 2), suggesting that the enzyme is not stably assembled. This is further supported by its thermostability profile (Fig. 3). It is perhaps not surprising that the L122stop mutation shares properties with the quinone-binding site mutants, because it removes transmembrane segment III of Sdh3p, and we postulate that a quinone-binding site is associated with the loop connecting transmembrane segments II and III.

The H106L/D117V mutant enzyme has the very interesting property of having a reduced turnover number in the succinate-PMS reductase assay (Table I), suggesting that these SDH3 mutations propagate their effects into the catalytic dimer. We imagine that Asp-117 is located at the interface between the catalytic and the membrane domains. (The H106L lesion is expected to be within the bilayer; see Fig. 5.) Alternatively, Asp-117 may be near and perturb the PMS binding site in the catalytic dimer. Asp-117 is the first membrane domain residue proposed to modulate electron transport of the catalytic domain in this family of enzymes. We will further explore Asp-117 by creating additional mutations at this residue and in its vicinity.

The turnover numbers of the H106Y mutant enzyme for succinate-DB reductase, succinate-cytochrome \( c \) reductase, and succinate oxidase activities were all decreased by 40%, whereas the apparent \( K_m \) was only slightly elevated compared with the wild type. This suggests that His-106 does not play a direct

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**Table IV**

| Strain      | \( K_{1s} \)  | \( K_{2s} \) |
|-------------|---------------|---------------|
| MH125       | 0.060 ± 0.005* | 0.67 ± 0.03   |
| H113Q       | 0.19 ± 0.03   | 0.70 ± 0.01   |
| H106Y       | 0.11 ± 0.01   | 0.69 ± 0.02   |

*a* Values represent the mean of triplicate determinations ± S.E.
role in quinone binding or reduction. Notably, the H106Y mutation caused the largest reduction of cytochrome \(b_{562}\) of all the mutants (Table I). Interestingly, the H106Y mutation also produced the enzyme with the highest succinate-DB reductase activity per heme content, a value that is almost twice that of the wild type enzyme (not shown).

Is His-106 an axial ligand for cytochrome \(b_{562}\)? In a multiple sequence alignment of SDH and FRD membrane subunits, the yeast Sdh3p His-106 aligns with an absolutely conserved histidine residue present in the second transmembrane segments of these enzymes (4). In some cases, such as His-70 of the Bacillus subtilis SDH and His-84 of the E. coli SDH, this histidine has been experimentally shown to be a heme ligand (30–33). The conclusion that His-106 is a heme liganding residue in Sdh3p can only be tentative because the two mutations involving this position (H106Y and H106L/D117V) do not completely eliminate cytochrome \(b_{562}\) assembly. Cytochrome assembly may be impaired but not prevented by the loss of a single ligand. In some cases, a natural ligand is apparently replaced by an alternative residue, as suggested by the reconstitution of the bovine cytochrome \(b_{562}\) in either purified QPs1 or QP3 subunits (16, 34). Further studies will be necessary to clarify the role of His-106.

The F103V, H113Q, and W116R quinone-binding site mutations may reduce the heme contents of the respective enzymes because of structural changes that perturb cytochrome or oxidations, and solid lines indicate truncations. IMS, intermembrane space. Residue numbering is based on the mature protein sequence.

Photoaffinity labeling experiments have also identified the amino-terminal end of the first transmembrane segment of the E. coli SdhC subunit as required for quinone binding (18). This region corresponds to the location of the first three FrdC mutations mentioned above. It seems likely that residues close to the amino- and carboxyl-terminal ends of transmembrane segments I and II, respectively, contribute to the formation of a quinone-binding site. Our data do not exclude the presence of additional Sdh3p residues involved in quinone binding. Our screening procedure is biased against completely respiratory deficient cells and thus might have prevented the isolation of mutations in another region of Sdh3p, such as close to transmembrane segment I.

Recently, we reported that the quinone analog s-BDNP inhibits the yeast SDH in a biphasic, noncompetitive manner (21). Similar inhibition patterns are seen with the E. coli FRD and the bovine SDH (17). We observed biphasic inhibition of the wild type enzyme that can most simply be explained by two inhibitor sites with a 10-fold difference in affinities. The H113Q and H106Y mutants also showed biphasic inhibition patterns, but with significant increases in their apparent \(K_i\) values, suggesting a perturbation in the high affinity inhibitor-binding site. If the s-BDNP inhibitor does bind to two sites, then two quinone-binding sites are likely present in the yeast SDH. Similarly, two sites are proposed for the E. coli FRD and for the bovine SDH (14, 17, 20, 35). In our previous work (21), mutations involving the carboxyl terminus of Sdh4p affected the apparent \(K_i\) values for s-BDNP, indicating that the two inhibitor sites and, by extension, the two quinone-binding sites, are spatially distinct in the yeast SDH. We cannot identify which of the two putative quinone-binding sites, Qs or Q3, is affected in the Sdh3p mutants or whether the inhibitor binding sites physically correspond to those sites, although the topological model for Sdh3p is consistent with it containing the Qb site.

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