The Quinone-binding Sites of the *Saccharomyces cerevisiae* Succinate-ubiquinone Oxidoreductase*

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The *Saccharomyces cerevisiae* succinate dehydrogenase (SDH) of the mitochondrial electron transport chain oxidizes succinate and reduces ubiquinone. Using a random mutagenesis approach, we identified functionally important amino acid residues in one of the anchor subunits, Sdh4p. We analyzed three point mutations (F69V, S71A, and H99L) and one nonsense mutation (Y89OCH) that truncates the Sdh4p subunit at the third predicted transmembrane segment. The F69V and the S71A results in greatly impaired respiratory growth *in vivo* and quinone reductase activities *in vitro*, with negligible effects on enzyme stability. In contrast, the Y89OCH and the H99L mutations elicit large structural perturbations that impair assembly as evidenced by reduced covalent FAD levels, membrane-associated succinate-phenazine methosulfate reductase activities, and thermal stability. We propose that the Phe-69 and the Ser-71 residues are involved in the formation of a quinone-binding site, whereas the His-99 residue is at the interface of the peripheral and the membrane domains. In addition, the properties of the Y89OCH mutation are consistent with the interpretation that the third transmembrane segment is not involved in catalysis but rather plays an important structural role. The mutant enzymes are differentially sensitive to a quinone analog inhibitor, providing further evidence for a two-quinone binding model in the yeast SDH.

Succinate dehydrogenase (SDH), also known as complex II or succinate-ubiquinone oxidoreductase, participates in the mitochondrial electron transport by oxidizing succinate to fumarate and transferring the electrons to ubiquinone (1–5). The mitochondrial respiratory chain carries out a series of vectorial reactions that generate an electrochemical potential across the inner mitochondrial membrane, which is then used to drive the synthesis of ATP (6–9). Membrane-bound fumarate reductases are structurally and functionally related enzymes and are present in anaerobic organisms respiring with fumarate as the terminal electron acceptor (1–4, 10). FRD catalyzes the reduction of fumarate to succinate coupled to the oxidation of quinol, the reverse of the reaction catalyzed by SDH. Both enzymes can catalyze their respective reverse reactions *in vitro*, and in some cases, *in vivo*. However, SDH and FRD are physiologically distinct enzymes (2, 4, 11).

Generally, SDH is made up of two distinct domains: a dimeric peripheral domain and a monomeric or a dimeric membrane-intrinsic domain. In the yeast *Saccharomyces cerevisiae*, the peripheral domain, which contains the active site for succinate oxidation, comprises the 67-kDa Sdh1p subunit to which is covalently attached an FAD cofactor (12–16) and the 28-kDa Sdh2p subunit, which contains three iron-sulfur clusters (17, 18). The membrane-intrinsic domain is composed of two hydrophobic subunits, Sdh3p and Sdh4p, of 16.7 and 16.6 kDa, respectively (19–21). The anchor domain contains a b-type heme and the active site for ubiquinone reduction (22–25). The anchor domain was proposed to contain a core of four anti-parallel helices comprising helices I, II, IV, and V (4, 26). This model is now supported by two crystal structures (10, 27, 28).

Several lines of experimentation have shown that the membrane domain contains the binding sites for quinone substrates. First, functional anchor polypeptides are required for interaction with quinone substrates (1, 3). Second, a thermodynamically stable interacting ubisemiquinone pair has been observed in the vicinity of iron-sulfur center 3 in SDH from *Bos taurus* (1, 3, 4), *Neurospora crassa* (29), and some green algae (30). The stability of ubiquinone in such a hydrophobic milieu is indicative of interaction with protein. Photoaffinity-labeling experiments (31–35), mutagenesis (36, 37), and inhibitor (1, 4, 38) studies have shown that SDH and FRD contain at least two putative quinone-binding pockets, which are located on the opposite sides of the membrane. The crystal structure of the *Escherichia coli* FRD (27) revealed two bound quinones. However, the crystal structure of the *Wolinella succinogenes* FRD (28) shows no such bound quinone, although there are two distal cavities in subunit C that could potentially bind quinone.

We have carried out several studies to identify functionally important residues in the yeast SDH (22, 24, 25). Residues in the unusual carboxyl-terminal extension of the yeast Sdh4p subunit are necessary for maintaining a stable conformation of the anchor domain required for quinone reduction (22, 24). We have identified residues in the yeast Sdh3p that are involved in quinone reduction (25). The location of these residues and inhibitor sensitivity analysis suggested that the yeast SDH contains at least two quinone-binding sites. The present paper extends these studies to the Sdh4p subunit and provides additional evidence for the two quinone-binding site hypothesis. We isolated and characterized four mutations in the *SDH4* gene. The mutants were characterized *in vivo* for their abilities to support respiratory growth and *in vitro* for quinone reduction, enzyme assembly and stability, and inhibitor sensitivity. The data show that a distal quinone-binding site (Qd) is associated with the cytoplasmic side of the membrane. In addition, the low

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The abbreviations used are: SDH, succinate dehydrogenase; FRD, fumarate reductase; DCPIP, 2,6-dichlorophenol indophenol; FMS, phenazine methosulfate; DB, 2,3-dimethoxy-5-methyl-6-decy1,4-benzoquinone; Qp, proximal quinone-binding site; Qd, distal quinone-binding site; s-BDNP, 2-sec-butyl-4,6-dinitrophenol; TMS, transmembrane segment.

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Succinate dehydrogenase (SDH), also known as complex II or succinate-ubiquinone oxidoreductase, participates in the mitochondrial electron transport by oxidizing succinate to fumarate and transferring the electrons to ubiquinone (1–5). The mitochondrial respiratory chain carries out a series of vectorial reactions that generate an electrochemical potential across the inner mitochondrial membrane, which is then used to drive the synthesis of ATP (6–9). Membrane-bound fumarate reductases are structurally and functionally related enzymes and are present in anaerobic organisms respiring with fumarate as the terminal electron acceptor (1–4, 10). FRD catalyzes the reduction of fumarate to succinate coupled to the oxidation of quinol, the reverse of the reaction catalyzed by SDH. Both enzymes can catalyze their respective reverse reactions *in vitro*, and in some cases, *in vivo*. However, SDH and FRD are physiologically distinct enzymes (2, 4, 11).

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and the high affinity inhibitor-binding sites, which may correspond to the two quinone-binding sites, map to the opposite sides of the membrane. A histidine residue in the matrix-facing loop between helices V and VI is proposed to interact with the catalytic domain.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Culture Conditions**—The parental yeast strain, MH125, and the E. coli strain, DH5α, have been described previously (24). The SDH4 knockout strain, sdh4w2, is an isogenic derivative of MH125 (MH125, SDH4::TRP1) and has been described previously (22). All the yeast media used in this study (YPD, YPG, SG, SD, YPDP, and YPGal) and yeast culture conditions have been described (22, 25). Plasmid loss was routinely monitored by plating out aliquots on YPD and selectable SD media. The proportion of rho− strains in the culture was determined by mating the colonies with the rho° strain, MS10 (mata his-l his-11 leu2-3 can1 rhrho3), and testing for 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.

**Random Mutagenesis**—Random mutagenesis was carried out essentially as described previously (25) with the following modifications. A plasmid-borne SDH4 gene, pSDH4–17, was transformed into DH5α. Cells were irradiated with ultraviolet light of 254 nm with a dose rate of 1.4 J m−2 s−1 on LB plates in the dark to achieve 1–5% survival. Mutagenized plasmids were isolated from the bacteria and transformed into the sdh4w2 strain, sdh4w2. Transformants were tested for growth on YPDG, SG, and YPG to identify respiratory-deficient colonies. Respiratory-deficient but rho− colonies were further analyzed. In order to ensure that any observed respiratory defect is plasmid-mediated, plasmids were rescued from yeast and transformed into transformed into the sdh4w2 strain. Mutations were identified by sequencing the entire SDH4 gene. Sequencing was performed by the Department of Biochemistry Core DNA Facilities, University of Alberta (Edmonton, Alberta, Canada). To ensure that any phenotype observed is due to mutation in the SDH4 gene (and not to mutation of vector sequence), the SDH4 cassette was subcloned into a non-irradiated pRS416 vector and reintroduced into sdh4w2.

**Membrane Preparation and Enzyme Assays**—YPGal-grown station any phase cells were harvested by centrifugation, lysed in a French pressure cell, and submitochondrial particles prepared by differential centrifugation as described previously (22, 24, 25). 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 (DB)-mediated reduction of 2,6-dichlorophenol indophenol (DCPIP). The succinate-dependent, phenazine methosulfate (PMS)-mediated reduction of DCPIP was determined as a measure of the membrane-associated Sdh1p/Sdh2p dimer. This assay only requires that the catalytic dimer be membrane-associated but does not require a catalytically competent membrane domain. Quinone reduction was also directly monitored as the reduction of DB using the wavelength pair, 280 and 325 nm, with a Hewlett Packard 8453 diode array spectrophotometer. The absorption coefficient is 16 nmol cm−1 cm−1. Other assays have been described (22, 24, 25).

**Kinetic Analysis**—The effect of quinone on the initial velocity of the succinate-DB reductase activity was measured by varying the concentrations of DB at fixed saturating concentrations of succinate and DCPIP. We evaluated the apparent kinetic parameters, and from double-reciprocal plots as described previously (22, 24, 25). Sensitivities to the quinone analog inhibitor, 2-sec-butyl-4,6-dinitrophenol (s-BDNP), were determined by measuring initial velocities of succinate-DB reductase in the presence of fixed concentrations of the inhibitor. The apparent inhibitor constants, , and , were evaluated by fitting data to the equation for noncompetitive inhibition having two nonequivalent constants (39),

$$
\frac{1}{y} = \frac{1}{V_{max,1}} \left(1 + IC_{50,1}\right) + \frac{1}{V_{max,2}} \left(1 + IC_{50,2}\right).
$$

(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 the inhibitor, and, are the high affinity and the low affinity inhibition constants, respectively.

**Thermal Stability**—Membrane fractions (20 mg/ml) were divided into equal aliquots (50 μl) in 1.5-ml Eppendorf tubes and placed on ice. Using a variable temperature block heater, each sample was incubated for 10 min in the absence of succinate and quinone, at temperatures between 22 and 70 °C. After incubation, the sample was cooled to 22 °C and the succinate-DB reductase activity determined. Residual activity was compared with the activities of unheated samples.

**Miscellaneous Methods**—Preparation of mitochondrial membranes, measurements of covalently bound flavin and protein contents, E. coli transformation, and recombinant DNA methods have been described (22, 24, 25).

**RESULTS**

**Mutagenesis of the SDH4 Gene**—An E. coli strain carrying the plasmid-borne SDH4 gene was mutagenized with ultraviolet light and the mutated plasmids were reintroduced into the yeast SDH4-deficient strain, sdh4w2. Mutagenesis in E. coli, rather than in yeast, greatly reduces the proportion of yeast mitochondrial petite mutations isolated. About 6000 Ura+ sdh4w2 transformants were tested for respiratory growth on YPDG and SG. Approximately 1% were impaired for respiratory growth. We isolated and sequenced the SDH4 genes from 20 respiratory-deficient strains that were Ura+ and rho−. Sixteen of these mutants contained frameshift mutations that resulted in null phenotypes. Four mutants contained single base alterations, resulting in the substitutions of Phe-69 with Val (F69V), Ser-71 with Ala (H99L), and Tyr-89 with a stop codon (T89OCH). The Y89OCH mutation truncates Sdh4p by removing the third predicted transmembrane segment, TMS-III (Fig. 1). The mutated SDH4 genes were reintroduced into the SDH4 knockout strain, sdh4w2, and assayed for respiratory growth on media containing the non-fermentable carbon sources, ethanol, glycerol, or lactate. All the mutant strains were greatly impaired for growth on these media. In order to determine the residual respiratory abilities of the mutant strains, growth on semisynthetic medium containing 0.1%, 0.2%, 0.3%, 0.4%, or 0.5% galactose was also monitored spectrophotometrically. Fig. 2 depicts the growth yields monitored as the optical densities at 600 nm of late stationary phase cultures. Growth of yeast strains in this medium is biphasic; an initial fermentative growth is followed by a respiratory phase when the fermentable carbon source is limiting (22, 40). Both the wild type and the mutant strains show similar growth rates during the fermentative phase (data not shown). The growth yields of the parental strain, MH125, and the sdh4w2 strain carrying a plasmid-borne wild type SDH4 gene, pSDH4–17, are similar. As expected, sdh4w2 achieves a growth yield of 10 ± 2% by fermentation alone. The growth yields of the F60V, the S71A, the
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Measurements of SDH assembly in yeast mitochondrial membranes

Each value represents the mean of triplicate independent determinations ± S.E.

| Yeast strain            | Covalent FAD<sup>a</sup> | Specific activity<sup>b</sup> | Turnover no.<sup>c</sup> |
|-------------------------|--------------------------|------------------------------|--------------------------|
| MH125                   | 30 ± 4                   | 102 ± 6                      | 3400 ± 200               |
| sdh4W2/SDH4–17          | 27 ± 2                   | 86 ± 4                       | 3200 ± 150               |
| F69V                    | 22 ± 2                   | 72 ± 5                       | 3000 ± 200               |
| S71A                    | 22 ± 3                   | 84 ± 3                       | 2900 ± 120               |
| H99L                    | 14 ± 3                   | 19 ± 1                       | 1400 ± 80                |
| Y89OCH                  | 12 ± 1                   | 12 ± 1                       | 1000 ± 45                |
| sdh4W2                  | ND<sup>d</sup>           | ND<sup>d</sup>              | ND<sup>d</sup>           |

<sup>a</sup> Covalent flavin contents are expressed as pmol FAD mg of protein<sup>–1</sup>

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

<sup>c</sup> Turnover numbers are expressed as μmol of PMS-mediated DCPIP reduced min<sup>–1</sup> nmol of covalent FAD<sup>–1</sup>

<sup>d</sup> ND, not detectable.

Not determined.

The succinate-PMS reductase activity of the S71A enzyme. These data confirm that the primary defects in the F69V and S71A are not due to impaired assembly. In contrast, the H99L and the Y89OCH enzymes show significant reductions in their succinate-PMS reductase activities, indicating assembly defects.

Quinone Reductase Activities of the SDH4 Mutants—We assayed mitochondrial membranes for malonate-sensitive succinate dependent quinone reduction, using quinones of the SDH4 analog DB and the reporter DCPIP (Table II). The succinate-DB reductase activities of F69V, S71A, H99L, and Y89OCH are severely reduced (30%, 26%, 20%, and 18% of the wild type turnover number, respectively). Succinate-DB reductase activity is not detectable in sdh4W2. These values are consistent with the residual levels of respiratory growth seen on limiting galactose (Fig. 2).

We also measured the malonate-sensitive, succinate-dependent cytochrome c reductase and the succinate-oxidase activities of mutant and wild type mitochondrial membranes (Table II). The former assay depends on complexes II and III of the respiratory chain, whereas the latter assay requires complexes II–IV. Both assays rely on the reduction of endogenous quinone and are more stringent assays of the integrity of the SDH anchor polypeptides. The succinate-cytochrome c reductase and the succinate oxidase activities of the mutant enzymes parallel the succinate-DB reductase activities, confirming that these mutant enzymes are impaired for quinone reduction. Neither activity is detectable in the sdh4W2 membranes. To rule out the possibility that the SDH4 mutations have pleiotropic effects on other respiratory enzymes of the mitochondrial inner membrane, we measured the NADH oxidase and the glycerol-1-phosphate cytochrome c reductase activities (Table II). As shown in Table II, the SDH4 mutations do not affect these activities; the respiratory deficiencies are limited to SDH.

Kinetics of Exogenous Quinone Reduction—In our previous studies (22, 24), we observed that some defects in quinone reduction could be partially rescued by preincubation with quinone. We incubated the mutant membranes with a 10-fold excess of DB (0.5 mM) at 22 °C for 5 min and determined the succinate-DB reductase activity (data not shown). The activities of the F69V and the S71A membranes could be stimulated by 25% and 20%, respectively, indicating lower affinities of these enzymes for quinone. The H99L and the Y89OCH membranes showed only slight stimulation in their succinate-DB reductase activities (10% and 8%, respectively). Longer incubations could not restore these activities to wild type levels. Stimulation of the succinate-DB reductase activities was not

FIG. 2. Growth of yeast strains on galactose media. Yeast strains were grown at 30 °C on semisynthetic liquid medium containing 1%, 0.2%, 0.3%, 0.4%, and 0.5% galactose, and the optical densities at 600 nm were measured. Precultures were prepared on selective minimal medium containing 2% galactose and 0.01% glucose. To compare their enzymatic activities, we determined the turnover numbers, based on the covalent FAD levels. The activity of the F69V enzyme is not significantly different from the wild type or the SDH4–17 enzymes. There is a slight reduction in the succinate-PMS reductase activity of the S71A enzyme. These data confirm that the primary defects in the F69V and S71A are not due to impaired assembly. In contrast, the H99L and the Y89OCH enzymes show significant reductions in their succinate-PMS reductase activities, indicating assembly defects.

Assembly of Mutants and Wild Type SDH Enzymes—We examined the possibility that the SDH4 mutations affected the anchoring role of the Sdh4p subunit by determining the covalent FAD contents of mitochondrial membranes prepared from wild type and mutant strains (Table I). In the yeast S. cerevisiae, SDH is the major protein with covalently bound FAD and the covalent flavin levels of mitochondrial membranes quantitatively reflect SDH assembly (41). There is no significant difference between the covalent FAD levels of the F69V mutant and the wild type strains, MH125 and SDH4–17. The S71A membranes have slightly reduced covalent FAD levels compared with the wild type values. In contrast, the covalent FAD levels of the H99L and the Y89OCH strains are significantly reduced, suggesting that these mutations induce structural perturbations that impair assembly into the membrane.

Table I also shows the succinate-PMS reductase activities of mutant and wild type enzymes. This activity requires that the membrane domain be competent for anchoring but not for quinone reduction (1, 3). To compare their enzymatic activities, we determined the turnover numbers, based on the covalent FAD contents. As expected, the succinate-PMS reductase activities closely parallel the covalent FAD levels. The activity of the F69V enzyme is not significantly different from the wild type or the SDH4–17 enzymes. There is a slight reduction in the succinate-PMS reductase activity of the S71A enzyme. These data confirm that the primary defects in the F69V and S71A are not due to impaired assembly. In contrast, the H99L and the Y89OCH enzymes show significant reductions in their succinate-PMS reductase activities, indicating assembly defects.
Quinone-binding Sites in the Yeast SDH

Enzymatic activities requiring quinone reduction

|                     | MH125 | sdh4w2 | pSDH4–17 | F69V | S71A | H99L | Y89OCH | sdh4w2 |
|---------------------|-------|--------|----------|------|------|------|--------|--------|
| Succinate-DB reductase | 3200 ± 200  | 3100 ± 120 | 960 ± 40  | 830 ± 30 | 640 ± 30 | 576 ± 10 | ND*     |
| Succinate-cytochrome c reductase | 2250 ± 50   | 2200 ± 100 | 670 ± 30  | 550 ± 10  | 450 ± 20  | 400 ± 20  | ND      |
| Succinate oxidase | 1690 ± 80   | 1660 ± 50  | 500 ± 20  | 380 ± 10  | 330 ± 7   | 300 ± 4   | ND      |
| NADH oxidase | 4000 ± 200  | 3980 ± 120 | 3700 ± 180| 3700 ± 200| 3500 ± 80 | 3550 ± 100| 1100     |
| Glycerol-1-P-cytochrome c reductase | 2700 ± 100  | 2700 ± 50  | 2400 ± 150| 2460 ± 90 | 2280 ± 80 | 2200 ± 90 | 81%    |

*Activities are expressed as μmol of DB-mediated DCPIP reduced min^−1 nmol of covalent FAD^−1.
*Each value represents the mean of triplicate independent determinations ± S.E.
*Activities are expressed as μmol of covalent FAD reduced min^−1 nmol of covalent FAD^−1.
*ND, not detectable or less than 4% of wild type activity.
*Activity is expressed as ng atoms of oxygen min^−1 mg protein^−1.
*Activity is expressed as nmol of covalent c reduced min^−1 mg protein^−1.

Kinetically parameters were determined by varying the concentrations of DB at fixed concentrations of succinate and DCPIP. $K_m$ and $V_{max}$ were calculated from a nonlinear regression fit to the Michaelis equation using initial estimates from the Lineweaver-Burk plots. Each value represents the mean of triplicate independent determinations ± S.E.

| Strain                  | $K_m$ | $V_{max}$ | $k_{cat}$ |
|------------------------|-------|-----------|-----------|
| MH125                  | 3.7 ± 0.2 | 108 ± 6 | 3580 ± 180 |
| sdh4w2/SDH4–17         | 4.1 ± 0.3 | 94 ± 3  | 3470 ± 100 |
| F69V                   | 14.2 ± 0.8 | 48 ± 1  | 2000 ± 90  |
| S71A                   | 15.2 ± 0.4 | 34 ± 2  | 1550 ± 80  |
| H99L                   | 8.0 ± 0.5  | 140 ± 3 | 1040 ± 90  |
| Y89OCH                 | 19.5 ± 0.3 | 9.6 ± 0.4| 800 ± 40   |

*Values are expressed in μmol.
*Values are expressed as μmol of DB-mediated DCPIP reduced min^−1 mg of protein^−1.
*Values are expressed as μmol of DB-mediated DCPIP reduced min^−1 nmol of covalent FAD^−1.

Succinate oxidase—An enzyme which transfers electrons from the quinone to the cytochrome c. This enzyme is involved in oxidizing quinones to their oxidized forms.

Sensitivities of SDH4 Mutants to a Quinone Analog Inhibitor—The 4,6-dinitrophenol derivatives are quinone analogs (43) that inhibit electron transfer in many respiratory proteins due to succinate activation, since the incubation was done in the absence of this substrate. The stimulation did not result from the higher quinone concentration used because membranes pre-incubated with 50 μM DB (the concentration used for the non-stimulated assay), showed similar levels of stimulation (data not shown). Pre-incubation with DB has no effect on the activities of the wild type, the SDH4–17, or the sdh4w2 enzyme activities.

We measured the apparent kinetic parameters, $K_m$ and $V_{max}$, using the reporter DCPIP in a coupled enzyme assay that measures the malonate-sensitive, succinate-dependent reduction of DB. Mitochondrial membranes containing mutant and wild type enzymes were pre-incubated with varying concentrations of DB at 22 °C for 5 min, and the reactions were initiated by the addition of succinate and DCPIP. The apparent Michaelis constants were calculated and are summarized in Table III. The apparent $K_m$ values of the F69V and the S71A enzymes are increased 3–4-fold, whereas the $K_m$ values of the Y89OCH enzyme exhibit thermal denaturation profiles that are comparable to those of the wild type enzyme. We also measured the succinate-PMS reductase activities of the heated membranes (Fig. 3B). Interestingly, the F69V and the S71A mutant enzymes are more stable for this activity, whereas the H99L and the Y89OCH enzymes are labile. These differential effects of thermal inactivation on the two enzyme activities suggest that the structural perturbations produced by the F69V and the S71A mutations are more localized to the membrane domain, the site of quinone reduction, whereas the H99L and the Y89OCH mutations induce structural perturbations that are propagated to the catalytic dimer. Since preincubation with quinone stimulates the activities of the mutant enzymes (data not shown), we also tested whether quinone could stabilize the enzymes at elevated temperatures. As shown in Fig. 3C, preincubation with DB restores the stability of the F69V and the S71A mutant enzymes to wild type levels, strongly suggesting that these residues are close to a quinone-binding site. In contrast, preincubation with DB has no detectable effect on the activities of the other mutant enzymes, suggesting that impaired quinone reduction may not be the primary defects in these enzymes. Similarly, preincubation with DB has no effect on the thermal denaturation profile of wild type enzyme.
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First, the F69V and the S71A mutations cause a severe impairment of respiratory growth (Fig. 2). However, residual respiratory activity remains, indicating that the mutant SDH enzymes are assembled and partially functional in vivo. Second, the two mutants have near normal levels of covalent flavin and of succinate-PMS reductase activity (Table I). These results demonstrate that the mutations do not significantly affect the anchoring function of Sdh4p and that the mutant enzymes are assembled into the membrane in normal amounts. Third, enzyme activities requiring quinone reduction (succinate-DB reductase, succinate-cytochrome c reductase, and succinate oxidase) are significantly reduced in the two mutants. Fourth, incubation of the two mutant enzymes at elevated temperatures has only a minor effect on the stability of succinate-DB reductase activity (Fig. 3A). Stability can be restored by preincubation with DB (Fig. 3C). Furthermore, the mutations do not affect the thermal denaturation profile of the succinate-PMS reductase activity (Fig. 3B). These data strongly suggest that the F69V and the S71A mutations specifically affect quinone binding in the membrane domain. Fifth, the mutations decrease the affinities of the enzymes for quinone substrate (Table III). The significant decrease in catalytic efficiency (kcat/Km) strongly favors this interpretation. Sixth, the kinetics of s-BDNP inhibition (Table IV) show that only the low affinity inhibitor-binding site, Kd(2), is affected. This inhibitor-binding site likely corresponds to a quinone-binding site (38, 44). Consistent with our previous observations (22, 24, 25), s-BDNP inhibits the yeast SDH in a non-competitive (Fig. 4A) and non-linear (Fig. 4B) manner. Accordingly, we evaluated the apparent high affinity and low affinity inhibitor constants (Kd(1) and Kd(2) respectively) as described previously (22, 25).

The anchor polypeptides of SDHs and FRDs show considerable variability in cofactor composition and primary structure (1, 3, 4, 10, 26, 45). It is likely that this variability contributes to the unique properties and functions of these enzymes in different biological systems. Intensive searches for quinone-binding sites in SDHs (31, 32, 46, 47) and FRDs (36, 37) have been carried out. In addition, the crystal structures of the E. coli and the W. succinogenes FRDs have recently been described (27, 28), revealing considerable structural similarity at the tertiary level. The membrane domains, as expected, contain the most dissimilar regions. The E. coli FRD contains two bound quinones without heme, whereas the W. succinogenes FRD has two bound hemes without quinone. The yeast SDH differs from these two cases, having a single heme and two quinone-binding sites.

As part of our ongoing effort to identify functionally important residues in the anchor polypeptides of the yeast SDH (22, 24, 25), we have randomly mutagenized the SDH4 gene. Several lines of experimental evidence presented in this study show that the two amino acid residues Phe-69 and Ser-71 are important in the formation of a quinone-binding site in SDH. First, the F69V and the S71A mutations cause a severe impairment of respiratory growth (Fig. 2). However, residual respiratory activity remains, indicating that the mutant SDH enzymes are assembled and partially functional in vivo. Second, the two mutants have near normal levels of covalent flavin and of succinate-PMS reductase activity (Table I). These results demonstrate that the mutations do not significantly affect the anchoring function of Sdh4p and that the mutant enzymes are assembled into the membrane in normal amounts. Third, enzyme activities requiring quinone reduction (succinate-DB reductase, succinate-cytochrome c reductase, and succinate oxidase) are significantly reduced in the two mutants. Fourth, incubation of the two mutant enzymes at elevated temperatures has only a minor effect on the stability of succinate-DB reductase activity (Fig. 3A). Stability can be restored by preincubation with DB (Fig. 3C). Furthermore, the mutations do not affect the thermal denaturation profile of the succinate-PMS reductase activity (Fig. 3B). These data strongly suggest that the F69V and the S71A mutations specifically affect quinone binding in the membrane domain. Fifth, the mutations decrease the affinities of the enzymes for quinone substrate (Table III). The significant decrease in catalytic efficiency (kcat/Km) strongly favors this interpretation. Sixth, the kinetics of s-BDNP inhibition (Table IV) show that only the low affinity inhibitor-binding site, Kd(2), is affected. This inhibitor-binding site likely corresponds to a quinone-binding site (38, 44). Finally, close proximity of the residues Phe-69 and Ser-71

![Fig. 3. Thermal stability profiles of succinate-DB reductase activities of mutant and wild type enzymes.](http://www.jbc.org/) Values represent the activities of mutant and wild type enzymes. Consistent with our previous observations (22, 24, 25), s-BDNP inhibits the yeast SDH in a non-competitive (Fig. 4A) and non-linear (Fig. 4B) manner. Accordingly, we evaluated the apparent high affinity and low affinity inhibitor constants (Kd(1) and Kd(2) respectively) as described previously (22, 25).

The apparent high affinity and low affinity inhibitor constants (Kd(1) and Kd(2) respectively) as described previously (22, 25). The apparent Kd(1) for the F69V and the S71A mutant enzymes show only a minimal increase compared with the wild type values, whereas their Kd(2) values are increased 3-fold. In contrast, both the apparent Kd(1) and Kd(2) values for the H99L and the Y89OCH mutant enzymes are considerably increased.

**DISCUSSION**

The anchor polypeptides of SDHs and FRDs show considerable variability in cofactor composition and primary structure (1, 3, 4, 10, 26, 45). It is likely that this variability contributes to the unique properties and functions of these enzymes in different biological systems. Intensive searches for quinone-binding sites in SDHs (31, 32, 46, 47) and FRDs (36, 37) have been carried out. In addition, the crystal structures of the E. coli and the W. succinogenes FRDs have recently been described (27, 28), revealing considerable structural similarity at the tertiary level. The membrane domains, as expected, contain the most dissimilar regions. The E. coli FRD contains two bound quinones without heme, whereas the W. succinogenes FRD has two bound hemes without quinone. The yeast SDH differs from these two cases, having a single heme and two quinone-binding sites.

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**FIG. 4. Inhibition of quinone reduction by 2-sec-butyl-4,6-dinitrophenol.**

A, double-reciprocal plots showing non-competitive inhibition of succinate-DB reductase activity of the wild type enzyme by the inhibitor. The inhibitor concentrations are: 0 mM (closed squares), 0.05 mM (triangles), 0.1 mM (open squares), 0.2 mM (closed circles), and 0.4 mM (open circles).

B, secondary re-plots of intercepts (reciprocal maximal velocities) against inhibitor concentration (MH125, open circles; SDH4–17, filled circles; F69V, open squares; S71A, filled squares; H99L, open diamonds; Y89OCH, filled diamonds). The data were fitted to Equation 1 by non-linear least squares using the quasi-Newton algorithm.
dropathy analysis using the Kyte-Doolittle algorithm (54). TMS-I, -II, topological model for the anchor polypeptides was developed after interpretation of the direct effect on the quinone-binding site. Similarly, the im

\[ K_{m} \]

... whereas the other site is in dynamic equilibrium with the this. One of these sites stabilizes a ubisemiquinone radical, involving proteins utilize two quinone-binding sites to accomplish discrete univalent steps (8, 48–53); hence, most quinone-bind

\[ K_{i} \]

(2), whereas the H106Y and the H113Q mutations in the Sdh3p subunit affect mainly the low affinity constant, \( K_{i(2)} \), whereas the H106Y and the H113Q mutations in the Sdh3p subunit affect mainly the high affinity inhibition constant, \( K_{i(1)} \). In addition, deletion of the Sdh4p carboxyl terminus exerts major effects on \( K_{i(2)} \) and Lys-132 was identified as a functionally important residue in this region. These observations strongly argue for the presence of two spatially distinct quinone-binding sites in the yeast SDH, similar to the E. coli FRD structure (27, 36, 37). The effects of the Sdh3p W116R mutation on inhibitor binding could not be evaluated due to low DB reductase activities but its location is consistent with an effect on \( Q_{o} \). Similarly, the Sdh3p mutation F103V specifically affected the enzyme’s affinity for quinone but inhibitor studies were not performed. Its location near the center is consistent with a role in either \( Q_{o} \) or \( Q_{D} \). Further investigation is required to clarify this. Taken together, the data suggest that the high affinity inhibitor-binding site (\( K_{i(1)} \)) is equivalent to the \( Q_{o} \) site whereas the low affinity inhibitor-binding site (\( K_{i(2)} \)) is equivalent to the \( Q_{D} \) site.

The coupling of one-electron redox chemistry (such as the iron-sulfur proteins) to two-electron donor/acceptor systems (such as quinones) presents an interesting challenge to biological systems. Electron transfer to quinones proceeds in two discrete univalent steps (8, 48–53); hence, most quinone-binding proteins utilize two quinone-binding sites to accomplish this. One of these sites stabilizes a ubisemiquinone radical, whereas the other site is in dynamic equilibrium with the quinone pool.

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**TABLE IV**

| Strain   | \( K_{i(1)} \) | \( K_{i(2)} \) |
|----------|----------------|----------------|
| MH125    | 0.045 ± 0.002a | 0.64 ± 0.03    |
| sdh4W2/SDH4-17 | 0.050 ± 0.001 | 0.60 ± 0.02    |
| H99L     | 0.100 ± 0.005  | 1.40 ± 0.06    |
| S71A     | 0.060 ± 0.003  | 2.00 ± 0.02    |
| F69V     | 0.060 ± 0.002  | 1.80 ± 0.08    |
| Y989CH   | 0.150 ± 0.006  | 2.00 ± 0.07    |

\( a \) The \( K_{i(1)} \) and the \( K_{i(2)} \) values are expressed in molarity. Each value represents the mean of triplicate independent determinations ± S.E.

**Fig. 5.** A model for quinone-binding sites in the yeast SDH. The topological model for the anchor polypeptides was developed after hydrophathy analysis using the Kyte-Doolittle algorithm (54). TMS-I, -II, -IV, and -V are proposed to form a bundle as in the E. coli and the W. succinogenes FRDs (4, 27, 28). The two quinone-binding sites are designated \( Q_{o} \) and \( Q_{D} \), (using the nomenclature developed for the E. coli FRD) (27). Residues in ovals are proposed to mediate quinone binding, whereas residues in rectangles are more likely involved in intersubunit interactions.

strongly argues for their involvement in a common function, which we propose is the formation of a quinone-binding site in the vicinity of the loop connecting the Sdh4p transmembrane segments I and II. The observed effects of the H99L mutation are explicable in terms of a larger structural perturbation of the Sdh4p subunit that is propagated to the catalytic domain. The H99L mutation leads to a reduced covalent FAD content and a reduced turnover number for succinate-FMS reductase activity, strongly suggesting that enzyme assembly is compromised. This conclusion is further strengthened by the enzyme’s thermal stability profiles. The H99L mutant is unstable for both the succinate-DB reductase (Fig. 3A) and the succinate-FMS reductase (Fig. 3B) activities. In a previous study (25), we proposed that Asp-117 in the Sdh3p subunit, is at the interface of the peripheral and the membrane domains. The Sdh4p H99L mutation mimics many of the effects of the Sdh3p D117V mutation, suggesting that they may perform a similar role. The H99L mutation leads to a 2-fold increase in the \( K_{m} \) for DB reduction; this is the smallest effect on \( K_{m} \) observed for all the mutants examined in this study. Since this mutation affects enzyme assembly, we do not consider that the elevated \( K_{m} \) reflects a direct effect on the quinone-binding site. Similarly, the impaired assembly of the H99L enzyme precludes a simple interpretation of the s-BDNP inhibition data. However, it is interesting to note that the H99L mutation has equal effects on the \( K_{i(1)} \) and the \( K_{i(2)} \) values, consistent with the mutation having more global effects on the Sdh4p subunit.

Deletion of Sdh4p subunit TMS-III does not lead to a complete loss of respiratory growth and the truncated enzyme still possesses significant quinone reductase activity in vitro. This suggests that TMS-III is not required for catalysis but rather plays an important role in maintaining the structural integrity of the Sdh4p subunit. The mutation causes the greatest reduction in catalysis as well as in thermal stability. This conclusion is consistent with our previous observations on the role of the Sdh4p COOH-terminal extension (22). It is also consistent with the location of TMS-III of the E. coli FRD D subunit outside the helix bundle that forms the quinone-binding pockets (10, 27, 28).

Based on the results obtained in this and in previous studies (22, 24, 25), we propose a two-site model for quinone-protein interaction (Fig. 5). Mutations whose effects appear to be limited to quinone binding are located on opposite sides of the membrane. The Sdh4p residues Phe-69 and Ser-71 are on TMS-II, toward the cytoplasmic side of the membrane, whereas the functionally important residues, His-113, Trp-116, and His-106, in the Sdh3p subunit are clustered toward the matrix side. Apart from their asymmetric locations, two of the residues also have differential effects on s-BDNP binding. The F69V and S71A mutations in the Sdh4p subunit affect mainly the low affinity constant, \( K_{i(2)} \), whereas the H106Y and the H113Q mutations in the Sdh3p subunit affect mainly the high affinity inhibition constant, \( K_{i(1)} \). In addition, deletion of the Sdh4p carboxyl terminus exerts major effects on \( K_{i(2)} \) and Lys-132 was identified as a functionally important residue in this region. These observations strongly argue for the presence of two spatially distinct quinone-binding sites in the yeast SDH, similar to the E. coli FRD structure (27, 36, 37). The effects of the Sdh3p W116R mutation on inhibitor binding could not be evaluated due to low DB reductase activities but its location is consistent with an effect on \( Q_{o} \). Similarly, the Sdh3p mutation F103V specifically affected the enzyme’s affinity for quinone but inhibitor studies were not performed. Its location near the center is consistent with a role in either \( Q_{o} \) or \( Q_{D} \). Further investigation is required to clarify this. Taken together, the data suggest that the high affinity inhibitor-binding site (\( K_{i(1)} \)) is equivalent to the \( Q_{o} \) site whereas the low affinity inhibitor-binding site (\( K_{i(2)} \)) is equivalent to the \( Q_{D} \) site.

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Quinone-binding Sites in the Yeast SDH
