Ubiquinone-binding Site Mutations in the *Saccharomyces
cerevisiae* Succinate Dehydrogenase Generate Superoxide
and Lead to the Accumulation of Succinate*

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The mitochondrial succinate dehydrogenase (SDH) is an essential component of the electron transport chain and of the tricarboxylic acid cycle. Also known as complex II, this tetrameric enzyme catalyzes the oxidation of succinate to fumarate and reduces ubiquinone. Mutations in the human *SDHB*, *SDHC*, and *SDHD* genes are tumorigenic, leading to the development of several types of tumors, including paraganglioma and pheochromocytoma. The mechanisms linking SDH mutations to oncogenesis are still unclear. In this work, we used the yeast SDH to investigate the molecular and catalytic effects of tumorigenic or related mutations. We mutated Arg47 of the Sdh3p subunit to Cys, Glu, and Lys and Asp88 of the Sdh4p subunit to Asn, Glu, and Lys. Both Arg47 and Asp88 are conserved residues, and Arg47 is a known site of cancer causing mutations in humans. All of the mutants examined have reduced ubiquinone reductase activities. The Sdh3 R47K, Sdh4 D88E, and Sdh4 D88N mutants are sensitive to hyperoxia and paraquat and have elevated rates of superoxide production in *vivo* and in *vivo*. We also observed the accumulation and secretion of succinate. Succinate can inhibit prolyl hydroxylase enzymes, which initiate a proliferative response through the activation of hypoxia-inducible factor 1α. We suggest that SDH mutations can promote tumor formation by contributing to both reactive oxygen species production and to a proliferative response normally induced by hypoxia via the accumulation of succinate.

functions to oxidize succinate to fumarate and reduce ubiqui-
nonone (Q) to ubiquinol (1–3). The yeast SDH, like its mamma-
lian counterpart, consists of four nuclear encoded subunits (Sdh1p–Sdh4p in yeast and SDHA–SDHD in mammals) (2), a covalently attached flavin adenine dinucleotide (FAD) (4), three iron-sulfur centers, a *b*-type heme, and two ubiquinone-bind-
ing sites referred to as the proximal and distal sites (*Q*1 and *Q*2), respectively (5, 6). Sdh1p and Sdh2p form the catalytic domain, the site of succinate oxidation. Electrons flow through the cat-
alytic domain to the membrane domain, consisting of Sdh3p and Sdh4p, where quinone reduction occurs.

Mutations in the human *SDHA* gene can result in Leigh syn-
drome, an infantile-onset progressive neurodegenerative dis-
ease (7, 8). Mutations in the human *SDHB*, *SDHC*, and *SDHD* genes lead to inheritable forms of cancer such as pheochromocytomas (catechol-secreting tumors commonly occurring in the adrenal medulla), paragangliomas (benign vascularized tumors in the head and neck), and renal cell carcinoma (9–11). More recently, it was reported that loss of the *SDHH* gene may contribute to gastric and colon cancers (12). These observations highlight the importance of *SDH* genes as mitochondrial tumor suppressors (13). Moreover, the SDHC and SDHD sub-
units are also implicated as regulators of apoptosis (14, 15).

There are currently three models that link SDH deficiencies and oncogenesis. The first model suggests that mitochondria-
generated reactive oxygen species (ROS) result in oxidative damage of nuclear and mitochondrial DNA, mutagenesis, and

tumorigenesis. Expression of an *SDHC* gene with a V69E muta-
tion in a mouse cell line leads to ROS overproduction and the

ability to form benign tumors when injected into nude mice (15). The mutant SDH leaks electrons to molecular oxygen and

forms superoxide anions. SDH is not normally thought of as a

major source of ROS, but evidence of oxidative stress, short-
ened lifespan of model organisms, genomic instability, and

tumorigenesis associated with SDH mutations is accumulating

(15–19).

The second model linking SDH dysfunction and oncogen-
esis also involves mitochondrial ROS, but in this case, the

ROS act as signaling molecules that lead to the stabilization of

the transcription factor hypoxia-inducible factor 1α (HIF-
1α) (20). Under normoxic conditions, the levels of HIF-1α are very low because of its constant degradation. HIF-1α degradation is signaled by its hydroxylation on two prolyl residues, a reaction catalyzed by prolyl hydroxylase (PH) enzymes. PH enzymes catalyze the conversion of proline resi-
dues, O2, and α-ketoglutarate to hydroxyproline, carbon
dioxide, and succinate using ascorbate and Fe\(^{2+}\) as cofactors. Under hypoxic conditions, PH activity is reduced, and HIF-1\(\alpha\) is stabilized. HIF activates a set of genes involved in angiogenesis, proliferation, cell survival, and glycolysis. High levels of ROS can induce a pseudo-hypoxic response by promoting the oxidation of Fe\(^{2+}\) to Fe\(^{3+}\), limiting PH activity and HIF-1\(\alpha\) modification and degradation. In the third model, the signaling pathway involves succinate rather than ROS (21–23). Succinate accumulates as a result of SDH dysfunction and moves out of the mitochondria via the dicarboxylate carrier. It induces a pseudo-hypoxic response and HIF-1\(\alpha\) stabilization via product inhibition of the PH enzymes. Mutations in the fumarate hydratase gene \(FH\), which encodes another enzyme of the tricarboxylic acid cycle, are also associated with tumorigenesis activated by a pseudo-hypoxic response; in the case of \(FH\) mutations, fumarate accumulates and inhibits PH activity (23, 24). High levels of succinate and the induction of HIF-1\(\alpha\) have also been observed in \(SDHA\)-deficient fibroblasts, although tumorigenesis has not been observed, probably because the expression of a second isoform of \(SDHA\) in paragangliomas precludes succinate buildup (25).

In this study, we used the yeast SDH to gain further insight into the molecular mechanisms that link SDH function and tumorigenesis. We focused our attention on residues lining the Q\(_P\) site for two reasons. First, residues near the Q\(_P\) site are frequently mutated in paraganglioma and pheochromocytoma. In addition, the \(mev-1\) mutation, also in the Q\(_P\) site, shortens lifespan, promotes apoptosis, and increases superoxide production in \(Caenorhabditis\) elegans; the \(mev-1\) mutation is also tumorigenic in a murine model (15, 17, 19). Second, we wished to further our understanding of the mechanism of ubiquinone reduction.

The human \(SDHC\) R72C mutation affects a conserved arginine residue located in the Q\(_P\) site (26, 27). We mutated the equivalent residue Arg\(^{77}\) in the yeast \(SDH3\) gene to Cys, Glu, and Lys. Similarly, Asp\(^{113}\) is a conserved amino acid in the human \(SDHD\) gene, also located in the Q\(_P\) binding site. Single amino acid substitutions of Asp\(^{113}\) have not been reported in the SDH mutation data base (28), but an Asp\(^{113}\) mutation in combination with a frameshift mutation is associated with tumor formation (11, 29). We mutated the equivalent yeast \(SDH4\) residue Asp\(^{98}\) to Asn, Glu, and Lys. The \(SDH3\) R47C and R47E mutations and the \(SDH4\) D88K mutation result in a loss of respiratory competence. The \(SDH3\) R47K and the \(SDH4\) D88E and D88N mutations render the cells hypersensitive to oxygen and paraquat. Using \textit{in vivo} and \textit{in vitro} assays, we determined that these mutations increase the production of superoxide anions. Thus, \(SDH3\) and \(SDH4\) mutations can be a significant source of oxidative stress. Finally, all of the mutants secrete succinate, leading to highly elevated succinate to fumarate ratios in the medium. Our results confirm that the SDH Q\(_P\) site should be considered a significant source of ROS production and that mutations that reduce SDH activity lead to elevated levels of succinate. These observations suggest that elevated levels of both ROS and succinate may participate in delivering an oncogenic signal in human tumors.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, Culture Conditions, and Isolation of Submitochondrial Particles—**The \(Saccharomyces\) \(cerevisiae\) strains \(sdh3\)W1 (MH125, \(sdh3::TRP1\)) and \(sdh4\)W2 (MH125, \(sdh4::TRP1\)) and the \(Escherichia\) coli strain DH5\(_{a}\) have been described (30, 31). \(sdh3\)W1/4K6 (YPH499, \(sdh3::TRP1\), \(sdh4::LYS2\)) double knock-out strain was constructed by targeted gene disruption (32). Briefly, a 0.13-kb KpnI fragment from the \(SDH3\) gene was replaced with a 0.83-kb KpnI fragment containing the \(TRP1\) gene in the yeast strain, \(sdh4\)K6 (YPH499, \(sdh4::LYS2\)) (33). The double knock-out strain was verified by PCR and complementation analysis. The yeast media (YPD, YPG, SG, SD, and YPGal) have been described (30). Acid secretion was assessed using YPDBP medium (1% yeast extract, 2% peptone, 0.5% glucose, 30 \(\mu\)g/ml bromocresol purple, pH 7.0) containing 2.5 mm potassium phosphate, pH 7.0 (34). All of the site-directed mutants were confirmed by sequencing the entire gene as described (35). Oxygen sensitivity was measured on plates incubated at 30 °C in a jar continuously flushed with pure oxygen at ~20 ml/min. Yeast precultures were grown on SD medium for 2 days to select for plasmid retention. YPGal medium was inoculated to a starting \(A_{590}\) of 0.1 and grown at 30 °C for 3 days. The cells were harvested and lysed in a French pressure cell for the preparation of submitochondrial particles (36).

**Free Radical Determination—**Intracellular ROS production was examined using dihydroethidium (DHE) and MitoSOX Red (Molecular Probes). MitoSOX is a lipid-soluble cation that accumulates in the mitochondrial matrix where it can be oxidized to a fluorescent product by superoxide (37). DHE is uncharged and is not selectively accumulated in the mitochondrial matrix. As such, the fluorescent DHE oxidation product reports cytoplasmic and intermembrane space superoxide levels, although DHE may also be oxidized by hydrogen peroxide (37, 38). Both probes are cell-permeant, and the red fluorescence of the oxidation products is enhanced by intercalation into DNA (38).

Yeast strains were grown in SG medium containing 0.05% glucose to an optical density at 600 nm of ~2. Aliquots of \(10^7\) cells were washed twice with phosphate-buffered saline and incubated for 45 min at 25 °C in either 5 \(\mu\)M DHE or MitoSOX in the dark. Unlabeled cells were used to determine intrinsic fluorescence levels. The cells were washed three times and resuspended in phosphate-buffered saline. Fluorescence was measured using a LS 50B luminescence spectrometer (Perkin Elmer) with excitation and emission wavelengths of 510 and 580 nm, respectively.

**Enzyme Assays—**SDH is normally isolated partially inhibited by oxaloacetate (39). To activate the enzyme, submitochondrial particles were preincubated in 20 mm succinate for 15 min at 30 °C immediately prior to analysis. The malate-sensitive succinate-dependent reduction of ubiquinone was monitored spectrophotometrically as the decylubiquinone-mediated reduction of dichlorophenol indophenol (DCPIP) (40). The succinate-dependent, phenazine methosulfate (PMS)-mediated malonate-sensitive reduction of DCPIP is used as a measure of the membrane-associated catalytic dimer. Succinate and glyceral phosphate cytochrome \(c\) reductase assays were performed as
Quinone-binding Site Mutants in the Yeast SDH

FIGURE 1. Positions of the Sdh3p Arg47 and Sdh4p Asp88 residues at the QP site. Ubiquinone-2 was docked into the yeast SDH model (6). Only side chains are shown. Included are residues Sdh2p His217 and Sdh4p Tyr89, which interact with the quinone examined in this study. Distances (shown in Å) between the side chains and the quinone are indicated by the dashed yellow lines.

described (5, 19). Superoxide generation was assayed as the superoxide dismutase-sensitive (from bovine erythrocytes; catalog number 190117, MP Biochemicals) and malonate-sensitive reduction of cytochrome c in the presence of 1 mM potassium cyanide (41). Ninety units of superoxide dismutase/ml were added to both sample and reference cuvettes. All of the assays were performed at room temperature.

1H NMR Analysis of Secreted Acids—Yeast strains were grown overnight at 30 °C in YP medium containing 0.25% glucose. The cells were removed by centrifugation, and the medium was subjected to trichloroacetic acid precipitation and centrifugation. The supernatant was recovered, adjusted to pH 7 with NaOH, flash frozen in liquid N2, and stored at −80 °C until it could be lyophilized for 2 days and stored dry at 4 °C. The lyophilized material was dissolved in 570 μl of D2O (99.9%; Isotec Inc., Miamisburg, OH). Thirty μl of 5 mM 2,2-dimethyl-2-sila 3,3,4,4,5,5-hexadeuteropentane sulfonic acid were added as a chemical shift reference and used as a concentration standard for determining absolute metabolite concentrations. pH was also recorded for calibration purposes. The samples were centrifuged at 14,000 × g for 3 min to remove particulate matter, and 500 μl of the supernatant were transferred to a 5 mm NMR tube. All of the spectra were acquired on a 600-MHz Inova NMR spectrometer at 25 °C using the tn noesy pulse sequence (carna Vnmr 6.1B software, Varian Inc). All of the spectra had an acquisition time of 4 s, a preacquisition delay of 1 s, a mixing time of 0.1 s, a sweep width of 7200 Hz, and 256 transients (42). All of the spectra were analyzed using the Chenomx NMR Suite Professional software version 4.5.

Miscellaneous Methods—Measurements of FAD and protein contents, yeast and E. coli transformations and recombinant DNA methods have been described (33).

RESULTS

Choice of Mutations—We chose to mutate residues in the yeast SDH Qp site proposed to have important roles in ubiquinone binding or reduction (Fig. 1) (6, 43). We mutated SDH3 Arg47 to Cys, Glu, and Lys. The R72C mutation in the equivalent, conserved arginine of the human SDHC gene results in paraganglioma (26, 27). Mutation of Arg31, the equivalent residue in the E. coli SdhC subunit, results in respiratory deficiency and in the loss of succinate-quinone reductase activity (44, 45). We also mutated SDH4 Asp88 to Asn, Glu, and Lys. Mutation of the equivalent E. coli SdhD Asp-82 abolishes succinate-quinone reductase activity and respiratory growth (45). Both SDH4 Asp88 and SDH3 Arg47 may be functionally important for the protonation of ubiquinone upon reduction (43, 46).

Growth Phenotypes of the Mutants—The mutant SDH3 genes were cloned into the single copy shuttle vector YCplac33 and transformed into the SDH3 knockout strain sdh3W1. Similarly, mutant SDH4 genes were cloned into the vector pRS416, and the resulting plasmids were introduced into sdh4W2. To quantify the ability of each strain to respire, growth yield assays on semisynthetic medium containing 0.1–0.5% galactose, and the optical densities at 600 nm were measured. The relative growth yields were calculated using the final absorbance values on 0.5% galactose.

FIGURE 2. Growth yields on galactose media. Yeast strains were grown at 30 °C on semisynthetic liquid medium containing 0.1–0.5% galactose, and the optical densities at 600 nm were measured. The relative growth yields were calculated using the final absorbance values on 0.5% galactose. A, growth of SDH3 mutant strains. Squares, sdh3W1/pYCSDH3; crosses, R47K; diamonds, R47E; circles, R47C and sdh3W1/pYCplac33. B, growth of SDH4 mutant strains. Squares, sdh4W2/pSDH4–21; diamonds, D88E; crosses, D88N; circles, D88K and sdh4W2/pRS 416.
its diauxic shift when cultured on galactose; an initial fermentative growth is followed by a respiratory phase when the fermentable carbon source is limiting (5, 31, 47). The growth yield of the sdh3W1 strain carrying a wild-type plasmid-borne SDH3 gene, sdh3W1/pYCSDH3 was set at 100% (Fig. 2A). The strain sdh3W1/pYCplac33 (empty vector) achieved by fermentation alone a growth yield of 12 ± 1%. The SDH3 R47K mutant retained significant respiratory capacity with a 70 ± 6% growth yield. In contrast, the mutants R47C and R47E showed more severely impaired or abolished respiratory capacities with yields of 35 ± 4 and 12 ± 1%, respectively. We have previously noted that strains with a growth yield below ~30% are unable to grow on minimal glycerol media. Thus, the yeast R47C mutation and likely its counterpart human R72C mutation that results in paraganglioma are strong alleles that severely impair SDH activity.

The SDH4 deletion strain containing the empty vector sdh4W2/pRS416 achieves a growth yield of 10 ± 1% of the wild type (Fig. 2B). A similar growth yield for the D88K mutant indicates a complete loss of respiratory growth. In contrast, the D88E and D88N mutants have yields of 80 ± 7 and 72 ± 9%, respectively, demonstrating that they have retained significant respiratory capacity.

We examined the abilities of the mutant strains to grow when glycerol, a nonfermentable carbon source, is available (Figs. 3, A and C, and 4, A and C). The SDH3 R47C and R47E mutants were unable to grow, whereas the R47K mutant grew as well as the wild type. Consistent with the galactose growth yield assays, the SDH4 D88K mutant is unable to grow on glycerol, whereas the D88E and D88N mutants displayed growth comparable with that of the wild type.

**The Mutant Enzymes Assemble and Have Reduced Quinone Reductase Activity—**We used two measures to assess the effects of the mutations on enzyme assembly. First, we determined the membrane-associated succinate-dependent PMS-mediated reduction of DCPIP in isolated submitochondrial membranes as a measure of enzyme assembly. This activity is a measure of the membrane-associated catalytic dimer; it does not require catalytically competent Sdh3p and Sdh4p subunits, but the subunits must be present to mediate membrane association of the Sdh1p/Sdh2p catalytic dimer (33, 36). We also determined the levels of covalent FAD; SDH is the major covalent flavoprotein in *S. cerevisiae* (48). All of the mutant enzymes are efficiently assembled (Table 1); the FAD contents of the mutants are similar to those of wild type, with exception of the SDH3 R47E and SDH4 D88K mutants, which have somewhat reduced levels. The PMS-mediated DCPIP reductase activities of the mutants are also comparable with wild type, indicating the Sdh3p and Sdh4p subunits are efficiently anchoring the catalytic domain to the membrane. The SDH3 and SDH4 deletion strains display a residual level of activity (<5% of wild type) consistent with the absence of assembled enzyme (data not shown). In contrast, the cytochrome c and the decylubiquinone reductase activities of the mutants are significantly reduced. Thus, the mutations impair catalytic activity involving quinone reduction rather than enzyme assembly. Even the mutations that do not abolish respiratory growth, such as the SDH3 R47K and SDH4 D88E mutations, impair catalytic activity involving quinone reduction rather than enzyme assembly.
and D88N mutations, show substantial losses of quinone reductase activity. These results are consistent with previously characterized Qp site mutants (5, 19, 31, 49).

The Mutant Enzymes Confer Hypersensitivity to Oxidative Stress—Paraquat (methyl viologen) is an herbicide believed to induce oxidative stress through the generation of superoxide ions either in the cytoplasm (50) or in the mitochondria (51). The SDH3 R47K mutation renders the strain hypersensitive to 1.0 mM paraquat (Fig. 3B), whereas the SDH4 D88E and D88N mutations confer mild sensitivity to this agent (Fig. 3D).

We also tested the effects of hyperoxia on respiratory growth. Wild-type SDH3 or SDH4 genes support slightly impaired growth in 100% oxygen as compared with normoxia (Fig. 4). The SDH3 R47K and SDH4 D88N mutations abolish growth in the oxygen atmosphere, whereas the SDH4 D88E mutation confers a milder sensitivity to hyperoxia (Fig. 4D). We believe the mutant enzymes increase ROS production under normoxic conditions but that cellular defense mechanisms can detoxify the ROS. However, the imposition of an additional exogenous stress such as paraquat or hyperoxia overwhelms these defense mechanisms.

To more directly test this hypothesis, in vivo ROS generation was assessed with the oxidation-sensitive probes DHE and MitoSOX. We observed significantly elevated levels of DHE and MitoSOX fluorescence in all three mutants compared with their corresponding wild-type strains (Fig. 5). The SDH3 R47K mutant displayed a 1.7-fold increase in DHE fluorescence and a 2.2-fold increase in MitoSOX fluorescence. The SDH4 D88E mutant displayed 1.7- and 2.3-fold increases, whereas the D88N mutant displayed 1.4- and 1.5-fold increases, respectively. As a positive control, DHE and MitoSOX fluorescence were examined in wild-type cells grown in the presence of 0.5 mM paraquat (Fig. 5). The addition of more superoxide dismutase to the assays did not further decrease activity (data not shown). In wild-type mitochondrial membranes, the superoxide dismutase-sensitive portion of the cytochrome c reductase activity is small (<3.5% of the total cytochrome c reductase activity) and not statistically significant. In contrast, the superoxide-mediated portion of the cytochrome c reductase activity is greater for the mutant enzymes and is statistically significant. Although the mutant enzymes are much less efficient catalysts, they divert a larger fraction of their electron flow to the production of superoxide.

### TABLE 1

| Strain    | Covalent FAD* | DCPIP reductasea | Cytochrome c reductaseb | Decylubiquinone reductasec |
|-----------|---------------|------------------|-------------------------|---------------------------|
| SDH3 WT   | 48 ± 1        | 4300 ± 200       | 2300 ± 100              | 4300 ± 400                |
| SDH3 R47C | 42 ± 2        | 4500 ± 500 (106%)| 370 ± 30 (16%)          | 310 ± 30 (7%)             |
| SDH3 R47E | 35 ± 1        | 3500 ± 600 (82%) | 280 ± 30 (12%)          | 130 ± 50 (3%)             |
| SDH3 R47K | 44.3 ± 0.9    | 4900 ± 600 (115%)| 780 ± 90 (34%)          | 740 ± 40 (17%)            |
| SDH4 WT   | 41 ± 2        | 3800 ± 100       | 2300 ± 100              | 3700 ± 200                |
| SDH4 D88E | 42 ± 2        | 3600 ± 200 (95%) | 1590 ± 80 (60%)         | 870 ± 80 (24%)            |
| SDH4 D88K | 20 ± 1        | 4400 ± 400 (116%)| 280 ± 30 (12%)          | 440 ± 100 (10%)           |
| SDH4 D88N | 39 ± 1        | 4700 ± 100 (124%)| 890 ± 40 (39%)          | 600 ± 40 (16%)            |

a The values are the means of five trials ± S.D. FAD contents are expressed as pmol of FAD mg of protein⁻¹.

b The activities are expressed as μmol of DHE reduced min⁻¹ μmol of covalent FAD⁻¹. The values represent the means of at least 10 trials ± S.D.

c The values are the means of at least 12 trials ± S.D.

d The values in parentheses represent the percentage activities of the mutant enzymes compared with their respective wild-type controls.
Complex III is a recognized source of ROS (52), and it has been shown that SDH and complex III have close structural and genetic interactions in yeast (53, 54). To determine whether the SDH mutations we created affected complex III-mediated ROS generation, we measured the glycerol phosphate-dependent reduction of cytochrome c in the presence and absence of superoxide dismutase. Glycerol phosphate is oxidized by the mitochondrial glycerol-phosphate dehydrogenase, an FAD-linked enzyme that, like SDH, reduces ubiquinone to ubiquinol (55). After the formation of ubiquinol, the pathway of electron transfer to cytochrome c is identical to that for the succinate-dependent reaction. If complex III function has been perturbed by the SDH mutations, we would also expect a large increase in superoxide production associated with glycerol-phosphate cytochrome c reductase activity. As shown in Table 3, the addition of superoxide dismutase has no significant effect on this activity in wild-type or mutant mitochondrial membranes. We conclude that SDH is directly responsible for the production of superoxide.

**Enzymes Possessing Double Mutations Are Catalytically Inactive**—We examined the effects of combining the SDH3 and SDH4 mutations in the same strain. Both of the double mutants we constructed (R47K/D88E and R47K/D88N) were unable to support respiratory growth (Fig. 6A). The PMS-mediated DCPIP reductase activities of the double mutants indicate that the double mutant enzymes can assemble, although less efficiently than the wild type, and are catalytically defective (Fig. 6B).

**SDH Mutations Lead to the Accumulation and Secretion of Succinate**—Inhibition of enzymes of the tricarboxylic acid cycle leads to the secretion of acid, which can be detected using pH-sensitive indicator dyes (34). Human SDH mutations that result in tumor formation have been shown to result in the accumulation of succinate (21, 23). Our data suggest that all of the mutations we created reduce or eliminate SDH activity, possibly leading to the accumulation and secretion of succinate. We qualitatively examined acid secretion using YPDBP plates, which contain bromocresol purple (Fig. 7). Wild-type and mutant strains were spotted onto YPDBP plates along with the Δtem62 strain, a previously characterized acid secreting strain (56). All of the mutant strains secreted acid as indicated by the halo of yellow color surrounding the colonies. In contrast, the area surrounding the wild-type colonies remained purple. These results suggest that SDH mutations cause the secretion of organic acids.

Metabolite concentrations were determined from the one-dimensional 1H NMR spectra using the Chenomx software. Individual metabolites were identified by comparison with...
Quinone-binding Site Mutants in the Yeast SDH

**A**

| Strain | Succinate | Fumarate | Succinate:fumarate |
|--------|-----------|----------|-------------------|
| **WT** | 490 ± 100 | 7.7 ± 1.6 | 70 ± 20          |
| **SDH3 WT** | 1200 ± 150 | 7.3 ± 0.9 | 160 ± 10          |
| **SDH3 (R47C)** | 1340 ± 90 | 8.4 ± 1.3 | 160 ± 20          |
| **SDH3 (R47K)** | 1200 ± 170 | 5.4 ± 0.8 | 230 ± 30          |
| **SDH3 KO** | 1200 ± 110 | 8.0 ± 1.3 | 160 ± 20          |
| **SDH4 WT** | 460 ± 180 | 8.6 ± 4.5 | 60 ± 30           |
| **SDH4 (D88E)** | 1020 ± 80 | 7.7 ± 3.9 | 160 ± 60          |
| **SDH4 (D88K)** | 1300 ± 330 | 9.9 ± 3.5 | 140 ± 30          |
| **SDH4 (D88N)** | 920 ± 100 | 4.3 ± 0.9 | 230 ± 60          |
| **SDH4 KO** | 1100 ± 380 | 6.3 ± 2.3 | 180 ± 30          |

**B**

| Strain | Succinate | Fumarate | Succinate:fumarate |
|--------|-----------|----------|-------------------|
| **WT** | 490 ± 100 | 7.7 ± 1.6 | 70 ± 20          |
| **R47C** | 1200 ± 150 | 7.3 ± 0.9 | 160 ± 10          |
| **R47E** | 1340 ± 90 | 8.4 ± 1.3 | 160 ± 20          |
| **R47K** | 1200 ± 170 | 5.4 ± 0.8 | 230 ± 30          |
| **Vec** | 1200 ± 110 | 8.0 ± 1.3 | 160 ± 20          |
| **Δtcm62** | 460 ± 180 | 8.6 ± 4.5 | 60 ± 30           |
| **D88E** | 1020 ± 80 | 7.7 ± 3.9 | 160 ± 60          |
| **D88K** | 1300 ± 330 | 9.9 ± 3.5 | 140 ± 30          |
| **D88N** | 920 ± 100 | 4.3 ± 0.9 | 230 ± 60          |
| **Δtcm62** | 1100 ± 380 | 6.3 ± 2.3 | 180 ± 30          |

**FIGURE 7.** SDH mutants secrete acid. SDH3 (A) or SDH4 (B) knock-out strains transformed with wild type (WT) or with empty vectors (Vec) or mutant SDH3 or SDH4 genes were grown overnight in SD Δtcm62 plates serves as a positive control for acid secretion (56). The cultures were diluted 1,000-fold in sterile H2O, and 10-μl aliquots were spotted onto YPDBP plates containing 2.5 mM potassium phosphate, pH 7.0. The plates were incubated at 30°C and photographed after overnight incubation.

**TABLE 4**

SDH mutants produce elevated amounts of succinate

| Strain     | Succinate | Fumarate | Succinate:fumarate |
|------------|-----------|----------|-------------------|
| *S. cerevisiae* WT | 490 ± 100 | 7.7 ± 1.6 | 70 ± 20          |
| *S. cerevisiae* R47C | 1200 ± 150 | 7.3 ± 0.9 | 160 ± 10          |
| *S. cerevisiae* R47E | 1340 ± 90 | 8.4 ± 1.3 | 160 ± 20          |
| *S. cerevisiae* R47K | 1200 ± 170 | 5.4 ± 0.8 | 230 ± 30          |
| *S. cerevisiae* KO | 1200 ± 110 | 8.0 ± 1.3 | 160 ± 20          |
| *S. cerevisiae* WT | 460 ± 180 | 8.6 ± 4.5 | 60 ± 30           |
| *S. cerevisiae* (D88E) | 1020 ± 80 | 7.7 ± 3.9 | 160 ± 60          |
| *S. cerevisiae* (D88K) | 1300 ± 330 | 9.9 ± 3.5 | 140 ± 30          |
| *S. cerevisiae* (D88N) | 920 ± 100 | 4.3 ± 0.9 | 230 ± 60          |
| *S. cerevisiae* KO | 1100 ± 380 | 6.3 ± 2.3 | 180 ± 30          |

*The values are expressed in μmol ΔA_{460}^-1 and represent the means of five trials ± S.D.

+ p < 0.001 compared with the wild-type ratio using a two-tailed unpaired Student’s t test.

+ p < 0.05 compared with the wild-type ratio using a two-tailed unpaired Student’s t test.

+ p < 0.01 compared with the wild-type ratio using a two-tailed unpaired Student’s t test.

spectra of standards, and then the absolute concentrations were calculated by comparison with the added reference peak (42). We identified succinate and fumarate in the 1H NMR spectra of the culture medium after growth of the strains. Their concentrations were normalized to the optical densities of the respective cultures. As a control, we also analyzed the starting medium concentration of succinate and fumarate. We found that the concentration of succinate does not change substantially with the growth of the wild-type strains, consistent with the YPDBP plate assay (Fig. 7). As seen in Table 4, the media used to grow the *SDH3* and *SDH4* deletion and mutant strains show significantly higher succinate to fumarate ratios when compared with media from their corresponding wild-type strains. These results confirm that SDH dysfunction can result in the accumulation and secretion of succinate.

**DISCUSSION**

To gain further insight into the molecular bases of pathogenic SDH mutations, we investigated the role of Qp site residues Sdh3p Arg47 and Sdh4p Asp88. Mutations around the Qp site of an eukaryotic SDH can generate significant amounts of superoxide and can be tumorigenic (15, 17, 19). Our results indicate that Sdh3p Arg47 and Sdh4p Asp88 are critical for quinone reduction. Substitution of these residues decreases quinone reductase activity (Table 1) and impairs respiratory growth (Fig. 2). The loss of enzymatic activity in the mutants is not due to the disruption of SDH assembly as judged by covalent FAD contents and by membrane-associated succinate-PMS/DCPIP activities (Table 1). The catalytic activities of the mutant enzymes were not stimulated by higher concentrations of decylubiquinone, suggesting that the decrease in activity of these mutants cannot be attributed to a lower affinity of the enzyme for ubiquinone (data not shown).

We observed an increase in superoxide production for the *SDH3* R47K and *SDH4* D88E and D88N mutants in comparison with the wild-type enzyme (Table 2). This superoxide generation was SDH-specific and was not the result of increased ROS production in complex III (Table 3). Our results are in agreement with previous data that suggest superoxide generation by SDH is not a flavin-mediated event but rather a quinone-mediated one (19). *In vitro*, the mutant enzymes produced superoxide at rates similar to the wild-type SDH; however, greater proportions of the total enzyme activity in the mutants were directed toward superoxide generation. We examined whether oxygen availability was limiting the rates of superoxide production. We repeated the cytochrome c reductase assays using oxygen-saturated buffer but found no increase in superoxide production (data not shown). We suggest that the rates of superoxide production *in vivo*, where succinate concentrations are not saturating as they are *in vitro* (57), may be considerably higher for the mutant enzymes or, alternatively, lower for the wild-type enzyme. The mutants are sensitive to oxidative stress and have higher rates of *in vivo* ROS production (Figs. 3–5).

The SDH mutations we introduced may increase superoxide production in one of two ways. They may perturb the Qp site allowing for increased electron flow out of the enzyme to oxygen, or they may extend the lifetime of the ubisemiquinone reaction intermediate, resulting in its premature release and subsequent reaction with oxygen to form superoxide. We cannot distinguish between these mechanisms but favor the latter. When the corresponding *E. coli* SDH residues SdhC Arg31 and SdhD Asp52 were mutated, succinate-quinone reductase and plumbagin-fumarate reductase activities were both greatly affected, suggesting that these residues are critical to the quinone chemistry of the enzyme whether operating in forward or in reverse directions (45).

Sdh3p Arg47 in yeast and SdhC Arg31 in *E. coli* are essential residues in their respective Qp sites; in *E. coli*, Arg31 has been proposed to modulate the pKₐ of SdhD Y83, which is probably a proton donor during quinone reduction (Fig. 1) (43, 58). The
R47K mutation is predicted to raise the $pK_a$ of the Tyr$^{89}$ hydroxyl group, reducing its propensity to donate a proton to the ubisemiquinone intermediate and increasing the lifetime of the intermediate. It was previously shown in the *E. coli* SDH that an arginine side chain is critical for proper quinone reduction and that substitution to lysine is inadequate (44).

Sdh4p Asp$^{88}$ does not interact directly with bound quinone but is an essential component of the Q$_o$ site. We propose that the D88E or the D88N mutations will perturb the electrostatic environment around Sdh3p Arg$^{47}$, resulting in the movement of Arg$^{47}$ away from Tyr$^{89}$. Asp$^{88}$ forms a hydrogen bond with Sdh2p His$^{317}$ and a salt bridge with Sdh3p Arg$^{47}$ (6, 46, 59). In *E. coli*, the equivalent histidine residue is believed to play an essential role in coordinating ubiquinone prior to electron transfer and protonation (60). The D88N substitution removes the charge and the salt bridge with Arg$^{47}$. The D88E mutation raises the $pK_a$ of that residue and reducing its ability to function as a proton donor. The effect is similar to that of the Sdh3p R47K substitution. For the Sdh3p R47K and the Sdh4p D88E and D88N substitutions, ubiquinone binding is maintained, allowing electron transfer to proceed, but protonation of the ubisemiquinone by Tyr$^{89}$ is slowed. These effects may contribute not only to the lower quinone reductase activities observed in the mutants but also their production of superoxide (Tables 1 and 2).

The SDH mutants we generated are acid secreting (Fig. 6). Using $^1$H NMR, we showed that succinate is secreted and that the ratio of succinate to fumarate is much higher in the media of the mutants than in the media of wild-type strains (Table 4). These observations are consistent with the third model of SDH-linked tumorigenesis in which SDH dysfunction leads to elevated levels of succinate and HIF-1$\alpha$ stabilization.

Oxidative stress may also contribute to the accumulation of succinate. In rat mitochondria treated with peroxides to induce oxidative stress, the nonenzymatic decarboxylation of $\alpha$-ketoglutarate results in the formation of succinate (61). A similar process may also be occurring in our mutants since the superoxide generated in the mitochondria is converted into hydrogen peroxide by the mitochondrial superoxide dismutase.

During embryogenesis, most sympathetic neuronal precursor cells undergo apoptosis as growth factors become limiting (62). This developmental apoptosis is mediated by the prolyl hydroxylase EglN3, whose activity is inhibited by succinate accumulation (62). It has been proposed that SDH mutations cause pheochromocytoma because neuronal precursor cells carrying mutations escape developmental apoptosis (62). HIF-1$\alpha$ activation will also confer a growth advantage to those surviving cells that go on to form tumors because they will be better adapted to the anaerobic state through enhanced vascularization and glycolytic capacity (63).

In conclusion, we have provided evidence that ROS production and succinate accumulation both occur during SDH dysfunction and that both may contribute to tumorigenesis. Their relative importance to tumor formation and tumor cell proliferation may depend on the particular nature of the SDH mutation. Our data also strongly suggest that the SDH Q$_o$ site is a likely source of superoxide.

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