The Carboxyl Terminus of the *Saccharomyces cerevisiae* Succinate Dehydrogenase Membrane Subunit, SDH4p, Is Necessary for Ubiquinone Reduction and Enzyme Stability*

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The succinate dehydrogenase (SDH) of *Saccharomyces cerevisiae* is composed of four nonidentical subunits encoded by the nuclear genes *SDH1*, *SDH2*, *SDH3*, and *SDH4*. The hydrophilic subunits, SDH1p and SDH2p, comprise the catalytic domain involved in succinate oxidation. They are anchored to the inner mitochondrial membrane by two small, hydrophobic subunits, SDH3p and SDH4p, which are required for electron transfer and ubiquinone reduction. Comparison of the deduced primary sequence of the yeast SDH4p subunit to SDH4p subunits from other species reveals the presence of an unusual 25–30 amino acid carboxyl-terminal extension following the last predicted transmembrane domain. The extension is predicted to be on the cytoplasmic side of the inner mitochondrial membrane. To investigate the extension's function, three truncations were created and characterized. The results reveal that the carboxyl-terminal extension is necessary for respiration and growth on nonfermentable carbon sources, for ubiquinone reduction, and for enzyme stability. Combined with inhibitor studies using a ubiquinone analog, our results suggest that the extension and more specifically, residues 128–135 are involved in the formation of a ubiquinone binding site. Our findings support a two-residues 128–135 are involved in the formation of a ubiquinone binding site model for the *S. cerevisiae* SDH.

Succinate dehydrogenase (SDH)³ and fumarate reductase (FRD) form a family of highly conserved and functionally related respiratory chain proteins. They are usually composed of four nonidentical subunits: a large flavoprotein subunit of about 70 kDa to which is covalently attached an FAD cofactor, an iron-sulfur protein subunit of about 27 kDa that contains three iron-sulfur clusters, and two smaller hydrophobic membrane subunits of about 17 and 13 kDa. The hydrophobic subunits are thought to contain two quinone binding sites and may oxidize succinate or reduce fumarate with artificial electron acceptors or donors, respectively. However, the oxidation or reduction of natural quinone substrates is only possible with the membrane anchor subunits (1–3).

The primary structures of the flavoprotein and the iron-sulfur subunits are conserved in SDHs and FRDs of different species, but this is not the case for the membrane anchor subunits (2–5). Thus, the anchor subunits may contribute to the unique properties of each enzyme. For example, the SDHs and FRDs from different sources interact with a variety of quinones and are differentially sensitive to quinone analog inhibitors (2, 3). Furthermore, the hydrophobic subunits may determine the preferred direction of electron flow in SDH and FRD enzymes. To date, our understanding of the molecular details of electron flow through the membrane subunits is rather limited.

In the *Saccharomyces cerevisiae* SDH, the flavoprotein, the iron-sulfur, and the membrane subunits are encoded by nuclear genes, *SDH1*, *SDH2*, *SDH3*, and *SDH4*, respectively (6–11). The two hydrophobic subunits, SDH3p and SDH4p, are predicted to each contain three membrane-spanning domains (10, 11). They provide the attachment for the catalytic domain to the inner mitochondrial membrane as well as for the electron transfer reaction with ubiquinone. Alignment of the SDH4p subunits from different organisms reveals the presence of a 25–30 amino acid extension at the carboxyl terminus of the *S. cerevisiae* SDH4p (Fig. 1). A search for the presence of functional or structural domains in the carboxyl-terminal extension failed to identify any significant features. We were curious to determine whether the carboxyl-terminal extension was required for SDH function in yeast. In this study, we investigate the role of the SDH4p carboxyl-terminal extension by deletion mutagenesis. Truncation of the carboxyl terminus reveals that it is necessary for respiration on non-fermentable carbon sources, for quinone reduction, and for enzyme stability.

**EXPERIMENTAL PROCEDURES**

*Strains and Media—* The parental yeast strain, MH125 and the *Escherichia coli* strain, DH5α, have been described previously (12). *sdh4Δ (MH125, *SDH4*:1::::TRP1)* was constructed by replacing the 0.8-kilobase *XhoI* to *SpeI* fragment containing the entire *SDH4* open reading frame with the *TRP1* gene by targeted gene disruption in MH125. The yeast media used are SD (0.67% yeast nitrogen base, 2% glucose), SG (0.67% yeast nitrogen base, 3% glycerol), YPD (1% yeast extract, 2% peptone, 2% glucose), YPGal (1% yeast extract, 2% peptone, 2% galactose), and semisynthetic galactose (0.3% yeast extract, 0.1% KH₂PO₄, 0.1% NH₄Cl, 0.05% NaCl, 0.06% MgSO₄·7H₂O, 0.05% CaCl₂, 0.003% FeCl₃, 2% galactose).

*Yeast Culture Conditions—* Cultures were grown on SD media for 2 days to select for plasmid retention, used to inoculate YPGal medium supplemented with 0.01% glucose to a starting *A₅₆₀* ~ 0.05, and grown at 30 °C to stationary phase. Cells were harvested and lysed in a French pressure cell for the preparation of submitochondrial particles (10). For the preparation of mitochondria, cultures were grown in a semisynthetic galactose to late logarithmic phase (*A₅₆₀* about 3), harvested, and lysed enzymatically as described (10).

*Mutant Construction—* By polymerase chain reactions with the

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³ The abbreviations used are: SDH, succinate dehydrogenase; FRD, fumarate reductase; DCPIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; Q, ubiquinone; α-BDNP, 2-sec-butyl-4,6-dinitrophenol; SSE, sum of squares error.
plasmid pSDH4–17 as template (10), and the following oligonucleotides, 5′-TACTCTGACGCGACATACCCTCTGGTTC-3′, 5′-TACCTCTGACCATAGACTTTTACTAAGC-3′, and 5′-TACTCTGACGCTTTGAAAGGACTCC-3′, stop codons, encoded within the XhoI restriction sites (underlined), were introduced into the SDH4 gene following the Val-127, Trp-135, and Lys-140 codons (Fig. 1) to produce the SDH4ΔC23, SDH4ΔC15, and SDH4ΔC10 constructs, respectively. The oligonucleotides introduced the amino acids aspartate and valine, valine, and valine at the carboxyl termini of the SDH4ΔC23, SDH4ΔC15, and SDH4ΔC10 constructs, respectively. The M13 forward primer, 5′-GTAAAACGACGGCCAGT-3′, was used in conjunction with each of the above primers. Amplifications were performed using the Expand Long Template PCR System kit (Boehringer Mannheim) according to the manufacturer’s instructions. The amplification products were purified, digested with XhoI and XbaI, and ligated into SalI- and XhoI-digested YEp1lac181 (13) to yield the plasmids, pSDH4ΔC23, pSDH4ΔC15, and pSDH4ΔC10. The deletions were confirmed by sequencing. Plasmids were introduced into sdh4W2 by lithium acetate-mediated transformation (14).

Enzyme Assays and Thermal Stability Measurements—The succinate-dependent reduction of ubiquinone was monitored spectrophotometrically at room temperature as the 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB)-mediated reduction of dichlorophenolindophenol (DCPIP (15)). The succinate-dependent, phenazine methosulfate (PMS)-mediated reduction of DCPIP was measured as a measure of the membrane-associated SDH1p/SDH2p dimer; this assay does not require catalytically competent hydrophobic subunits, but membrane association of the SDH1p/SDH2p dimer requires intact anchor subunits (10, 16). Other assays have been described previously (10, 16). For thermal stability measurements, submichaelisian particles (20 mg/ml) were incubated at 30 °C in 20 m mM HEPES-KOH, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, and aliquots were assayed at 5-min intervals.

Analysis of Kinetic Data—Double reciprocal plots (Lineweaver-Burk) were fitted by a weighted least squares algorithm in the EnzymeKinetixics software package (Trinity Software, Campton, NH) to evaluate the kinetic constants, respectively (17). The equation was compiled into proFit 5.0 (Quantum Soft, Zurich, Switzerland) and fitted by non-linear least squares using the quasi-Newton algorithm. In all cases, the fit with the lowest sum of squares error (SSE) was chosen to describe the inhibition patterns.

Miscellaneous Methods—Measurements of covalently bound flavin have been described (10).

RESULTS

Construction of a Chromosomal SDH4 Deletion Mutant—An SDH4 disruption mutant, sdh4K7, previously made by insertion of the LYS2 marker into the SDH4 open reading frame, still possessed low levels of succinate-quinone reductase activity (10). We surmised that the amino-terminal peptide of 65-amino acid residues might retain partial SDH4p function. A complete deletion of the SDH4 gene in the parent strain, MH125, was constructed in which the entire SDH4 open reading frame is replaced by the TRP1 marker gene by targeted gene disruption to produce the strain sdh4W2. The genotype was confirmed by Southern blot analysis (data not shown). As expected, sdh4W2 has lost the ability to use glycerol as the sole carbon source unless a wild type SDH4 gene is introduced.

In Vivo Characterization of SDH4 Carboxyl-terminal Deletion Mutants—The presence of the unusual C-terminal extension in the yeast SDH4p sequence (Fig. 1) prompted us to determine whether this extension is required for proper enzyme function. Truncated, plasmid-borne SDH4 genes were constructed and introduced into sdh4W2. Deletion of 23-amino acid residues in pSDH4ΔC23 severely impairs respiratory growth on the non-fermentable carbon sources, glycerol and lactate. That the impaired respiratory did not result from a mutation in the mitochondrial genome was verified by mating with a rho0 strain; the diploid is fully competent to grow on glycerol-containing media. These data indicate that the SDH4 carboxyl-terminal extension is essential for SDH function in vivo.

To define more precisely the critical sequences in the carboxyl-terminal extension that are required for SDH function, two shorter truncations were constructed (Fig. 2). The plasmids pSDH4ΔC10 and pSDH4ΔC15 both conferred the ability to grow on glycerol-containing media to sdh4W2.

To compare the respiratory growth abilities of the wild type and mutant strains, growth on semisynthetic medium containing 0.1, 0.2, 0.3, 0.4, or 0.5% galactose was monitored (Fig. 3). A growth phase is fermentative followed by a respiratory phase (18). Growth rates during the fermentative phases were similar for wild type and mutant strains (not shown) but were markedly different in the respiratory phases. The growth yields monitored as the optical densities at 600 nm of late stationary phase cultures reflect the respiratory capabilities of each strain and were plotted against the initial galactose concentrations. The growth yields of the parent strain, MH125, and sdh4W2 carrying the plasmid pSDH4-17 are similar. The deletion strain, sdh4W2, by fermentation alone achieves 11% of the growth yield of the respiration-proficient wild type on 0.5% galactose. The growth yield of 85% achieved with sdh4W2-pSDH4ΔC10 indicates that this deletion has only minor effects on respiratory growth. In contrast, the growth yield of sdh4W2-pSDH4ΔC23 is severely reduced (29%), whereas sdh4W2-pSDH4ΔC15 produces an intermediate value (59%). These data indicate that deletion of the carboxyl-terminal 23 amino acids of SDH4p severely impairs respiratory growth in vivo, while deletion of 15 residues results in partial impairment.

Enzymatic Characterization of Deletion Mutants—To determine whether the impaired growth we observed is due to a loss of SDH activity or to decreased assembly of the enzyme into the membrane, we prepared mitochondrial membrane fractions for biochemical analysis (Table I). sdh4W2-pSDH4ΔC23 mitochondrial membranes show greatly reduced turnover numbers for succinate-DB reductase, succinate-cytochrome c reductase, and malate-sensitive succinate oxidase activities. As expected, these activities are not detectable in sdh4W2 mitochondrial membranes. Interestingly, the succinate-PMS reductase activity in sdh4W2-pSDH4ΔC23 membranes is the same as that of MH125 membranes. These data indicate that the SDH4ΔC23 enzyme is correctly assembled into mitochondria in near normal amounts and is able to reduce the artificial electron acceptor, PMS, but is impaired in the reduction of ubiquinone or a ubiquinone analog (DB). To independently confirm enzyme assembly, we determined the trichloroacetic acid-precipitable flavin levels in mutant and wild type membranes. In S. cerevisiae,
the amount of acid-precipitable flavin directly reflects the levels of SDH1p (16). The covalent flavin levels of SDH4 membranes are only slightly reduced compared with the wild type, supporting our conclusion that membrane assembly is only slightly impaired in this mutant. In contrast, the succi-
nate-PMS reductase and covalent flavin levels in sdh4W2 membranes are not detectable. The levels of NADH oxidase and glycerol-1-phosphate-cytochrome c reductase activities are unaffected or even slightly elevated in sdh4W2-pSDH4C23, indicating that the carboxyl-terminal deletion specifically affects SDH. Taken together, the results strongly suggest that the carboxyl-terminal extension of SDH4p is not necessary for a membrane-anchoring function but is required for ubiquinone reduction.

As shown in Table I, truncation of 15 amino acids from the carboxyl terminus (SDH4ΔC15) had smaller effects on succinate-DB reductase, succinate-cytochrome c reductase, and succinate-oxidase activities, whereas the loss of 10 (SDH4ΔC10) amino acids had no appreciable effects; the results of the enzymatic assays are consistent with observed growth abilities in that the deletions of 10 or 15 amino acids do not greatly impair respiratory abilities. SDH4ΔC15 and SDH4ΔC10 membranes have normal levels of succinate-PMS reductase and covalent flavin. This suggests that the carboxyl-terminal decapetide (DNSQKIEAKK) is dispensable for membrane anchoring and ubiquinone reduction while residues 136 to 140, missing in the SDH4ΔC23 enzyme, are required for optimal quinone reduction activity.

The SDH4ΔC23 Enzyme Has a Lower Affinity for Quinones—Since the SDH4ΔC23 enzyme is partially active in quinone reduction, we tested whether its activity could be stimulated by higher quinone concentrations. We preincubated SDH4ΔC23 membranes at 22 °C with a 10-fold excess of DB (0.5 mM) before assaying succinate-DB reductase activity. A greater than 5-fold stimulation could be achieved (Fig. 4) with maximal stimulation occurring after a 5-min incubation. In contrast, preincubation with DB had no effect on the activities of the wild type, the SDH4ΔC15, or the SDH4ΔC10 enzymes (data not shown).

We measured the apparent kinetic parameters \( K_m \) and \( V_{\text{max}} \) for DB reduction using the reporter DCPIP in a coupled enzyme assay as previously reported (15). Succinate reduces exogenous DB directly without interference from endogenous \( Q_6 \) (19). In contrast to earlier observations (20), we did not observe a lag in enzyme activity. SDH4ΔC23 mitochondrial membranes were preincubated with varying concentrations of DB at room temperature, and reactions were initiated by the addition of succinate and DCPIP. The apparent Michaelis constants \( K_m \) and \( V_{\text{max}} \) were calculated and are summarized in Table II. The apparent \( K_m \) for DB of the SDH4ΔC23 enzyme (22 \( \mu \)M) is almost 7 times higher than that of the wild type enzyme (3.3 \( \mu \)M). The apparent \( K_m \) for the SDH4ΔC15 and the SDH4ΔC10 enzymes are only slightly higher than that of the wild type. We suggest that the higher apparent \( K_m \) of the SDH4ΔC23 enzyme is an indication of a reduced quinone binding affinity due to an altered binding site.

### Table I

<table>
<thead>
<tr>
<th>Activity</th>
<th>MH125</th>
<th>sdh4W2-pSDH4-17</th>
<th>sdh4W2-pSDH4ΔC10</th>
<th>sdh4W2-pSDH4ΔC15</th>
<th>sdh4W2-pSDH4ΔC23</th>
<th>sdh4W2</th>
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<tr>
<td>Succinate-DB reductase(^a)</td>
<td>3400</td>
<td>3500</td>
<td>3200</td>
<td>2600</td>
<td>600  ND</td>
<td>ND</td>
</tr>
<tr>
<td>Succinate-cytochrome c reductase(^c)</td>
<td>2300</td>
<td>3100</td>
<td>2500</td>
<td>1900</td>
<td>500  ND</td>
<td>ND</td>
</tr>
<tr>
<td>Succinate oxidase(^d)</td>
<td>2500</td>
<td>3100</td>
<td>2600</td>
<td>1700</td>
<td>700  ND</td>
<td>ND</td>
</tr>
<tr>
<td>Succinate-PMS reductase(^e)</td>
<td>4100</td>
<td>5100</td>
<td>4100</td>
<td>3700</td>
<td>4300</td>
<td>ND</td>
</tr>
<tr>
<td>NADH oxidase(^f)</td>
<td>3900</td>
<td>4400</td>
<td>4700</td>
<td>3700</td>
<td>5200</td>
<td>95' ND</td>
</tr>
<tr>
<td>Glycerol-1-P-cytochrome c reductase(^c)</td>
<td>2000</td>
<td>2000</td>
<td>2100</td>
<td>2600</td>
<td>2800</td>
<td>55' ND</td>
</tr>
<tr>
<td>Covalent flavin(^g)</td>
<td>32</td>
<td>25</td>
<td>29</td>
<td>27</td>
<td>21</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) Activities are expressed as \( \mu \)mol of DCPIP reduced min\(^{-1} \) \( \mu \)mol of FAD\(^{-1} \).

\(^b\) ND, not detectable.

\(^c\) Activities are expressed as \( \mu \)mol of cytochrome c reduced min\(^{-1} \) \( \mu \)mol of FAD\(^{-1} \).

\(^d\) Activities are expressed as \( \mu \)g atoms of oxygen min\(^{-1} \) \( \mu \)mol of FAD\(^{-1} \).

\(^e\) Activity is expressed as ng atoms of oxygen min\(^{-1} \) mg of protein\(^{-1} \).

\(^f\) Activity is expressed as nmol of cytochrome c reduced min\(^{-1} \) mg of protein\(^{-1} \).

\(^g\) Covalent flavin contents are expressed as pmol of FAD mg of protein\(^{-1} \).

**Stabilities of Mutant Enzymes**—Fig. 4 shows that preincubation of the SDH4ΔC23 enzyme with DB for longer than 5 min results in lower activities suggesting that the enzyme is unstable. We directly tested this possibility by incubating mitochondrial membranes at 30 °C in 50 mM potassium phosphate, 20 mM succinate, 1 mM EDTA, pH 7.4. Aliquots were withdrawn at 5-min intervals and assayed for succinate-DB reductase activity as described under "Experimental Procedures."
Function of the Yeast SDH4p Carboxyl Terminus

TABLE II
Apparent Michaelis constants for quinone reduction

<table>
<thead>
<tr>
<th>Strain</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH125</td>
<td>3.3 ± 0.2*</td>
<td>3500 ± 200</td>
</tr>
<tr>
<td>sdh4W2-pSDH4ΔC10</td>
<td>4.5 ± 0.1</td>
<td>3400 ± 40</td>
</tr>
<tr>
<td>sdh4W2-pSDH4ΔC15</td>
<td>5.1 ± 0.2</td>
<td>2900 ± 100</td>
</tr>
<tr>
<td>sdh4W2-pSDH4ΔC23</td>
<td>22 ± 1</td>
<td>3400 ± 100</td>
</tr>
</tbody>
</table>

*Values are expressed as μM.

**Values are expressed as μmol of DCPIP reduced min$^{-1}$ μmol of FAD$^{-1}$.

Values represent means of triplicate determinations ± S.E.

Fig. 5. Stabilities of mutant and wild type enzymes. Membrane fractions were incubated at 30 °C in 50 mM potassium phosphate, 20 mM succinate, 1 mM EDTA, pH 7.5, for up to 30 min. Succinate-DB reductase reactions were initiated with the addition of 50 μM DB and 75 μM DCPIP. The symbols used are: triangles, wild type; open squares, SDH4ΔC10; closed squares, SDH4ΔC15; open circles, SDH4ΔC23 incubated in the presence of 0.5 mM DB; closed circles, SDH4ΔC23.

of quinone oxidation and reduction (21, 22). The 4,6-dinitrophenol derivative, 2-sec-butyl-4,6-dinitrophenol (s-BDNP) effectively inhibits beef heart complex II (23). We tested whether the S. cerevisiae SDH was similarly sensitive. As with the bovine enzyme, we observed non-competitive inhibition of succinate-DB reductase activity with the yeast enzyme (Fig. 6A).

The secondary plots of the abscissa intercepts against the inhibitor concentrations for the wild type, the SDH4ΔC10, and the SDH4ΔC15 enzymes showed hyperbolic curvature (Fig. 6B). The data could best be described with Equation 2 for non-competitive inhibition with two non-equivalent $K_i$ values. The sum of squares error (SSE) values for the wild type, the SDH4ΔC10, and the SDH4ΔC15 enzymes are 1.7 × 10$^{-4}$, 1.6 × 10$^{-4}$, and 2.0 × 10$^{-4}$, respectively. This suggests a two-site model for inhibitor binding, with two non-equivalent inhibition constants. Table III presents the estimates of the affinities of both inhibitor sites. The affinities of the inhibitor sites in the wild type and the SDH4ΔC10 enzymes are similar with an approximate 10-fold difference between the 2 sites. For the SDH4ΔC15 enzyme, the high affinity inhibitor site (0.06 mM) remains unchanged while the low affinity site has a 2-fold lower affinity (1.14 mM), consistent with a small perturbation of the putative low affinity inhibitor binding site and a partial loss of quinone-reductase activity exhibited in growth and activity studies.

For the SDH4ΔC23 enzyme, non-competitive inhibition by

TABLE III
Apparent inhibition constants for quinone reduction

<table>
<thead>
<tr>
<th>Strain</th>
<th>$K_i$</th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH125</td>
<td>0.05 ± 0.01*</td>
<td>0.56 ± 0.06</td>
</tr>
<tr>
<td>sdh4W2-pSDH4ΔC10</td>
<td>0.06 ± 0.01</td>
<td>0.74 ± 0.1</td>
</tr>
<tr>
<td>sdh4W2-pSDH4ΔC15</td>
<td>0.06 ± 0.01</td>
<td>1.14 ± 0.12</td>
</tr>
<tr>
<td>sdh4W2-pSDH4ΔC23</td>
<td>0.08 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

*Inhibition constants are expressed as mM.

**Values represent means of triplicate determinations ± S.E.

s-BDNP is also observed (not shown), but the secondary plot of reciprocal maximal velocities against inhibitor concentrations is linear (Fig. 6B). This suggests the presence of only one inhibitor binding site in the SDH4ΔC23 enzyme (Table III). There is a good fit of the data to Equation 1 (SSE = 4.0 × 10$^{-4}$). The secondary inhibitor site may be non-functional, or its affinity for s-BDNP may be too low to measure. Interestingly, the apparent $K_i$ for the SDH4ΔC23 mutant enzyme (0.08 mM) is
close to the value obtained for the high affinity inhibitor site in the wild type enzyme.

DISCUSSION

The participation of ubiquinone in the mitochondrial electron transport chain is well established (2, 3). However, the molecular details of its interactions with SDH are poorly understood. Studies on the bovine SDH (2, 3), the E. coli SDH (24), and the E. coli FRD (25, 26) have revealed that quinone substrates interact via the membrane anchor subunits.

Our data clearly demonstrate that the S. cerevisiae SDH4p subunit is required for an anchoring role. The SDH4 deletion mutant, sdh4W2, is unable to grow on glycerol, has undetectable levels of the ubiquinone-dependent enzyme activities, succinate oxidase and succinate-cytochrome c reductase, and undetectable levels of acid-precipitable flavin (Table I), consistent with absence of membrane-associated SDH. Similarly, the SDH3p subunit is essential for SDH assembly (11).

Our data also establish a role for the yeast SDH4p subunit in ubiquinone reduction and more specifically for its terminal 23-amino acid residues. The loss of those 23 amino acids severely impairs SDH function \textit{in vivo} (Fig. 3). The lack of respiratory growth is not due to a loss of SDH4p anchoring function since mitochondrial membranes from sdh4W2-pSDH4C23 have normal levels of succinate-PMN reductase activity (Table I); this clearly indicates that the SDH1p and SDH2p subunits are membrane-associated and active. This is further supported by the covalent flavin levels in SDH4C23 membranes.

The SDH4C23 enzyme is only poorly able to reduce the ubiquinone analog, DB (Table I). Preincubation of the enzyme with 0.5 mmol DB for 5 min at room temperature stimulates the succinate-DB reductase activity 5-fold (Fig. 4) and achieves a turnover number similar to the wild type enzyme (Table II). Stimulation of activity by preincubation with DB is not observed for the wild type or for the other mutant enzymes. This indicates that the SDH4C23 enzyme is fully catalytically competent when provided with saturating substrate (DB) levels. The apparent affinity of the SDH4C23 enzyme for DB is about 7-fold lower than that of the wild type enzyme (Table II). The data demonstrate that the SDH4p carboxyl-terminal extension is involved in quinone reduction.

At the molecular level, at least two possibilities may account for these observations. The carboxyl-terminal extension may directly interact with DB and ubiquinone as part of a quinone binding site. Alternatively, it may stabilize protein conformational changes necessary for the formation of a high affinity quinone binding site. In either model, its loss impairs quinone binding and hence quinone reduction. Our data do not distinguish between these alternatives. Further insight should await a detailed structural analysis of the quinone binding sites in the \textit{S. cerevisiae} SDH.

The SDH4p residues 128–135, which are missing in the SDH4C23 enzyme but not in the SDH4C15 enzyme, are critical for the formation of a high affinity quinone binding site since it is only with the more extensive deletion that severely impaired quinone reduction is observed (Table I). Residues 136 to 140, missing in the SDH4C15 enzyme but not in the SDH4C10 enzyme, are necessary for optimal quinone reducing activity. The enzymatic activities of the SDH4C10 enzyme are comparable with those of the wild type enzyme, suggesting that the carboxyl-terminal decapeptide is dispensable.

In addition to the carboxyl-terminal extension’s role in quinone reduction, it also serves to stabilize the enzyme. At 30 °C, the SDH4C23 enzyme is extremely labile with a complete loss of succinate-DB reductase activity within 20 min (Fig. 5), whereas the wild type and the SDH4C10 enzymes are stable. The SDH4C15 enzyme is moderately unstable indicating that residues 136–140 are important for stability while the additional loss of residues 128–135 in the SDH4C23 enzyme can be compensated for by higher DB concentrations.

The mechanism of how an iron-sulfur center, a one-electron carrier, can fully reduce a two-electron acceptor like ubiquinone has been a focus of considerable attention. Studies on the photosynthetic reaction center (27) indicate that quinone reduction proceeds via two “one-electron” steps carried out at separate quinone binding sites, Q\textsubscript{A} and Q\textsubscript{B}. In this model, a tightly bound quinone at the Q\textsubscript{A} site cycles between the fully oxidized and the semiquinone states to mediate single-electron transfer between the enzyme and a dissociable quinone at the Q\textsubscript{B} site. A similar two-state model has been postulated for SDH and FRD based on thermodynamic considerations (28). During quinone reduction by beef heart SDH, two protein-stabilized ubisemiquinone radicals can be identified in the vicinity of Center 3 iron-sulfur cluster (29). Detailed mutational analysis of the \textit{E. coli} FRD hydrophobic subunits has led to a structural model for the locations of the Q\textsubscript{A} and Q\textsubscript{B} sites (25, 26).

Despite the absence of detailed structural information about the yeast anchor subunits, our observations can be rationalized in terms of a two-ubiquinone binding site model. The non-competitive inhibition pattern of the enzyme by s-BDNP, an analog of ubiquinone, is best explained by 2 inhibitor binding sites; these may correspond to the Q\textsubscript{A} and Q\textsubscript{B} sites. By analogy to the model for the FRD Q\textsubscript{A} and Q\textsubscript{B} sites (25), the SDH Q\textsubscript{A} site would be oriented toward the cytoplasmic side of the inner mitochondrial membrane and the SDH Q\textsubscript{B} site toward the matrix. The SDH4 carboxy-terminal extension is predicted to be on the cytoplasmic face of the membrane (Fig. 2). Our data do not identify which of the two putative quinone binding sites is affected in the SDH4C23 enzyme nor whether the inhibitor binding sites physically correspond to those sites, although the loss of quinone reductase activity is consistent with a perturbed Q\textsubscript{A} site and with the topological model for SDH4p (25).

This study clearly demonstrates that the SDH4 carboxy-terminal extension is necessary for SDH activity and stability. It is interesting to note that of the 8 additional residues deleted in the SDH4C23 enzyme as compared with the SDH4C15 enzyme, only one, lysine 132, is charged, and only two, lysine 132 and serine 133 are potentially involved in hydrogen bonding. These residues are thus candidates for direct interactions with ubiquinone or for protein-protein interactions that produce a competent quinone binding conformation. More detailed mutagenesis studies are in progress.

REFERENCES

Function of the Yeast SDH4p Carboxyl Terminus

The Carboxyl Terminus of the *Saccharomyces cerevisiae* Succinate Dehydrogenase Membrane Subunit, SDH4p, Is Necessary for Ubiquinone Reduction and Enzyme Stability

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