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The *Saccharomyces cerevisiae* succinate dehydrogenase anchor subunit, Sdh4p: mutations at the C-terminal Lys-132 perturb the hydrophobic domain

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Abstract

The yeast succinate dehydrogenase (SDH) is a tetramer of non-equivalent subunits, Sdh1p–Sdh4p, that couples the oxidation of succinate to the transfer of electrons to ubiquinone. One of the membrane anchor subunits, Sdh4p, has an unusual 30 amino acid extension at the C-terminus that is not present in SDH anchor subunits of other organisms. We identify Lys-132 in the Sdh4p C-terminal region as necessary for enzyme stability, ubiquinone reduction, and cytochrome b_{562} assembly in SDH. Five Lys-132 substituted *SDH4* genes were constructed by site-directed mutagenesis and introduced into an *SDH4* knockout strain. The mutants, K132E, K132G, K132Q, K132R, and K132V were characterized in vivo for respiratory growth and in vitro for ubiquinone reduction, enzyme stability, and cytochrome b_{562} assembly. Only the K132R substitution, which conserves the positive charge of Lys-132, produces a wild-type enzyme. The remaining four mutants do not affect the ability of SDH to oxidize succinate in the presence of the artificial electron acceptor, phenazine methosulfate, but impair quinone reductase activity, enzyme stability, and heme insertion. Our results suggest that the presence of a positive charge on residue 132 in the C-terminus of Sdh4p is critical for establishing a stable conformation in the SDH hydrophobic domain that is compatible with ubiquinone reduction and cytochrome b_{562} assembly. In addition, our data suggest that heme does not play an essential role in quinone reduction.

Keywords: Succinate dehydrogenase; Cytochrome b_{562}; Point mutation; Structural perturbation; Mitochondria; (*Saccharomyces cerevisiae*)

1. Introduction

Generally succinate:ubiquinone oxidoreductase (also called succinate dehydrogenase, SDH) is made up of two parts: a soluble catalytic dimer and a membrane-anchoring domain [1–4]. The catalytic domain comprises a large flavoprotein (Fp) subunit of about 70 kDa to which is covalently attached an FAD cofactor, and an iron–sulfur protein (Ip) subunit of about 27 kDa that contains three iron–sulfur clusters. This catalytic dimer can transfer electrons from succinate to artificial electron acceptors, such as phenazine methosulfate (PMS) or potassium ferricyanide. However, the reduction of natural quinones or quinone analogs requires the presence of the mem-

Abbreviations: SDH, succinate dehydrogenase; FRD, fumarate reductase; DCPIP, 2,6-dichlorophenol indophenol; PMS, phenazine methosulfate; DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone

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brane-anchoring domain [1,5–7], which is typically comprised of two hydrophobic subunits of about 17 and 13 kDa. This domain attaches the catalytic subunits to the inner membrane of mitochondria or the cytoplasmic membrane of bacteria. In addition, the membrane domain contains binding site(s) for quinone and may also ligand one or two *b*-type hemes. SDH occupies a unique position as a member of both the citric acid cycle and the respiratory chain and thus plays a key role in the generation of chemical energy. Anaerobic cells respiring with fumarate as terminal electron acceptor contain the related enzyme, fumarate reductase (FRD) which is functionally and structurally similar to SDH [1–4,8].

Unlike the flavoprotein and iron–sulfur subunits that are well-conserved across species, there is considerable variability in subunit composition and primary amino acid sequence of the anchor polypetides [1,3–5]. Some SDHs have a single anchor subunit while others have two; some have two hemes in their anchor subunits, while others have one or none. The variability has made it difficult to identify residues that may be involved in quinone binding or subunit–subunit interactions through sequence comparisons. In the absence of high-resolution structural information, the yeast SDH promises to be a good model system for studying enzyme structure and the mechanism of ubiquinone-reduction through genetic approaches. *Saccharomyces cerevisiae* is able to grow either by fermentation, where functional SDH is not required, or by respiration, where its presence is essential [6,7,9,10].

In *S. cerevisiae*, SDH is composed of four subunits encoded by the nuclear *SDH1*, *SDH2*, *SDH3*, and *SDH4* genes [6,7,11–14]. The hydrophilic subunits, SDH1p and SDH2p, which comprise the catalytic domain and are involved in succinate oxidation, are anchored to the inner mitochondrial membrane by the small, hydrophobic subunits, Sdh3p and Sdh4p. These are believed to contain two quinone binding sites by analogy to the bovine SDH [1,3,4,15–19], the *Bacillus subtilis* SDH [20], and the *Escherichia coli* FRD [1,16,21]. They are predicted to each contain three membrane-spanning domains [6,7,22]. Our understanding of the Sdh3p and Sdh4p structural requirements for ubiquinone binding and reduction and for their anchoring roles are at best rudimentary. Although there is emerging evidence that the respiratory complexes may function as one supramolecular unit [23], understanding the molecular details of electron transfer and oxidative phosphorylation will depend on the study of the individual respiratory complexes.

The yeast Sdh4p subunit contains a matrix-localized amino terminus, three transmembrane helices, and a carboxyl terminus of about 30 amino acids that extends into the intermembrane space [6,22]. The C-terminal extension of the yeast subunit is not shared by most other SDH or FRD anchor subunits [4,5,24]. Recently, we showed that the carboxyl terminus of the yeast Sdh4p subunit is necessary for respiratory growth on non-fermentable carbon sources, for ubiquinone reduction, and for enzyme stability [22]. Through a stepwise truncation, residues 128–135 were shown to be critical for the formation of a quinone binding site. Here, we further characterize the role of the Sdh4p C-terminus by identifying Lys-132 as a key residue in the establishment of a stable conformation compatible with heme assembly and quinone binding in the SDH hydrophobic domain.

2. Materials and methods

2.1. Strains and media

The yeast strains, MH125 and sdh4W2 and the *E. coli* strain, DH5α, have been described [22,25]. The yeast media used are SD (0.67% yeast nitrogen base, 2% glucose), SG (0.67% yeast nitrogen base, 3% glycerol), YPGal (1% yeast extract, 2% peptone, 2% galactose) and semisynthetic galactose (0.3% yeast extract, 0.1% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl, 0.06% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2$, 0.003% $FeCl_3$, 2% galactose).

2.2. Yeast culture conditions

Cultures were grown on SD for 2 days to select for plasmid retention, used to inoculate YPGal medium supplemented with 0.01% glucose to a starting $OD_{600} = 0.05$, and grown at 30°C to stationary phase. Cells were harvested and lysed in a French pressure cell for the preparation of submitochondrial particles [22]. For the preparation of mitochondria, cultures were grown in semisynthetic galactose to late loga-

rithmic phase (OD_{600} about 3), harvested, and lysed enzymatically as described [22].

2.3. Mutant construction

Five point mutations were constructed at the *SDH4* Lys-132 codon with the Altered Site II in vitro Mutagenesis Kit (Promega, Madison, WI). Briefly, an *Xho*I–*Spe*I fragment of *SDH4* was ligated into the *Sal*I–*Xba*I sites of pAlter-I vector and used as a template for the mutagenesis reaction. The mutagenic oligonucleotides used were: (1) 5′-CCCATAGACTT(**T/A/C**)(**C/G**)TACTAAACCAAC-3′, a degenerate oligonucleotide designed to generate the six codons, GAA, GTA, GGA, CAA, CTA, CGA coding for Glu, Val, Gly, Gln, Leu, and Arg, respectively; (2) 5′-CCCATAGACTT(**T/A/C**)**C**TACTAAACCAAC-3′, a degenerate oligonucleotide designed to generate the three codons, GAA, GTA, and GGA coding for Glu, Val and Gly, respectively; and (3) 5′-CCCATAGACTT**CG**TACTAAACCAAC-3′, designed to generate the codon CGA coding for Arg. The mutagenesis reactions were performed as described by the manufacturer. The mutations were confirmed by sequencing. The *SDH4* mutants were moved as *Eco*RI-*Hin*dIII fragments into the yeast shuttle vector, YCplac111 [26] to yield the plasmids, pSDH4K132E, pSDH4K132G, pSDH4K132Q, pSDH4K132R, and pSDH4K132V.

2.4. Enzyme assays and thermal stability measurements

Unless otherwise stated, the succinate-dependent reduction of quinone was monitored spectrophotometrically at 22°C as the 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB)-mediated reduction of dichlorophenol indophenol (DCPIP) [22]. The succinate-dependent, PMS-mediated reduction of DCPIP was determined as a measure of the membrane-associated SDH1p/SDH2p dimer; this assay does not require catalytically competent Sdh3p and Sdh4p subunits, but membrane association of the SDH1p/SDH2p dimer requires intact anchor subunits [6,10]. Quinone reduction was also directly monitored as the reduction of DB using the wavelength pair, 280 and 325 nm [27], with a Hewlett Packard 8453 diode array spectrophotometer. The absorption coefficient is 16 mM^{-1} cm^{-1}. Other assays have been described [22]. For thermal stability measurements, membrane fractions (20 mg ml^{-1}) were incubated at temperatures ranging from 25 to 65°C in 50 mM potassium phosphate, 50 μM EDTA, pH 7.4, 2 mM KCN for 10 min and assayed immediately for succinate-DB reductase activity. The reactions were initiated with the addition of 50 μM DB and 20 mM succinate. Aliquots were also assayed for succinate-PMS reductase activity.

2.5. Measurement of SDH-associated cytochrome b content

The Complex II cytochrome *b* content was determined spectrally as the dithionite-reduced minus the dithionite-reduced/fumarate-oxidized difference spectrum [27,28]. Mitochondria were isolated from strains grown on semisynthetic galactose medium, suspended to 185 pmol FAD ml^{-1} (2.5–3.2 mg protein ml^{-1}) in 0.65 M sorbitol, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 0.3% fatty acid-free bovine serum albumin, pH 6.5. Lauryl maltoside (Boehringer Mannheim) was added to a final concentration of 0.1% to reduce spectral noise. Spectra were recorded at 22°C on a Hewlett Packard 8453 diode array spectrophotometer with a 1-cm path length.

2.6. Miscellaneous methods

Measurements of covalently bound flavin and protein contents, *E. coli* transformation, and recombinant DNA methods have been described [6]. Determination of the apparent Michaelis parameters, K_m and V_{max} was as previously described [22].

3. Results

3.1. In vivo characterization of SDH4 point mutants

Lys-132 falls within the C-terminal sequence of Sdh4p (residues 128–135) that was recently shown to be necessary for quinone reduction and for enzyme stability through a stepwise truncation of the C-terminus [22]. Since deletion mutagenesis has the potential for causing large structural perturbations, we further explored the role of the C-terminus by

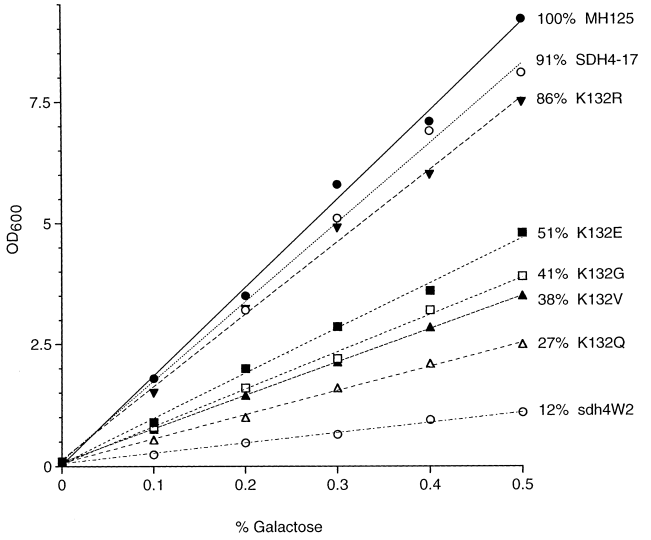

Fig. 1. Growth of yeast strains on galactose media. Precultures were prepared on selective minimal medium containing 2% galactose and 0.01% glucose and used to inoculate the main culture to a starting OD_{600} of 0.1. Growth was at 30°C on semisynthetic liquid medium containing 0.1, 0.2, 0.3, 0.4, or 0.5% galactose and the optical densities at 600 nm were measured [22]. The cultures were allowed to reach late stationary phase (approximately 100 h). The relative growth yields were calculated using the final absorbance values reached on 0.5% galactose.

creating five-point mutants in Lys-132. Lys-132 is the only charged amino acid residue within this sequence and was replaced by Arg, Glu, Gln, Gly and Val (K132R, K132E, K132Q, K132G, and K132V). These substitutions have the potential for preserving the positive charge (K132R), removing the charge, but preserving the potential for hydrogen bonding (K132Q), introducing a negative charge (K132E), and removing both the charge and hydrogen-bonding capabilities (K132G, and K132V). The mutant *SDH4* genes were introduced into the *SDH4* knockout strain, sdh4W2, and assayed for respiratory growth. Replacement of Lys-132 with Gln abolishes growth on minimal glycerol (SG) or lactate media, while the Glu, Gly, and Val substitutions lead to greatly impaired growth. In contrast, the K132R substitution grows almost as well as the wild-type.

In order to compare quantitatively the respiratory growth abilities of the wild-type and mutant strains, growth on a semisynthetic medium containing 0.1, 0.2, 0.3, 0.4, or 0.5% galactose was monitored. An initial growth phase is fermentative followed by a respiratory phase [29]. Growth rates during the fermentative phases were similar for wild-type and mutant strains (data 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 are plotted against the initial galactose concentrations (Fig. 1). A growth yield of less than about 30% is usually associated with a respiratory deficiency severe enough to prevent growth on minimal glycerol medium. The growth yields of the parent strain, MH125, sdh4W2 carrying the wild-type *SDH4* plasmid, pSDH4-17, or sdh4W2 with the pSDH4K132R plasmid are similar. sdh4W2 achieves a 12% growth yield on 0.5% galactose by fermentation alone. The growth yields of sdh4W2 pSDH4K132E, sdh4W2 pSDH4K132G, sdh4W2 pSDH4K132V, and sdh4W2 pSDH4K132Q strains are severely reduced (51, 41, 38, and 27%, respectively). We conclude that Lys-132 is critical for SDH function and respiratory competence.

3.2. Membrane-association of the catalytic dimer

In order to examine the possibility that the mutation of Lys-132 compromises the anchoring role of Sdh4p, we determined the amounts of catalytic dimer on the mitochondrial membranes of the mutants. The SDH1p subunit contains a covalently attached FAD and its presence is a measure of SDH assembly. The membrane-associated covalent flavin levels in sdh4W2 pSDH4-17 and sdh4W2 pSDH4K132R are similar to the wild-type level, while those of sdh4W2 pSDH4K132E, sdh4W2 pSDH4K132G, sdh4W2 pSDH4K132Q, and sdh4W2 pSDH4K132V are slightly reduced (Table 1). We also measured the succinate-PMS reductase activities of mutant and wild-type enzymes; this activity does not require that the anchor subunits be functional for quinone reduction. The turnover numbers in all the mutant strains are comparable to that of the wild-type enzyme (Table 1). As expected, sdh4W2 membranes have undetectable levels of succinate-PMS reductase activity and covalent FAD. Taken together, the results indicate that membrane-association of SDH1p/SDH2p dimer proceeds normally with all the Lys-132 substituted Sdh4p subunits.

Table 1
Succinate dehydrogenase activities of mitochondrial membranes

Strain	Covalent flavin[a]	Specific activity[b]	Turnover numbers[c]
MH125	33 ± 5	135 ± 7	4100 ± 205
sdh4W2 pSDH4-17	31 ± 2	121 ± 5	3900 ± 156
sdh4W2 pSDH4K132E	25 ± 4	97 ± 5	3900 ± 195
sdh4W2 pSDH4K132G	22 ± 5	85 ± 3	3900 ± 117
sdh4W2 pSDH4K132Q	20 ± 4	78 ± 5	3900 ± 234
sdh4W2 pSDH4K132R	28 ± 3	112 ± 4	4000 ± 160
sdh4W2 pSDH4K132V	21 ± 3	82 ± 4	3900 ± 214
sdh4W2	ND	ND	ND

Each value represents the mean of triplicate determinations ± S.E.M. ND, not detectable or less than 4% of that of the wild-type.
[a]Covalent flavin contents are expressed as pmol of FAD mg of protein^{-1}.
[b]Specific activities are expressed as μmol of PMS-mediated DCPIP reduced min−1 mg of protein^{-1}.
[c]Turnover numbers are expressed as μmol of PMS-mediated DCPIP reduced min^{-1} μmol FAD^{-1}.

3.3. Respiratory activities of Sdh4p point mutants

Table 2 shows the quinone-dependent respiratory chain activities of mitochondrial membranes isolated from mutant and wild-type strains. The SDH4K132E, SDH4K132G, SDH4K132Q, and SDH4K132V enzymes show 2–3-fold decreases in turnover numbers for succinate-DB reductase, succinate-cytochrome *c* reductase, and succinate oxidase activities. In contrast, the SDH4K132R enzyme activities are unaffected. sdh4W2 membranes lacking Sdh4p have undetectable levels of these enzymatic activities. NADH oxidase and glycerol-1-P-cytochrome *c* reductase activities are normal in all the mutants (Table 2). This clearly demonstrates that Lys-132 substitutions do not affect other respiratory complexes that may be associated with SDH.

3.4. Kinetics of exogenous quinone reduction

The succinate-DB reductase activities of all the mutant enzymes, except that of the K132R mutant, can be stimulated significantly when incubated with higher concentration of DB at room temperature (data not shown). This suggests a possible structural perturbation in the vicinity of the quinone binding site(s). To further explore this, we measured the apparent Michaelis parameters, K_m and V_{max}, of the

Table 2
Enzymatic activities of mitochondrial membranes

	MH125	sdh4W2 pSDH4-17	sdh4W2 K132E	sdh4W2 K132G	sdh4W2 K132Q	sdh4W2 K132R	sdh4W2 K132V	sdh4W2
Succinate-DB reductase[b]	3500[a]	3400	1700	1500	1100	3300	1400	ND
Succinate-cytochrome *c* reductase[c]	2200	2200	1000	900	700	2100	800	ND
Succinate oxidase[d]	2000	2000	900	800	700	2000	700	ND
NADH oxidase[d]	4000	3800	3800	3700	3500	3900	3600	110[e]
Glycerol-1-P-cytochrome *c* reductase[c]	2700	2600	2200	2300	2400	2500	2300	65[f]

[a]Values represent the means of triplicate determinations. In all cases the S.E.M. are within 5%. ND, not detectable or less than 4% of wild type activity.
[b]Activities are expressed as μmol of DB-mediated DCPIP reduced min^{-1} μmol FAD^{-1}.
[c]Activities are expressed as μmol of cytochrome *c* reduced min^{-1} μmol FAD^{-1}.
[d]Activities are expressed as μatoms of oxygen min^{-1} μmol FAD^{-1}.
[e]Activity is expressed as ng atoms of oxygen min^{-1} mg protein^{-1}.
[f]Activity is expressed as nmol of cytochrome *c* reduced min^{-1} mg protein^{-1}.

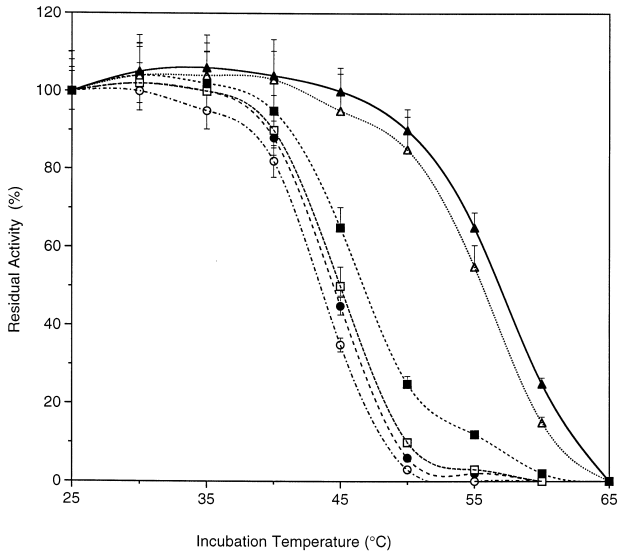

Fig. 2. Thermal stability profiles of succinate-DB reductase activities of mutant and wild-type enzymes. DB reduction was monitored spectrophotometrically at the wavelength pair of 280 and 325 nm after incubating mitochondrial membranes at the indicated temperatures for 10 min. Activities are expressed as percentages of turnover numbers observed at 25°C for each strain. The symbols used are: open circles, K132Q; closed circles, K132V; open squares, K132G; closed squares, K132E; open triangles, K132R; closed triangles, MH125. Error bars represent mean of triplicate determinations ± S.E.M.

mutant and wild-type enzymes by incubating membranes with different DB concentrations at room temperature for 5 min. Since the mutants have variable levels of covalent FAD, we also determine the k_{cat} (maximal turnover number). We observed approximately 3-fold increases in the apparent K_m for DB reduction of the SDH4K132G, SDH4K132Q, and SDH4K132V enzymes, and a 2-fold increase in that of the SDH4K132E enzyme (Table 3). The apparent K_m for the SDH4K132R enzyme is similar to the plasmid-borne wild-type *SDH4* gene, SDH4-17 and the parental strain, MH125. When normalized for covalent FAD contents, all mutant enzymes have maximal turnover numbers (k_{cat}) of at least 70% of the wild-type when supplied with a saturating amount of DB (Table 3). We conclude that the Lys-132 mutations induce a structural defect in SDH that alters the local environment of a quinone binding pocket.

3.5. Thermal stability profiles of mutant and wild-type enzymes

The structural perturbations that lead to the differences in apparent K_m values for DB reduction in the mutant enzymes might also be expected to affect enzyme stability. We tested this possibility by incubating mitochondrial membranes at temperatures ranging from 25 to 65°C (Fig. 2). Membranes were assayed for succinate-DB reductase activities by directly monitoring DB reduction at the wavelength pair 280 and 325 nm (Fig. 2) [27] or for succinate-PMS reductase activities (Fig. 3). The SDH4K132E, SDH4K132G, SDH4K132Q, and SDH4K132V mutant enzymes are significantly more thermolabile than the wild-type enzyme or the SDH4K132R enzyme. Interestingly, there are no significant differences between the thermostability profiles for the succinate-PMS reductase activities of mutant and wild-type enzymes (Fig. 3). This indicates a temperature-

Table 3
The apparent Michaelis constants for the reduction of exogenous quinone analog

Strain	K_m^a	V_{max}^b	k_{cat}^c
MH125	3.5 ± 0.1	118.8 ± 4.9	3600 ± 150
sdh4W2 pSDH4-17	3.7 ± 0.2	108.5 ± 1.9	3500 ± 60
sdh4W2 pSDH4K132E	7.8 ± 0.1	75.0 ± 6.3	3000 ± 250
sdh4W2 pSDH4K132G	11.5 ± 0.6	63.8 ± 4.4	2900 ± 200
sdh4W2 pSDH4K132Q	13.2 ± 0.1	50.0 ± 5.2	2500 ± 260
sdh4W2 pSDH4K132R	3.9 ± 0.2	98.0 ± 2.8	3500 ± 100
sdh4W2 pSDH4K132V	12.2 ± 0.3	58.8 ± 4.2	2800 ± 200

The Michaelis constants were determined from double reciprocal plots as described in Section 2. Each value represents the mean of triplicate determinations ± S.E.M.
[a]Values are expressed as μmol of DB.
[b]Values are expressed as μmol of DCPIP reduced min^{-1} mg of $protein^{-1}$.
[c]Values are expressed as μmol of DCPIP reduced min^{-1} μmol of FAD^{-1}.

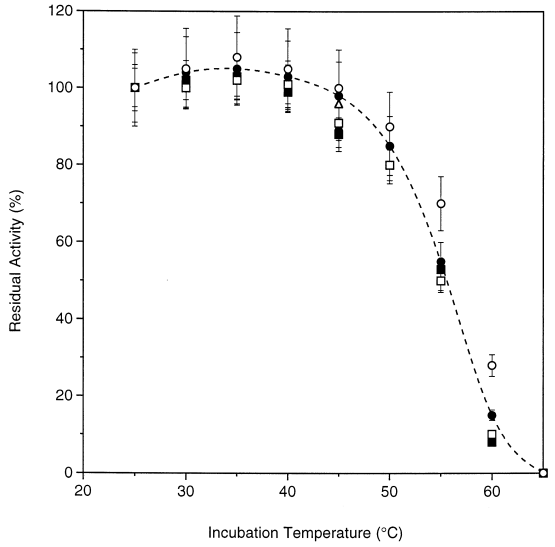

Fig. 3. Thermal stability profiles of succinate-PMS reductase activities of mutant and wild-type enzymes. Mitochondrial membranes were incubated at the indicated temperatures for 10 min and assayed immediately for succinate-PMS reductase activity. Activities are expressed as percentage of turnover numbers observed at 25°C for each strain. The symbols used are: open circles, wild-type; closed circles, SDH4K132R; open squares, SDH4K132E; closed squares, SDH4K132G; open triangles, SDH4K132V closed triangles, SDH4K132Q. Error bars represent means of triplicate determinations ± S.E.M.

sensitive impairment of the quinone binding site in the hydrophobic domains of the *SDH4* mutants.

3.6. SDH cytochrome b_{562} levels in mutant and wild-type mitochondria

The cytochrome *b* from the mammalian SDH has the unique property of being selectively oxidized by fumarate after reduction with dithionite [30]. Therefore, a dithionite-reduced minus dithionite-reduced/fumarate-oxidized difference spectrum corresponds to the absorption spectrum of SDH cytochrome b_{562} [27,28,30]. We used this approach to determine cytochrome b_{562} spectra of mutant and wild-type mitochondria (Fig. 4). Spectra were normalized to covalent FAD levels. As expected, cytochrome b_{562} is not detected in the strain lacking the *SDH4* gene, sdh4W2. A 2-fold reduction in cytochrome b_{562} levels is observed in mitochondria from SDH4K132E, and a greater than 2-fold reduction in mitochondria from SDH4K132G, SDH4K132Q, and SDH4K132V compared to the wild-type. In contrast, there is no significant difference between the cytochrome b_{562} contents of the SDH4K132R and the wild-type mitochondria.

4. Discussion

Although relatively simple in subunit composition, the structure of SDH is still poorly understood due to a lack of information on the requirements for quinone binding and on intersubunit interactions. The variability in anchor subunit composition and primary amino acid sequence amongst SDH family members has made it difficult to compare results between the many SDH and FRD model systems. Yeast Sdh4p is unusual in its possession of a 30 amino acid extension at its C-terminus, suggesting that it may have an unusual function in this organism. The results presented in this report demonstrate the importance of Lys-132 of the Sdh4p C-terminus to the structure of the SDH hydrophobic domain.

We show that residue 132 of the yeast Sdh4p is important in the formation of a native conformation compatible with ubiquinone reduction, cytochrome b_{562} assembly, and enzyme stability. Of the five amino acid substitutions investigated, only the Arg substitution is benign, suggesting the need for a basic residue at this position. The K132E, K132G, K132Q, and K132V substitutions greatly impair SDH function in vivo (Fig. 1). The impaired respiratory growth is not due to a loss of Sdh4p anchoring function, since mitochondrial membranes from mutant strains have normal levels of succinate-PMS reductase activities and near normal levels of covalent FAD (Table 1). In *S. cerevisiae*, SDH is the only protein with covalently bound flavin. The covalent flavin content of submitochondrial membranes provides an accurate measure of the membrane-associated SDH1p [10,21,31,32]. This directly reflects the assembly of the catalytic dimer, SDH1p and SDH2p, since the latter is not assembled in the absence of the former and vice versa [33].

Replacing Sdh4p Lys-132 by Glu, Gln, Gly, or Val leads to significant decreases in the ability of SDH to reduce an exogenous quinone analog, DB (Table 2). However, when supplied with saturating DB concentrations, the mutant enzymes can achieve maximal turnover numbers approaching that of the wild-

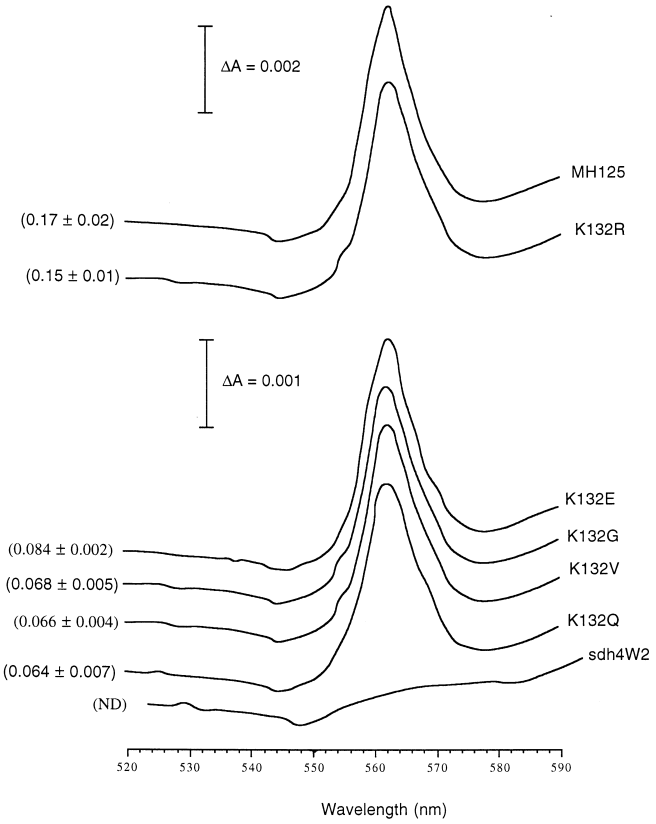

Fig. 4. Spectra of SDH-associated heme in mitochondria from wild-type and mutant strains. Mitochondria were normalized to a covalent FAD level of 185 pmol ml^{-1} and the dithionite-reduced minus the dithionite-reduced/fumarate-oxidized difference spectra were determined. Spectra were recorded after adding a few grains of dithionite and 40 mM fumarate to the reference cuvette and a few grains of dithionite to the sample cuvette. Enclosed in parentheses are cytochrome concentrations (nmol per 185 pmol covalent FAD) calculated from the spectra, using the absorption coefficient of 24 mM^{-1} cm^{-1}. Values represent means of triplicate determinations ± S.E.M.

type enzyme (Table 3). The Michaelis constant, K_m, is a very complex parameter. The differences in the apparent K_m's do not lead to concomitant changes in the k_{cat}'s (the maximal turnover numbers), suggesting that the rate of quinone reduction is not affected once the enzyme is supplied with a saturating amount of quinone. This raises the interesting question of how a residue located on the cytoplasmic side of the membrane can affect functions that are carried out in the membrane. Currently, the amino acid residues constituting the quinone binding site(s) of the yeast SDH are not known. By analogy to the bovine SDH and the *E. coli* FRD, yeast SDH has been proposed to contain two quinone binding sites, Q_A and Q_B, located towards the matrix and the cytoplasmic sides of the inner mitochondrial membrane, respectively [1,3,4,15–19]. It seems unlikely that Lys-132 serves as a quinone ligand in a quinone binding site. Our data favor the alternative possibility that the alteration of Lys-132 to a non-basic amino acid induces a structural perturbation that is propagated to the quinone binding sites in the membrane. The increased thermolability of the succinate-DB reductase activities and the unaffected thermostability of the succinate-PMS reductase activities of mutant enzymes (Fig. 2) support this conclusion. Thus, the structural changes induced by the mutations at Lys-132, although large enough to affect SDH enzyme stability, apparently only affect the hydrophobic domain. Furthermore, the differential thermal stability of the succinate-DB reductase and the succinate-PMS reductase activities indicates that an intact quinone binding site is not necessary for the transfer of electrons from succinate to the artificial

electrons acceptor, PMS. The site of PMS reduction by the SDH1p/SDH2p dimer is unknown. From the data in Table 2, it is clear that the mutations have no effects on other respiratory chain complexes including the ubiquinol-cytochrome *c* reductase which may be intimately associated with SDH [23,34].

In addition to the effects on quinone reduction and enzyme stability, all the amino acid substitutions except the K132R substitution significantly impair cytochrome b_{562} assembly in SDH. Measurement of the fumarate-oxidizable spectrum provides a convenient means for detecting SDH-associated *b*-type heme in yeast [35]. This spectrum is usually masked by that of the more abundant cytochrome *b* of complex III in the classical reduced minus oxidized difference absorption spectrum of mitochondria. The cytochrome contents in the mutant strains are about half that of the wild-type, indicating that structural perturbations are propagated to the heme ligands since it is unlikely that Lys-132 participates in heme coordination. An alternative possibility is that the mutations significantly raise the midpoint potential of the heme such that it is less easily oxidizable by fumarate and not detected spectrophotometrically under our assay conditions. Deletion of the nuclear *ABC1* gene in yeast has been shown to lead to a drastic decrease in the amount of cytochrome b_{562} associated with SDH [28]. However, ours is the first report of point mutants in yeast SDH that affect the cytochrome.

At present, the role of heme in electron transfer from succinate to ubiquinone is not clear. Our data indicate that heme does not play an essential role in quinone reduction. For example, in membranes of the K132Q mutant, which contain only 38% (0.064/0.17 nmol heme per 185 nmol of FAD) the amount of heme per covalent FAD as wild-type membranes (Fig. 4), we find 31% (1100/3500 μmol DCPIP min^{-1} μmol of FAD^{-1}) succinate-DB reductase activity (Table 2). However, activity can be stimulated to 69% (2500/3600 μmol DCPIP min^{-1} μmol of FAD^{-1}) under saturating DB concentrations (Table 3). The presence of approximately 2-fold more succinate-DB reductase activity under saturating DB conditions than heme content is also seen with the K132E, K132G, and K132V mutants. This observation is significant in that it suggests that heme is not involved in electron transfer from succinate to ubiquinone. This is in agreement with a recent observation that heme is not necessary for the catalytic function of the *E. coli* SDH [36]. Recently, it has been postulated that the cytochrome in complex II interacts with the ubisemiquinone radical to reduce the production of superoxide free radicals [37]. Further studies will be required to test these possibilities.

The impairments in quinone reduction, enzyme stability, and cytochrome *b* assembly strongly suggest that a structural change has occurred in SDH due to the substitutions at Lys-132 of Sdh4p. Our results are consistent with the possibility that Lys-132 of Sdh4p may be a determinant of structural integrity and conformation probably through salt-bridge formation. Of the five amino acid substitutions investigated, only the Arg substitution preserves both the charge and hydrogen bonding properties of Lys, and only the Arg substitution is benign at this position. We imagine that Lys-132 is part of either an intramolecular or an intermolecular salt-bridge with an acidic residue that stabilizes the native conformation of SDH hydrophobic domain. It should be possible to identify the interacting residue by the isolation of second-site revertants that restore wild-type enzyme function. Such studies are currently in progress.

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