The Iron-Sulfur Clusters in *Escherichia coli* Succinate Dehydrogenase Direct Electron Flow

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Succinate dehydrogenase is an indispensable enzyme involved in the Krebs cycle as well as energy coupling in the mitochondria and certain prokaryotes. During catalysis, succinate oxidation is coupled to ubiquinone reduction by an electron transfer relay comprising a flavin adenine dinucleotide cofactor, three iron-sulfur clusters, and possibly a heme electron transfer relay comprising a flavin adenine dinucleotide oxidation is coupled to ubiquinone reduction by an mitochondria and certain prokaryotes. During catalysis, suc -volved in the Krebs cycle as well as energy coupling in the mitochondrial function, we monitored aerobic growth in succinate minimal medium, anaerobic growth in glycerol-fumarate minimal medium, non-physiological and physiological enzyme activities, and heme reduction. It was discovered that a decrease in midpoint potential of the succinate oxidizing catalytic dimer (SdhAB) that is anchored to the membrane by a hydrophobic ubiquinone-reducing dimer (SdhCD). Succinate is oxidized to fumarate at the flavin adenine dinucleotide (FAD) active site in SdHA. Electrons from this oxidation are then shuttled through an ET relay comprising three [Fe-S] clusters in SdhB to the membrane anchor domain (SdhCD) where ubiquinone (UQ) is reduced to ubiquinol (UQH2). A b-type heme (b566) is sandwiched between SdhC and SdhD and has been proposed to play a role in the suppression of reactive oxygen species (ROS) produced during enzyme turnover (1). The reduction couple for the succinate → fumarate reaction is +15 mV and that for UQ → UQ2 is +100 mV. The cofactors of the Sdh ET relay have E_m values of −80 mV (FAD), +10 mV ([2Fe-2S], FS1), −175 mV ([4Fe-4S], FS2), +65 mV ([3Fe-4S], FS3) and +35 mV (heme b566) (11–13).

Control of [Fe-S] cluster E_m values by the protein environment has been studied extensively in bacterial ferredoxins (14–21), but only a handful of studies have been done on [Fe-S] clusters in respiratory chain enzymes such as the bc1 complex (9, 22) and yet even fewer studies on “simple” respiratory chain enzymes that utilize a single non-bifurcated electron pathway dehydrogenase; UQ/UQH2, ubiquinone/ubiquinol; WT, wild-type; MOPS, 4-morpholinepropanesulfonic acid.
(6, 7). Site-directed mutagenesis experiments that alter the cysteinylligands of [Fe-S]clusters have been performed on the E. coli fumarate reductase (23, 24), nitrate reductase (25), and dimethyl sulfoxide reductase (26). In most cases, alteration of the [Fe-S] cluster ligands resulted in enzymes that were not assembled or targeted correctly. These mutated enzymes often had undetectable enzyme activity; thus, changes in midpoint potential could not be consistently correlated with changes in catalysis.

In this study, emphasis was placed on hydrophobic residues around the [4Fe-4S]cluster (FS2) of Sdh. Using the recently published high resolution x-ray crystallography structure of Sdh (1), we generated site-directed mutants of SdhB-Ile-150 and SdhB-Leu-220 and determined their effects on $E_m$ values of all three [Fe-S] clusters and the resultant changes in enzyme activity and rates of heme reduction. Because Sdh has been shown to produce ROS (27), we also addressed whether changes in enzyme turnover are consistent with changes in ROS production. Based on results obtained in this study, we propose a novel role for [Fe-S] clusters in ET relays whereby their midpoint potentials dictate the direction of electron transfer.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—**E. coli strain DW35 (Δfrd-ABCD, sdhC::kan) (28) was used for all enzyme expression and growth studies. Expression of wild-type and mutant Sdh was anaerobically induced by using the plasmid pFAS, which encodes the sdh operon under the control of the fumarate reductase promoter (29). Laboratory strain TG1 (supE hsd $\Delta S$ thi $\Delta (lac-proAB)$ F’ [traD36 proAB+ lacIq lacZΔ15]); GE Healthcare) and plasmid pTZ18R (Ampr lacZ; GE Healthcare) were used for molecular cloning and mutagenesis.

**Cloning—**The 3.7-kb KpnI-Sphl fragment of pFAS was subcloned into pTZ18R, and this recombinant plasmid was used for site-directed mutagenesis. Mutagenic oligonucleotides were designed with the addition/deletion of restriction sites in the DNA sequence to facilitate the screening process and were purchased from Sigma. Mutants were generated using the QuikChange site-directed mutagenesis kit from Stratagene. DpnI was purchased from Invitrogen, and DNA purification kits were purchased from Qiagen. Mutants were verified by DNA sequencing (DNA core facility, Department of Biochemistry, University of Alberta), and the smaller 1.3-kb XhoI-Sphl fragment cloned back into the pFAS expression vector. The mutant plasmids were then transformed into DW35 for biochemical studies. Preparation of competent cells and transformations of plasmids into competent cells were carried out as described (30).

**Enzyme Expression and Preparation—**Wild-type and mutant SdhCDAB were expressed in DW35 cells. A 20-ml starter culture of Terrific Broth grown at 37 °C for 8 h was used to inoculate 2 liters of Terrific Broth in a 4-liter flask. The culture was grown at 37 °C for ~18 h with shaking (160 rpm). All media contained 100 μg ml$^{-1}$ ampicillin, 100 μg ml$^{-1}$ streptomycin, and 40 μg ml$^{-1}$ kanamycin. Cells were harvested by centrifugation followed by resuspension in 50 mM MOPS/5 mM EDTA buffer, pH 7.0. Phenylmethylsulfonyl fluoride was added to a final concentration of 0.2 mM, and cells were lysed using a French press. Membranes were prepared by differential centrifugation as previously described (7). Enriched membranes were prepared by centrifugation on a 55% sucrose cushion at 150,000 × g for 90 min and were diluted to 25 ml. Malonate was added to a final concentration of 1 mM, and membranes were incubated at 30 °C for 15 min to activate the Sdh enzyme. Two subsequent spins at 150,000 × g for 90 min were carried out to wash the membranes using 50 mM MOPS/5 mM EDTA/1 mM malonate buffer. The final membrane preparations were resuspended in 4 ml of the above buffer and flash frozen with liquid N$_2$. All steps were carried out at 4 °C unless otherwise specified.

**Growth Assays—**Anaerobic growth in minimal medium was carried out as described (31) using 50 mM fumarate as the terminal electron acceptor and a Klett-Summerson colorimeter equipped with a number 6 filter to monitor growth. Aerobic growth in succinate minimal medium was carried out as the anaerobic growth experiment with the following exceptions: final volume decreased to 25 ml, glycerol and fumarate were omitted, and succinate was added to a final concentration of 25 mM.

**SDS-PAGE—**Protein concentrations were estimated by the Lowry method (32) with the inclusion of 1% (w/v) sodium dodecyl sulfate in the mixture (33). 30 μg of protein was incubated at 100 °C for 5 min before separation by SDS-PAGE (30) and visualized by Coomassie Blue staining. Low molecular mass markers from Bio-Rad included phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

**Flavin Quantification—**Fluorometric quantification of the covalent flavin of Sdh was carried out as described (34), using 5 mg of protein as starting material.

**Redox Titration and EPR Spectroscopy—**Redox titrations were carried out anaerobically under argon at 25 °C on Sdh-enriched membranes at a total protein concentration of ~30 mg ml$^{-1}$ in 50 mM MOPS/5 mM EDTA, pH 7.0. The following redox mediators were used at a concentration of 25 μM: quinhydrone, 2,6-dichloroindophenol, 1,2-naphthoquinone, tolulylene blue, phenazine methosulfate, thionine, duroquinone, methylene blue, resorufin, indigotrisulfonate, indigodisulfonate, anthraquinone-2-sulfonic acid, phenosafranine, benzyl viologen, and methyl viologen. All samples were prepared in 3-mm internal diameter quartz EPR tubes and were rapidly frozen in liquid nitrogen-chilled ethanol before storing in liquid nitrogen. EPR spectra were recorded using a Bruker Elexys E500 EPR spectrometer equipped with an Oxford Instruments ESR900 flowing helium cryostat. $E_m$ values for FS1 and FS3 were determined at 12 K and 20 milliwatt while that of FS2 were determined at 40 K and 2 milliwatt, all at a frequency of 9.38 GHz. All spectra were corrected for internal EPR tube diameters. Presented data are representative of two independent potentiometric titrations for each Sdh mutant. Samples for spin quantitation measurements were prepared by oxidizing with 160 μM potassium ferricyanide or reducing with 8 mM sodium dithionite. Calculations were done using 1 mM copper-EDTA as standard.
Fe-S Cluster Midpoint Potentials and Electron Transfer

Enzyme Assays—Succinate-dependent reduction of MTT (ε = 17 mm⁻¹ cm⁻¹) was measured spectrophotometrically at 570 nm in the presence of 750 μM phenazine methosulfate (PMS) and 0.1% Triton X-100 (12). Anaerobic succinate-dependent reduction of Q₁₀ (ε = 0.73 mm⁻¹ cm⁻¹) was monitored at 410 nm; anaerobicity was achieved by saturating the assay buffer with N₂ and the addition of glucose (20 mM) and glucose oxidase (8.8 units ml⁻¹) to the reaction cuvette. Anaerobic oxidation of plumbagin (ε = 3.95 mm⁻¹ cm⁻¹) in the presence of fumarate was measured at 419 nm as described (35) in N₂-saturated buffer. Turnover numbers were calculated based on covalent flavin concentrations.

Heme Reduction Assays—Stopped flow experiments were carried out using a Sequential Bio SX-17MV stopped flow spectrophotometer (Applied Photophysics Ltd., Leatherhead, UK) with a 1-cm path length at 25 °C. Pre-steady state heme reduction was monitored by subtracting A₅₇₅ from A₅₆₀. Sdh-enriched membranes at a total concentration of 1 mg ml⁻¹ in N₂-saturated 100 mM MOPS/5 mM EDTA, pH 7.0, buffer were used. The assay was performed anaerobically in the presence of 50 mM KCN to eliminate overlapping heme absorbances from cytochromes b₅ and b₆. Heme reduction was achieved by addition of 10 mM succinate, and a time window of 10 s was monitored. Temporal changes in absorbance were averaged and fitted to the double exponential equation ΔAbs = A₁e⁻ᵏ₁t + A₂e⁻ᵏ₂t + b where A₁ and A₂ are amplitudes, k₁ and k₂ are rate constants, t is time, and b is the end point of the data trace.

ROS Assay—Generation of ROS was monitored at A₅₅₀ using the superoxide-dependent cytochrome c reduction assay (εFe²⁺-cytochrome c) — εFe³⁺-cytochrome c) = 21 mm⁻¹ cm⁻¹) as described (27, 36). Essentially, Sdh enzyme, cytochrome c, Q₁₀, KCN, superoxide dismutase, and succinate were added in the order listed to final concentrations of 1 μg ml⁻¹, 10 μM, 50 μM, 10 mM, 30 units ml⁻¹, and 10 mM, respectively.

RESULTS

Using the published Sdh structure (Protein Data Bank file 1NEN) (1), we elected to study two conserved hydrophobic residues located within 4 Å of the [4Fe-4S] cluster in SdhB, Ile-150 and Leu-220 (Fig. 1). These two residues were chosen based on their proximity to FS2 with the objective of examining the role of the low potential [4Fe-4S] cluster in Sdh. Three mutants, I₁₅₀E, I₁₅₀H, and L₂₂₀S, were generated using site-directed mutagenesis and were analyzed by EPR spectroscopy and enzymatic assays.

We first examined whether mutation of a hydrophobic residue to a charged or polar residue in the middle of the electron transfer subunit had an impact on the proper assembly and targeting of Sdh. Membrane preparations containing Sdh were analyzed by SDS-PAGE, and the covalent flavin content was determined. The Coomassie Blue-stained gel is depicted in Fig. 2 and shows that although the SdhB subunits of mutant enzymes run anomalously compared with the wild-type (WT), mutant enzymes are assembled and correctly targeted to the cytoplasmic membrane. Table 1 shows the flavin content determined for each membrane preparation, indicating virtually no difference between the amount of mutant and WT Sdh enzymes targeted to the cytoplasmic membrane. DW35 mem-
TABLE 1
Quantitation of cofactors and midpoint potentials (mV) of [Fe-S] clusters in Sdh

Membrane fractions containing over expressed wild-type or mutant SdhB were assayed for covalent flavin content as described under “Experimental Procedures.” Concentrations of FS1 and FS3 were determined by spin quantitation. \( E_m \) values were determined by redox titration in combination with EPR spectroscopy. The FS1 and FS3 signals were monitored at 12 K and 20 mW, while the signal for FS2 was followed at 40 K and 2 mW. EPR spectra were all obtained at a frequency of 9.38 GHz with a modulation amplitude of 10 Gpp at 100 KHz. The average and range of \( E_m \) values from two independent redox titrations are shown.

| Membranes | Quantitation of Sdh | Midpoint Potentials | Flavin | FS1 | FS3 | FS1 | FS2 | FS3 |
|-----------|---------------------|---------------------|--------|-----|-----|-----|-----|-----|
|           | \( \text{nmol/mg protein} \) | \( mV \) |        |     |     |     |     |     |
| WT        | 3.2                 | -15 \( \pm \) 15     | -213 \( \pm \) 3 | 55 \( \pm \) 15 |
| I150E     | 3.2                 | -33 \( \pm \) 13     | -285 \( \pm \) 10 | 55 \( \pm \) 10 |
| I150H     | 3.3                 | -30 \( \pm \) 5      | -338 \( \pm \) 17 | 35 \( \pm \) 0  |
| L220S     | 2.6                 | -18 \( \pm \) 8      | -223 \( \pm \) 3  | -20 \( \pm \) 10 |

FIGURE 3. Aerobic growth with succinate as electron donor (A) and anaerobic growth with fumarate as electron acceptor (B). Cells were grown as described under “Experimental Procedures.” Klett readings are plotted as a function of time for the DW35 strain ( ), and the DW35 strain harboring the wild-type Sdh ( ). The I150E mutant ( ), the I150H mutant ( ), and the L220S mutant ( )

branes were not included in the flavin assay as no covalently bound flavin was expected to be present. The concentrations of FS1 and FS3 as determined by spin quantitation are also shown in Table 1. The concentrations of FS2 are not shown due to the high intrinsic error in its determination (37).

Fig. 3A shows the ability of the SdhB mutants to grow aerobically on succinate minimal medium at 37 °C. Clearly, the SdhB mutants were able to complement E. coli DW35 and support growth on succinate, with neither the growth rate nor the maximum growth density affected. It is well documented that Sdh can function as a fumarate reductase under anaerobic conditions (29), and we tested the ability of the mutants to support anaerobic growth on fumarate. E. coli DW35 did not grow on glycerol-fumarate minimal medium (Fig. 3B) but grew well when complemented with the pFAS plasmid containing the WT Sdh. Both SdhB-Ile-150 mutants did not support growth on glycerol-fumarate minimal medium, whereas the SdhB-L220S mutant showed growth similar to DW35/pFAS.

Potentiometric titrations and EPR spectroscopy were performed on the WT and mutant Sdh enzymes to determine the \( E_m \) values of the [Fe-S] clusters in SdhB. \( E_m \) values of the FAD and heme were not determined as the mutations in SdhB were not expected to alter their redox behaviors. Individual spectra and redox titration data are shown in supplemental information. Table 1 lists the average \( E_m \) values determined for the WT and mutant enzymes in two independent experiments. Redox titration of the WT enzyme yielded \( E_m \) values of \(-15, -213, \) and \(+55 \text{ mV for FS1-FS3, respectively, which are in reasonable agreement with previously published values (11). In the SdhB-I150E mutant, the } E_m \text{ value of FS2 was decreased by } -70 \text{ mV to } -285 \text{ mV with no major changes in the } E_m \text{ values of FS1 and FS3. A similar case was observed with the SdhB-I150H mutant, where } E_m \text{ values of FS1 and FS3 were unchanged while an average } E_m \text{ value of } -338 \text{ mV was recorded for FS2, a decrease of } 125 \text{ mV compared with the WT enzyme. The SdhB-L220S mutant did not alter } E_m \text{ values of FS1 or FS2 but, surprisingly, did alter the } E_m \text{ value of FS3 by lowering it to } -20 \text{ mV.}

Steady state activity assays using the non-physiological acceptor PMS/MTT to measure the assembly and quantity of enzyme in the membrane showed that the Ile-150 mutants and the WT had comparable succinate:PMS/MTT activity and so were assembled to similar levels (Fig. 4). The L220S mutant showed a 50% decrease in PMS/MTT activity after taking into account the covalent flavin contents. Using the succinate:Q0 assay as a measure of physiological Sdh activity, all three mutants showed a decrease in activity. The turnover
rate decreased ~65% for the Ile-150 mutants and almost 80% for the L220S mutant. When plumbagin was used to measure the fumarate reductase activity of the mutant enzymes, we found the I150E mutant to have the smallest effect and the L220S mutant the largest decrease in activity.

The ability of the heme b to be reduced by succinate was measured using the stopped flow technique (Fig. 5). Activated enzyme was reduced by addition of succinate, and the A_{460} was monitored on a 10-s time window. Triplicate traces were averaged and fitted to a double exponential equation from which we found $k_1$ to be significantly decreased in the Ile-150 mutants while $k_2$ and $k_3$ were $0$ in the L220S mutant. $k_1$ was decreased from 6.44 s$^{-1}$ (WT enzyme) to 1.05 and 1.35 s$^{-1}$ in the I150E and I150H mutants, respectively. $A_1$ for the WT, I150E, and I150H enzymes were $-4.82 \times 10^{-3}$, $-5.12 \times 10^{-3}$, and $-3.09 \times 10^{-3}$, respectively. No changes in $K_2$ and $A_2$ were observed in the Ile-150 mutants.

Finally, we examined the ability of the SdhB mutants to produce ROS during enzyme turnover. Because it has been demonstrated that both Sdh and E. coli fumarate reductase can produce ROS, the former enzyme at the Q site and the latter enzyme at the FAD site (27, 38), we wondered whether a bottleneck in the electron transfer rate in Sdh affects ROS production. Also, we wanted to confirm whether the decrease in enzyme turnover in the SdhB mutants is indeed due to slower electron transfer rate or whether it is a side effect of short-circuiting the electron pathway by increased solvent exposure when the hydrophobic residues were replaced by hydrophilic amino acids. Fig. 6 shows that in all the SdhB mutants, ROS generation during physiological turnover was decreased compared with the WT enzyme, which correlates with the decreased rates of succinat-Q$_0$ turnover.

**DISCUSSION**

In this study, we examined the role of hydrophobic residues around the low potential [4Fe-4S] cluster of Sdh, as well as the intimate relationship between enzyme turnover, electron transfer rate, and cofactor midpoint potential. Extensive studies on bacterial ferredoxins have yielded insights into how the $E_m$ of [Fe-S] clusters are generally controlled by the surrounding amino acids and degree of solvent exposure (14–21). Specifically, it was found that in the *Azotobacter vinelandii* ferredoxin I protein, mutation of the Glu residues to His can increase $E_m$ values of nearby [Fe-S] clusters by 100–200 mV (21). We found that conversion of SdhB-Ile-150 to Glu lowered the $E_m$ value of FS2 from $-213$ to $-285$ mV; when the same residue was altered to Hist, the $E_m$ value of FS2 surprisingly showed an additional decrease in $E_m$ value to $-338$ mV. This is the opposite effect to that observed in the ferredoxin I protein by Chen *et al.* (21). We strongly believe that there is a functional relationship between Ile/Glu/His and a nearby [Fe-S] cluster as we have also observed similar decreases in $E_m$ values of a [Fe-S] cluster in *Me$_2$SO* reductase when a nearby Ile residue is mutated to Glu and His. An alternative explanation may be offered by residue SdhB-Cys-154, which has been proposed to H-bond to FS2 such that it raises the apparent $E_m$ value of the cluster in Sdh (6). In the x-ray crystallography structure (1), Cys-154 is a possible H-bond donor to one of the cysteine ligands of FS2, but is 7.5 Å from Ile-150. Glu and His mutants of Ile-150 may cause local perturbations in the structure such that it H-bonds to Cys-154, thus eliminating the C154-S, H-bond to FS2 and thereby decreasing the $E_m$ of the [4Fe-4S] cluster. H-bonds to [Fe-S] clusters have been shown to contribute between 50 and 200 mV to the overall $E_m$ of [Fe-S] clusters (9, 21, 39).

The observation that the L220S mutant has no significant effect on FS2 but instead decreased the $E_m$ value of FS3 is much harder to rationalize. In the published Sdh structure, the Leu-220 residue is close to FS3 but is also pointing away from FS3. It is possible that the L220S mutant induces some conformational change in the electron transfer subunit that affects the redox behavior of FS3, but not that of FS1 or FS2. An x-ray crystallography structure of the L220S mutant would be needed to completely understand why FS3 was unexpectedly affected in this mutant.

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*V. W. T. Cheng, R. A. Rothery, and J. H. Weiner, unpublished data.*
When we examined the enzymatic activities of the SdhB-Ile-150 mutants, it was found that both had decreased succinate:Q₀ and plumbagin:fumarate activities despite having similar succinate:PMS/MTT activities. In terms of FS2 Eₘ values, the WT enzyme had the highest midpoint potential, followed by I150E and I150H. This trend can be correlated to succinate:Q₀ and plumbagin:fumarate activities, where the WT enzyme has the highest turnover followed by the I150E and I150H mutants. Thus if we raise the energetic barrier against ET, we slow down the rate of electron flux or steady state turnover of the enzyme in both the forward and reverse directions. Thus far, we have been unable to raise the Eₘ value of FS2 to verify whether the converse is true. Although we have observed a direct relationship between Eₘ values of FS2 and turnover rates in Sdh, it is interesting to point out that an inverse relationship was recently observed between Eₘ values of FS2 and turnover in E. coli fumarate reductase (6). Increases in cofactor Eₘ values that result in increases in enzymatic activity have also been observed in the Rieske protein (9) and recently in the Blastochloris viridis reaction center (10).

The turnover rates observed in the L220S mutant were also decreased compared with the wild-type enzyme. We believe that the decreased activities are the result of altering the Eₘ value of FS3, which is adjacent to and may exchange electrons directly with the Q site. Experiments done on the Saccharomyces cerevisiae bc₂ complex, E. coli Me₂SO reductase, and E. coli fumarate reductase have shown that alteration of the Eₘ value of the [Fe-S] cluster adjacent to the Q site can cause major changes in enzyme activity (9, 26, 40).

The trend seen in the in vitro enzyme assays for the three SdhB mutants was not reflected in the aerobic or anaerobic growth in minimal medium. The mutant enzymes were able to complement E. coli DW35 for aerobic growth on succinate minimal medium, presumably because the overexpressed level of Sdh overcomes their decreased succinate:Q₀ activities (29). It is intriguing to note that, despite having the lowest plumbagin:fumarate turnover, the L220S mutant was able to support growth on glycerol-fumarate minimal medium whereas the Ile-150 mutants could not. We propose that this phenomenon arises from the energetic restrictions in electron transfer. In the WT enzyme, the ΔEₘ for the FS3 → FS2 transition is −268 mV. In the I150E and I150H mutants, this ΔEₘ value increases to −340 and −383 mV, respectively. Thus, the electrons have a higher energy barrier to surmount when the I150E and I150H mutants are functioning as a fumarate reductase and are apparently unable to do so in vivo. In the L220S mutant, however, the ΔEₘ for the FS3 → FS2 transition is now only −203 mV, less than the value calculated for the WT Sdh. We propose that the Eₘ values of the [Fe-S] clusters in Sdh are poised such that in vivo the forward reaction of succinate oxidation is favored compared with that of fumarate reduction reaction. Because the active sites of Sdh are already biased toward succinate oxidation and ubiquinone reduction (3, 13, 41), the [Fe-S] clusters exert an additional level of control over the directionality of electron transfer such that the succinate:UQ reaction is preferred in Sdh. This hypothesis can also be applied to the homologous E. coli fumarate reductase enzyme, wherein FS1-FS3 have Eₘ values of −35, −310, and −65mV, respectively (37). Thus, the ET relay in E. coli fumarate reductase would favor electron transfer from FS3 to FS1. The effects of Eₘ values in relation to electron transfer have also been previously examined in Me₂SO reductase (7).

In terms of ROS production, we did not see any significant change relative to the succinate:Q₀ activity in the SdhB mutants. The imposition of a higher energy barrier in the Ile-150 mutants slows down electron transfer, meaning that cofactors upstream of FS2 such as the FAD are more likely to be reduced. Because Sdh produces ROS at the Q site (27, 38), we might expect less ROS to be produced from the I150E and I150H mutants. Indeed, we saw decreases in ROS production in all three mutants that correlate with the decreases in physiological turnover rates. This suggests that ROS production only occurs during the UQ → UQH₂ reduction step, rather than a simple leakage of electrons to molecular oxygen from the ET chain.

In this study, the midpoint potentials of the [4Fe-4S] and [3Fe-4S] clusters of Sdh were individually lowered by point mutations of hydrophobic residues to charged and polar residues. Lowering the Eₘ values of these [Fe-S] clusters led to decreases in in vitro enzyme turnover rates. Based on results obtained in this study, we propose that the Eₘ values of the [Fe-S] clusters in Sdh act as a checkpoint such that the forward reaction of succinate oxidation is the preferred reaction over the fumarate reduction reaction.

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REFERENCES
1. Yankovskaya, V., Horsefield, R., Tornroth, S., Luna-Chavez, C., Miyoshi, H., Leger, C., Byrne, B., Cecchini, G., and IWata, S. (2003) Science 299, 700–704
2. Bertero, M. G., Rothery, R. A., Palak, M., Hou, C., Lim, D., Blasco, F., Weiner, J. H., and Strynadka, N. C. (2003) Nat. Struct. Biol. 10, 681–687
3. Cecchini, G., Schroder, I., Gunsalus, R. P., and Maklashina, E. (2002) Biochim. Biophys. Acta 1553, 140–157
4. Nicholls, D. G., and Ferguson, S. I. (2002) Bioenergetics Vol. 3, pp. 89–156, Academic Press, Boston
5. Page, C. C., Moser, C. C., Chen, X., and Dutton, P. L. (1999) Nature 402, 47–52
6. Hudson, J. M., Jeffron, K., Kotlyar, V., Sher, Y., Maklashina, E., Cecchini, G., and Armstrong, F. A. (2005) J. Am. Chem. Soc. 127, 6977–6989
7. Cheng, V. W., Rothery, R. A., Bertero, M. G., Strynadka, N. C., and Weiner, J. H. (2005) Biochemistry 44, 8068–8077
8. Jeuken, L. J., Jones, A. K., Chapman, S. K., Cecchini, G., and Armstrong, F. A. (2002) J. Am. Chem. Soc. 124, 5702–5713
9. Denke, E., Markowitz-Zahradnik, T., Hatzfeld, O. M., Snyder, C. H., Link, T. A., and Trumpower, B. L. (1998) J. Biol. Chem. 273, 9085–9093
10. Alric, J., Lavergne, I., Rappaport, F., Vermiglio, A., Matsuura, K., Shimada, K., and Nagashima, K. V. (2006) J. Am. Chem. Soc. 128, 4136–4145
11. Condon, C., Cammack, R., Patil, D. S., and Owen, P. (1985) J. Biol. Chem. 260, 9427–9434
12. Kita, K., Vibat, C. R., Meinhardt, S., Guest, J. R., and Gennis, R. B. (1989) J. Biol. Chem. 264, 2672–2677
13. Maklashina, E., Iverson, T. M., Sher, Y., Kotlyar, V., Andrelli, J., Mirza, O., Hudson, J. M., Armstrong, F. A., Rothery, R. A., Weiner, J. H., and Cecchini, G. (2006) J. Biol. Chem.
14. Adman, E., Watenpaugh, K. D., and Jensen, L. H. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 4854–4858
15. Langen, R., Jensen, G. M., Jacob, U., Stephens, P. J., and Warshel, A. (1992)...
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16. Rayment, I., Wesenberg, G., Meyer, T. E., Cusanovich, M. A., and Holden, H. M. (1992) J. Mol. Biol. 228, 672–686
17. Jensen, G. M., Warshel, A., and Stephens, P. J. (1994) Biochemistry 33, 10911–10924
18. Agarwal, A., Li, D., and Cowan, J. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9440–9444
19. Stephens, P. J., Jollie, D. R., and Warshel, A. (1996) Chem. Rev. 96, 2491–2514
20. Jang, S. B., Seefeldt, L. C., and Peters, J. W. (2000) Biochemistry 39, 641–648
21. Chen, K., Bonagura, C. A., Tilley, G. J., McEvoy, I. P., Jung, Y. S., Armstrong, F. A., Stout, C. D., and Burgess, B. K. (2002) Nat. Struct. Biol. 9, 188–192
22. Gray, K. A., Davidson, E., and Daldal, F. (1992) Biochemistry 31, 11864–11873
23. Manodori, A., Cecchini, G., Schroder, I., Gunsalus, R. P., Werth, M. T., and Johnson, M. K. (1992) Biochemistry 31, 2703–2712
24. Kowal, A. T., Werth, M. T., Manodori, A., Cecchini, G., Schroder, I., Gunsalus, R. P., and Johnson, M. K. (1995) Biochemistry 34, 12284–12293
25. Guigliarelli, B., Magalon, A., Asso, M., Bertrand, P., Frixon, C., Giordano, G., and Blasco, F. (1996) Biochemistry 35, 4828–4836
26. Rothery, R. A., and Weiner, J. H. (1991) Biochemistry 30, 8296–8305
27. Messner, K. R., and Imlay, J. A. (2002) J. Biol. Chem. 277, 42563–42571
28. Westenberg, D. J., Gunsalus, R. P., Ackrell, B. A., Sices, H., and Cecchini, G. (1993) J. Biol. Chem. 268, 815–822
29. Maklashina, E., Berthold, D. A., and Cecchini, G. (1998) J. Bacteriol. 180, 5989–5996
30. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: a Laboratory Manual, 3rd Ed., pp. 1.32–1.170, A8.40–A8.51, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31. Sambasivarao, D., and Weiner, J. H. (1991) J. Bacteriol. 173, 5935–5943
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
33. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) Anal. Biochem. 87, 206–210
34. Singer, T. P., and Edmondson, D. E. (1980) Methods Enzymol. 66, 253–264
35. Rothery, R. A., Chatterjee, L., Kiema, G., McDermott, M. T., and Weiner, J. H. (1998) Biochim. Biophys. Acta 1380, 545–551
36. Guo, J., and Lemire, B. D. (2003) J. Biol. Chem. 278, 47629–47635
37. Leggate, E. J., and Hirst, J. (2005) Biochemistry 44, 7048–7058
38. Rothery, R. A., Seime, A. M., Spiers, A. M., Maklashina, E., Schroder, I., Gunsalus, R. P., Cecchini, G., and Weiner, J. H. (2005) FEBS J. 272, 313–326
39. Cecchini, G., Maklashina, E., Yankovskaya, V., Iverson, T. M., and Iwata, S. (2003) FEBS Lett. 545, 31–38