The Quinone-binding Site in Succinate-ubiquinone Reductase from Escherichia coli

QUINONE-BINDING DOMAIN AND AMINO ACID RESIDUES INVOLVED IN QUINONE BINDING

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When purified ubiquinone (Q)-depleted succinate-ubiquinone reductase from Escherichia coli is photoaffinity-labeled with 3-azido-2-methyl-5-methoxy-[3H]6-geranyl-1,4-benzoquinone ([3H]azido-Q) followed by SDS-polycrylamide gel electrophoresis, radioactivity is found in the SdhC subunit, indicating that this subunit is responsible for ubiquinone binding. An [3H]azido-Q-linked peptide, with a retention time of 61.7 min, is obtained by high performance liquid chromatography of the protease K digest of [3H]azido-Q-labeled SdhC obtained from preparative SDS-polycrylamide gel electrophoresis on labeled reductase. The partial N-terminal amino acid sequence of this peptide is NH2-TIRFPI-TAIASTLHRVS-, corresponding to residues 17–33. The final amino acid sequence of this peptide is NH2-TIRFPI-TAIASTLHRVS-. The putative ubiquinone-binding region of SdhC were generated and characterized. The putative ubiquinone-binding domain in the proposed structural model of SdhC, constructed based on the hydrophathy plot of the deduced amino acid sequence of this protein, is located at the N-terminal end toward the transmembrane helix I. To identify amino acid residues responsible for ubiquinone binding, substitution mutations at the putative ubiquinone-binding region of SdhC were generated and characterized. E. coli NM256 lacking genomic succinate-Q reductase genes was constructed and used to harbor the mutated succinate-Q reductase genes in a low copy number pRK418 plasmid. Substitution of serine 27 of SdhC with alanine, cysteine, or threonine or substitution of arginine 31 with alanine, cysteine, or histidine yields cells unable to grow aerobically in LB medium. These results indicate that the hydroxyl group, the size of the amino acid side chain at position 27, and the guanidino group at position 31 of SdhC are critical for succinate-Q reductase activity and [3H]azido-Q uptake are detected in succinate-ubiquinone reductases prepared from these mutant cells grown aerobically in LB medium. These results indicate that the hydroxyl group, the size of the amino acid side chain at position 27, and the guanidino group at position 31 of SdhC are critical for succinate-ubiquinone reductase activity, perhaps by formation of hydrogen bonds with carbonyl groups of the 1,4-benzoquinone ring of the quinone molecule. The hydroxyl group, but not the size of the amino acid side chain, at position 33 of SdhC is also important, because Ser-33 can be substituted with threonine but not with alanine.

Escherichia coli succinate-ubiquinone (Q)1 reductase, which catalyzes electron transfer from succinate to ubiquinone during aerobic respiration, has been purified and characterized (1). The purified complex contains four protein subunits with apparent molecular masses of 64,000, 28,000, 19,500, and 17,500 Da (2) and has five redox prosthetic groups: one covalently bound FAD, three iron-sulfur clusters ([2Fe-2S], [4Fe-4S], and [3Fe-4S]), and one protoheme IX, b556. The two larger subunits (SdhA and SdhB) are succinate dehydrogenase. The 64-kDa subunit (Fp) houses FAD, and the 28-kDa subunit (Ip) houses the three iron-sulfur clusters. The two smaller subunits (SdhC and SdhD) are membrane-anchoring proteins that house cytochrome b556. The ligands for cytochrome b556 are histidines (3). Gene deletion studies have shown that both SdhC and SdhD are involved in heme ligation (4). Site-directed mutagenesis has identified His-84 of SdhC and His-71 of SdhD as ligands for cytochrome b556 (5).

The genes for E. coli succinate-Q reductase subunits are located in one operon (sdh), which is transcribed in the following sequence: sdhC, sdhD, sdhA (Fp), sdhB (Ip) (6, 7). This operon has been cloned and sequenced (6, 7). The amino acid sequences of Fp and Ip are highly conserved among species (8). For example, the Ip subunits from E. coli and from bovine heart mitochondria are approximately 50% identical, and the amino acid sequences of Fp and Ip from E. coli show 50% similarity to their counterparts in human liver succinate-Q reductase. The Fp and Ip subunits of E. coli succinate-Q reductase are also closely related to those of bacterial and mitochondrial fumarate reductases that catalyze the reverse reaction of succinate-Q reductase. On the other hand, the amino acid sequences of the membrane-anchoring subunits of succinate-Q reductase differ significantly among species (9).

Recently, the E. coli succinate-Q reductase has been resolved into two reconstitutively active fractions: succinate dehydrogenase and the membrane-anchoring fraction (SdhC-SdhD) (10). Isolated succinate dehydrogenase is soluble and can catalyze electron transfer from succinate to artificial acceptors, such as phenazinemethosulfate, but not to its physiological acceptor, Q. The addition of the membrane-anchoring fraction to isolated succinate dehydrogenase forms membrane-bound succinate-Q reductase, which catalyzes electron transfer from succinate to Q (10), indicating that SdhC-SdhD provides membrane docking for succinate dehydrogenase and Q binding in succinate-Q reductase. In fact, the membrane-anchoring protein fraction is named QPs (Q-binding protein in succinate-Q reductase).
ubiquinone reductase) in the mitochondrial system (11). The involvement of the membrane-anchoring fraction in the Q binding of \textit{E. coli} succinate-Q reductase is shown by detection of ubiquinonine radicals in intact and reconstituted succinate-Q reductases in the presence of exogenous Q. No ubiquinonine radical is detected with succinate dehydrogenase in the presence of Q 2.

To understand the protein-Q interaction and the mechanism of Q reduction in succinate-Q reductase requires knowledge of the Q-binding site. Photoaffinity labeling of beef heart mitochondrial succinate-Q reductase with \textsuperscript{3}H[Azido-Q] derivative identified all three QPs subunits as putative Q-binding proteins in this reductase. The Q-binding domain in QPs\textsubscript{1} is located at the loop connecting transmembrane helices II and III, which extrudes from the surface of the M-side of the mitochondrial inner membrane (12). The Q-binding domain in QPs\textsubscript{3} is located at the end of transmembrane helix I toward the C-side of the membrane (13). The finding that the Q-binding domains in QPs\textsubscript{3} and QPs\textsubscript{1} are on opposite sides of the membrane is in line with a two-Q-binding site hypothesis formulated from the inhibitor studies of this enzyme complex (14). The Q-binding domain(s) in other succinate-Q reductases has not been identified.

The amino acid residues in the putative Q-binding domain of QPs\textsubscript{1} and QPs\textsubscript{3}, responsible for Q binding, have not been identified. The photoaffinity labeling technique does not permit us to identify specifically the amino acid residue(s) directly involved in Q binding because of the rather long life and some-what nonspecific nature of the activated nitrene radical. The molecular genetic approach to identify amino acid residues involved in Q binding of bovine heart mitochondrial succinate-Q reductase requires site-directed mutagenesis of the QPs\textsubscript{1} or QPs\textsubscript{3} gene, expression and isolation of recombinant mutant protein, and reconstitution of recombinant mutant QPs\textsubscript{1} or QPs\textsubscript{3} with a QPs\textsubscript{1}- or QPs\textsubscript{3}-deficient succinate-Q reductase. Although bovine heart mitochondrial QPs\textsubscript{1} and QPs\textsubscript{3} have been cloned and overexpressed in \textit{E. coli} as glutathione S-transferase fusion proteins (15, 16), the functional activity of the recombinant protein cannot be assessed because of the unavailability of reconstitutively active, QPs\textsubscript{1}- and/or QPs\textsubscript{3}-depleted succinate-Q reductase. Since \textit{E. coli} succinate-Q reductase is structurally and functionally similar to the mitochondrial enzyme and is readily manipulable genetically, it provides an ideal system for the study of Q-binding site using photoaffinity labeling techniques in concert with site-directed mutagenesis. Herein, we report the identification of the Q-binding domain in \textit{E. coli} succinate-Q reductase by photoaffinity labeling, the deletion of most of the sdh gene, and the construction of QPs\textsubscript{1} or QPs\textsubscript{3} with a QPs\textsubscript{1}- or QPs\textsubscript{3}-deficient succinate-Q reductase. The resulting plasmid, SQR::Kn/pSELECT-1 was transformed into \textit{E. coli} chromosome by site-specific recombination, and the identification of amino acid residues involved in Q binding by site-directed mutagenesis.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Materials—}Dichlorophenolindophenol (DCPIP), polyoxyethylene-9-lauryl ether (E9C12), and FMN were obtained from Sigma. DEAE-Sephrose CL-6B was from Amersham Pharmacia Biotech. Other chemicals were of the highest purity commercially available.

\textit{pKRD418}, which was used for the expression of wild type and mutated \textit{sdh} genes, and pSL1180KnERV, which was used to provide the kanamycin-resistant (Kn\textsubscript{R}) gene, were constructed in our laboratory (17). Restriction endonucleases and DNA-modifying enzymes were purchased from Promega, Life Technologies, Inc., New England Biolabs, and Amersham Pharmacia Biotech. \textit{E. coli} strain JC7623 (recB21 recC22 sbcB15 sbcC201) and pgaeP1clr100 were generously provided by Dr. R. Eisenberg of Oklahoma State University. \textit{E. coli} strain NM256 (rec\textsuperscript{A}) was generously provided by Dr. J. R. Guest (Sheffield University, United Kingdom).

Ubiquinone derivatives 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q\textsubscript{4}), 3-azido-2-methyl-5-methoxy-\textsuperscript{3}H[14]-geranyl-1,4-benzoquinone (\textsuperscript{3}H[Azido-Q]), 5-azido-2,3-dimethoxy-\textsuperscript{3}H[14]-geranyl-1,4-benzoquinone (\textsuperscript{3}H[5-Azido-Q]), were synthesized in our laboratory as previously reported (18).

\textbf{Growth of Bacteria—}\textit{E. coli} cells were grown at 37 °C in LB medium or M9 minimal medium containing 20 mM succinate and required amino acids with vigorous shaking. Cell growth was monitored by measuring the increase of optical density at 660 nm. Where appropriate, ampicillin (100–125 \textmu g/ml), tetracycline (10–15 \textmu g/ml), kanamycin sulfate (30–50 \textmu g/ml), or trimethoprim (85–100 \textmu g/ml) was used for the selection of the mutants of \textit{E. coli}. Extra rich medium, TYP, was used in the procedures for rescue of single-stranded DNA (19).

\textbf{Construction of Mutations and Expression of Mutated Succinate-Q Reductase in \textit{E. coli}—}Mutations were constructed by site-directed mutagenesis using the Altered Sites System from Promega (20). Mutagenic oligonucleotides were used as follows: CTGCACTTACGCCATCCGTTCCC (T17A), CATACGACATTGCTTCCCATACCGCGG (R19A), CAGACCATCGGCTTCCCATACGGCGG (T23A), ACCGGGTAGAAGCGTCATTTCC (S27A), ACCGGGTAAGCCTGTTCCCATACGGCGG (R31A), TCCATTCTCCATCGTGCATTCT (S33C), TCCATTCTCCATCGTGCATTCT (S33C), TCCATTCTCCATCGTGCATTCT (S33C), TCCATTCTCCATCGTGCATTCT (S33C). The resulting plasmid (SQR::KnR) was used as template DNA for mutagenesis.

\textbf{Follow-up mutagenesis,} a 4.5-kg \textit{BamHI} fragment containing \textit{sdh} operon was inserted into the \textit{BamHI} site of pSELECT-1, which was used as template DNA for mutagenesis.

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ability to catalyze Q-stimulated DCPIP reduction by succinate. The assay was performed at room temperature in a Shimadzu UV2101PC spectrophotometer. An appropriate amount of enzyme was added to an assay mixture (1 ml) containing 50 mM sodium/potassium phosphate, pH 7.0, 50 μM DCPIP, 20 mM succinate, 1 mM EDTA, 0.01% Triton X-100, and 25 μM of Qb. For determination of apparent Ks, for Qb, various concentrations of Qb were used. The reduction of DCPIP was followed by measuring the absorbance decrease at 600 nm, using a millimolar extinction coefficient of 21 nm⁻¹ cm⁻¹.

Isolation of [³H]Azido-Q-labeled SdhC—8 ml of succinate-Q reductase, 2 mg/ml (10.6 μM cytochrome b₅₅₆)_c in 50 mM sodium/potassium phosphate buffer, pH 7.5, containing 0.1% E9C12 was incubated with 424 pmol of [³H]Azido-Q (5-fold molar excess to cytochrome b₅₅₆ at 0 °C for 10 min in the dark. The [³H]Azido-Q was added in 100 μl of ethanol. The specific radioactivity of azido-Q used was 1.6 × 10⁶ cpm/nmol in 95% ethanol and 6.6 × 10⁵ cpm/nmol in 50 mM sodium/potassium phosphate buffer, pH 7.5, containing 0.1% E9C12 in the presence of succinate-Q reductase. This mixture was transferred to an illuminating apparatus made from two quartz glasses sandwiched by a Teflon ring, which was immersed in ice in a Petri dish to maintain the temperature at 0 °C. The sample was illuminated with long wavelength UV light for 7 min at a distance of 5 cm from the light source.

To determine the amount of [³H]azido-Q incorporated into succinate-Q reductase, the illuminated [³H]azido-Q-treated samples were spotted onto a 3 mm paper and developed with a mixture of chloroform and methanol (2:1, v/v) to remove non-protein-bound [³H]Azido-Q. After the paper was air-dried, the origin spot was cut into small pieces and subjected to liquid scintillation counting.

The illuminated [³H]Azido-Q-treated succinate-Q reductase was precipitated by 50% ammonium sulfate saturation and centrifuged at 12,000 × g for 20 min. The precipitate was dissolved in 30 mM Tris-Cl buffer, pH 7.5, and dialyzed against double-distilled water, overnight, with one change of water. The dialyzed sample was subjected to organic solvent extraction as described previously (12), to remove the free azido-Q and detergent-azido-Q adducts, from the protein. The organic solvent remaining in the aqueous layer was evaporated under a stream of nitrogen gas before the solution containing the photolyzed protein was subjected to lyophilization. The lyophilized sample was dissolved in 20 mM Tris-Cl, pH 7.0, containing 1% SDS and 1% β-mercaptoethanol, and the solution was incubated at 37 °C for 2 h before being subjected to preparative SDS-PAGE. The SDS-PAGE gel was prepared according to Schagger and Jagow (24) except that 7% urea was used in the separating gel instead of 13% glycerol. For the reference wells, the digested sample with an electroeluter from Bio-Rad. The gel was stained with Coomassie Blue. The SdhC protein band was excised from the SDS-PAGE gel, and the protein was eluted with an electroduster from Bio-Rad.

Protease K Digestion of [³H]Azido-Q-labeled SdhC—Purified [³H]Azido-Q-labeled SdhC obtained by electroelution was subjected to a repeated dilution and concentration process using Centricon-10 with dilution buffer of 30 mM Tris-Cl, pH 7.5. The final protein concentration was about 2 mg/ml, with the SDS concentration around 0.5%. The protein was then digested with protease K at 37 °C for 6 h using a protease K:SdhC ratio of 1.50 (w/w).

Isolation of Ubiquinone-binding Peptides—100-μl aliquots of the protease K-digested SdhC were separated by high-performance liquid chromatography (HPLC) on a Synchron RPC-8 column (0.46 × 25 cm) using a gradient formed from 0.1% trifluoroacetic acid and 90% acetone containing 0.1% trifluoroacetic acid with a flow rate of 0.8 ml/min. 0.8-ml fractions were collected. The absorbance at 214 nm and the radioactivity of each fraction were measured. Peaks with high specific radioactivity were collected, dried, and subjected to peptide sequence analysis.

Other Biochemical Methods—The FAD contents in wild-type and mutant E. coli succinate-Q reductases were determined fluorometrically as previously reported (25). The content of cytochrome b₅₅₆ was determined from the reduced minus oxidized spectrum using the millimolar extinction coefficient of 22.8 cm⁻¹ nm⁻¹ for the wavelength pair 555-575 nm as described previously (2). Absorption spectra were recorded with a Shimadzu spectrophotometer, model UV2101PC.

RESULTS AND DISCUSSION

Identification of Q-binding Subunit in Succinate-Q Reductase by Photoaffinity Labeling with [³H]Azido-Q Derivatives—

Study of the Q-protein interactions in succinate-Q reductase, using synthetic Q derivatives, requires the prior removal of endogenous Q from the complex because the binding affinity of synthetic Q derivatives is weaker than that of endogenous Q. Since purified E. coli succinate-Q reductase contains less than 5% of endogenous Q₁₀ with respect to FAD (10) and is fully active upon the addition of exogenous Q, it is an ideal system for studying Q-protein interactions and for identifying the Q-binding site with azido-Q derivatives. When purified succinate-Q reductase was incubated with a 5-fold molar excess of [³H]Azido-Q or [³H]β-azido-Q for 10 min at 0 °C in the dark and then illuminated with long wavelength UV light for 7 min, only the [³H]Azido-Q-treated sample showed inactivation, indicating that [³H]Azido-Q is suitable for studying the Q-protein interactions in this reductase. Fig. 1 shows that when succinate-Q reductase was incubated with various concentrations of [³H]Azido-Q and illuminated, the activity decreased as the concentration of azido-Q was increased. Maximum inactivation of 35% was obtained when 5 μM of [³H]Azido-Q/mmol of cytochrome b₅₅₆ was used. Inactivation was not due to the inhibition of succinate-Q reductase by photolyzed products of azido-Q, because when azido-Q was photolyzed in the absence of reductase and then mixed with succinate-Q reductase, no inhibition was observed. Inactivation was also not due to damage of reductase protein by UV, because when the enzyme alone was illuminated, no activity loss was observed. These findings together with the finding that the amount of azido-Q uptake by succinate-Q reductase correlated with the extent of the enzymatic inactivation (about 0.35 mol of azido-Q uptake/mol of cytochrome b₅₅₆ at 35% inactivation) indicate that inactivation results from the binding of azido-Q to the Q-binding site. Since purified E. coli succinate-Q reductase contains less than 5% of endogenous Q, we were surprised to see a maximum uptake of 0.35 mol of azido-Q/mol of cytochrome b₅₅₆. This low azido-Q uptake may result from some Q-binding sites in the enzyme complex being partially masked by detergent E9C12 or from the azido-Q binding at one of the two putative Q-binding sites in the reductase (14).

Fig. 2 shows that in illuminated [³H]Azido-Q-treated succinate-Q reductase, radioactivity was found only in the SdhC subunit, suggesting that this subunit provides the Q-binding site. The lack of azido-Q labeling in the SdhD subunit is in
SdhC is about 2 mg/ml. Isolated [3H]azido-Q-labeled SdhC about 0.5%, while the concentration of [3H]azido-Q-labeled adducts. The SDS concentration in the final purification step is Two steps in the isolation procedure, organic solvent extraction, PAGE, electrophoretic elution, and membrane concentration.

organic solvent extraction, lyophilization, preparative SDS-dure involving ammonium sulfate fractionation, dialysis, protein.

band, from SDS-PAGE gel, to avoid contamination with SdhD yield of SdhC is probably due to very thin slicing of the SdhC contains 1 mol of SdhC/mol of reductase. This relatively low recovered in the final purification step, assuming that the

40% of the SdhC protein present in succinate-Q reductase was labeled SdhC be free from contamination with unbound [3H]azido-Q, and completely susceptible to proteolytic enzyme di-
labeled peptide, it is absolutely necessary that the isolated azido-Q-linked SdhC through isolation and sequencing of an azido-Q-linked peptide, it is absolutely necessary that the isolated azido-Q-labeled SdhC be free from contamination with unbound azido-Q and completely susceptible to proteolytic enzyme di-
gestion. [3H]Azido-Q-labeled SdhC was isolated from illumi-
nated, [3H]azido-Q-treated succinate-Q reductase by a procedure involving ammonium sulfate fractionation, dialysis, organic solvent extraction, lyophilization, preparative SDS-PAGE, electrophoretic elution, and membrane concentration. Two steps in the isolation procedure, organic solvent extraction and SDS-PAGE, are used to remove non-protein-bound azido-Q adducts. The SDS concentration in the final purification step is about 0.5%, while the concentration of [3H]azido-Q-labeled SdhC is about 2 mg/ml. Isolated [3H]azido-Q-labeled SdhC shows only one band, in SDS-PAGE, which corresponds to the third subunit of succinate-Q reductase (data not shown). About 40% of the SdhC protein present in succinate-Q reductase was recovered in the final purification step, assuming that the molecular mass of succinate-Q reductase is 117 kDa and that it contains 1 mol of SdhC/mol of reductase. This relatively low yield of SdhC is probably due to very thin slicing of the SdhC band, from SDS-PAGE gel, to avoid contamination with SdhD protein.

When the SDS present in the purified [3H]azido-Q-labeled SdhC was removed by cold acetone precipitation, the resulting protein was highly aggregated and resistant to proteolytic en-
zyme digestion. Inclusion of 0.1% SDS and 2 M urea in the digestion mixture does not increase proteolysis. Modification of SDS-free, [3H]azido-Q-labeled SdhC by reductive carboxymethylation followed by succinylation also does not render the protein susceptible to chymotrypsin or trypsin digestion. This is different from the mitochondrial system in which reductive carboxymethylation and succinylation effectively rendered pur-
fied, SDS-free, [3H]azido-Q-labeled QPs1 and QPs3 susceptible to chymotrypsin or trypsin digestion.

Since the SDS-free [3H]azido-Q-labeled SdhC, with or with-
out prior chemical modification, cannot be completely digested by proteolytic enzymes, an alternative way is to digest isolated [3H]azido-Q-labeled SdhC with a protease that is active even when SDS concentration is higher than 0.5%. Of the commer-
cially available proteolytic enzymes, only protease K was re-
ported to be active in 0.5% SDS and 1 M urea. Therefore, isolated [3H]azido-Q-labeled SdhC was subjected to protease K digestion at 37 °C using a protease K:SdhC ratio of 1:50 (w/w). To obtain the optimal digestion time, 100-μl aliquots were withdrawn from the digestion mixture at different time intervals, subjected to HPLC separation, and analyzed for peptide pattern and radioactivity recovery on HPLC chromatograms. A 6-h digestion time was found to be optimal. Fig. 3 shows the [3H] radioactivity distribution on HPLC chromatogram of [3H]azido-

Q-labeled SdhC digested with protease K for 6 h at 37 °C. Most of the radioactivity was found in a fraction with a retention time of 61.7 min (P-61.7). The radioactivity recovery is about 65% based on the radioactivity applied to the HPLC column.

The partial NH₂-terminal amino acid sequence of P-61.7 was found to be TIRFPITAIASILHRVS-, corresponding to amino acid residues 17–33 of SdhC. Judging from the gradually de-
creased amino acid recoveries in sequencing chromatograms, this peptide probably ends at residue 36. The Q-binding do-
main, using the proposed structure of SdhC (see Fig. 4), is located before the start of transmembrane helix I, toward the cytoplasmic side of the membrane. The proposed structural model of SdhC, shown in Fig. 4, is a modification of the one from Hagerhäll et al. (8) with Arg-31 remaining in the cy-
plasm instead of being in the transmembrane helix I. This modification is based on thermodynamic considerations. In this Q-binding region are several highly conserved amino acid res-
dues for type C anchors of succinate-Q reductase (8), such as Ser-27, His-30, Arg-31, and Ser-33. It should be noted that the
Q-binding domain identified in *E. coli* SdhC is different from that identified in bovine heart mitochondrial QPs₁ (12).

**Characterization of the *E. coli* Strain Lacking Succinate-Q Reductase**—After localization of the Q-binding domain in SdhC, it is necessary to identify the amino acid residues responsible for Q binding in this region in order to fully understand the Q-binding site. Since the *sdh* operon has been cloned, sequenced (6, 7), and overexpressed in *E. coli* NM256 strain (2), an effective and unambiguous way to attack this problem is by site-directed mutagenesis of the *sdhC* gene in the *sdh* operon followed by complementation of the mutated operon to an *E. coli* strain lacking succinate-Q reductase (ΔSQR).

Although there are a number of chromosomal *sdh* mutants available, all of them were constructed by insertion or point mutation to inactivate the *sdh* genes; in order to avoid possible recombinations, we chose to construct ΔSQR by genomic replacement of most of the *sdh* operon with a Kn⁴ gene in *E. coli* NM256. The replacement of *sdh* operon with the Kn⁴ gene in ΔSQR cells was confirmed by polymerase chain reaction, medium selection, and gene complementation. When genomic DNA was extracted from ΔSQR and used as template for polymerase chain reaction amplification using a forward primer, *AAAATCTCCTTTGTTATTACTG-3′*, and a reverse primer, *AATTCTCTTGGATTACTCATCTG-3′*, which is located at the end of the *sdhC* gene and a reverse primer, *AATTCTCT-GACTGGCAATTTCAGA-3′*, which is located at the beginning of *sdhA*, no polymerase chain reaction product was obtained. However, when genomic DNA prepared from the wild-type cell was used as template, a 420-bp polymerase chain reaction product was obtained, confirming that most of the *sdh* genes are absent from the chromosome of ΔSQR cells.

ΔSQR cells are unable to support aerobic growth on M₉ medium, supplemented with essential amino acids, using succinate as a carbon source (M₉/succinate medium). To confirm that the failure of ΔSQR to grow aerobically in M₉/succinate medium is due to deletion of *sdh* operon, the intact *sdh* operon contained in a 4.5-kb BamHI fragment on a broad range, low copy number plasmid pRK418 was transformed into the cell. The resulting strain (complement) grew aerobically in M₉/succinate medium at a rate similar to that of a wild-type strain harboring the pRK418. pRK418 plasmid carries tetracycline- and trimethoprim-resistant genes (17) while ΔSQR carries kanamycin-resistant gene. Thus transformants are selected for trimethoprim-, tetracycline-, and kanamycin-resistant phenotypes. It should be mentioned that pGS133 plasmid (2), a low copy number plasmid that was used previously to overexpress succinate-Q reductase in *E. coli* NM256, is not suitable for use in complementation of *sdh* operon in ΔSQR cells because the plasmid carries the same antibiotic-resistant gene, the Kn⁴ gene, as that in the chromosome of ΔSQR cell and thus makes selection of transformants difficult. The yield and activity of succinate-Q reductase obtained from ΔSQR harboring cloned *sdh* operon in pRK418 are comparable with those of succinate-Q reductase obtained from *E. coli* NM256 containing the *sdh* operon in pGS133.

Although ΔSQR cells cannot grow aerobically in M₉/succinate medium, they are able to support aerobic growth in LB medium at a rate slower than that of wild-type cells. No succinate-Q reductase activity was detected in membrane preparations obtained from ΔSQR cells grown aerobically in LB medium, indicating that *E. coli* can rely on various dehydrogenases to support aerobic growth.

**Identification of Amino Acid Residues Involved in Q Binding of SdhC**—The amino acid residues in the putative Q-binding domain of SdhC essential for Q binding were investigated by using site-directed mutagenesis coupled with biochemical and biophysical characterizations of succinate-Q reductase obtained from ΔSQR cells carrying cloned *sdh* operon with mutations in the *sdhC* gene in pRK418. Three-dimensional structural information and site-directed mutagenesis studies (26, 27) of the Q-binding sites in photosynthetic bacterial reaction centers have revealed two categories of amino acid residues involved in Q binding. One category is those having the ability to form hydrogen bonds with carbonyl groups of the 1.4-benzoquinone ring of quinone, such as His-M217 and Ala-M258 (where M represents M subunit) of *Rhodopseudomononas viridis* and Thr-M220 and Ala-M258 of *Rhodobacter sphaeroides* for the Qₐ site, and His-L190 and Ser-L223 (where L represents L subunit) of *R. viridis* for the Qₐ site. The other category is amino acids having the ability to stabilize Q binding through π-π interactions with the benzoquinone ring, such as Phe-L216 of *R. viridis* (27).

Assuming that the Q-binding site in SdhC of *E. coli* succinate-Q reductase resembles the one in the photosynthetic bacterial reaction center, Thr-17, Thr-23, Ser-27, and Ser-33 (through hydroxyl groups), His-30 (imidazole group), and Arg-19 and Arg-31 (guanidine group) may form hydrogen bonds.
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with the carbonyl groups of the 1,4-benzoquinone ring. Therefore, these amino acid residues were selected for mutagenesis. Serine, threonine, histidine, and arginine residues were mutated to alanine, in which the methyl group is not capable of forming hydrogen bonds with the carbonyl group of Q.

Table I summarizes the succinate-supported growth behavior of cells expressing the wild-type succinate-Q reductase (complement cells) and the T17A, R19A, F20L, T23A, S27A, S27C, S27T, H30A, R31A, R31K, R31H, S33A, S33C, and S33T-substituted succinate-Q reductases as well as the electron transfer activities and apparent \( K_m \) values for Q2 of purified succinate-Q reductases derived from these recombinant strains. Replacing Thr-17, Arg-19, or His-30 with alanine, or replacing Phe-20 with leucine yields cells capable of aerobic growth in M9/succinate medium at a rate similar to that of the complement cells. Since the electron transfer activities, the apparent \( K_m \) values for Q2, and the amount of \([\text{3H}]\)azido-Q incorporation into the SdhC (see Fig. 5) of T17A-, R19A-, H30A-, and F20L-substituted succinate-Q reductases are comparable with those of the complement reductase, Thr-17, Arg-19, His-30, and Phe-20 of SdhC are not involved in the Q binding.

Replacing Thr-23 with alanine yields cells capable of aerobic growth on M9/succinate medium at a rate slightly slower than that of complement strain. The specific activity of T23A-substituted succinate-Q reductase obtained from their respective mutant cells grown aerobically in LB medium has little succinate-Q reductase activity. The involvement of Ser-27 and Ser-33, and Arg-31 of SdhC are conserved residues in the Q-binding site (Fig. 5). These results indicate that both the hydroxyl group and the size of the amino acid side chain, at position 27 of SdhC, are critical for Q binding. Since the R31K or R31H substitution also yields cells unable to grow aerobically in the succinate/M9 medium and abolishes succinate-Q reductase activity and \([\text{3H}]\)azido-Q uptake in succinate-Q reductase (Fig. 5) from cells grown in LB medium, the guanidino group, rather than its positive charge, at position 31 of SdhC, is critical for succinate-Q reductase activity. The guanidino group of arginine occupies a much larger space than the primary amine of lysine, extends a longer distance, and may provide more chance for hydrogen bond formation (five hydrogen atoms versus two in lysine). Moreover, it may stabilize Q binding through π-π interactions between the guanidino group and the benzoquinone ring.

The structural importance of Ser-27 and Arg-31 in Q binding was further examined by replacing Ser-27 with cysteine and threonine and Arg-31 with lysine and histidine. Similar to S27A substitution, S27C or S27T substitution results in cells unable to support aerobic growth in succinate/M9 medium. Succinate-Q reductases prepared from these two mutant cells grown aerobically in LB medium have little succinate-Q reductase activity and no \([\text{3H}]\)azido-Q incorporation (Fig. 5). These results indicate that both the hydroxyl group and the size of the amino acid side chain, at position 27 of SdhC, are critical for Q binding. Since the R31K or R31H substitution also yields cells unable to grow aerobically in the succinate/M9 medium and abolishes succinate-Q reductase activity and \([\text{3H}]\)azido-Q uptake in succinate-Q reductase (Fig. 5) from cells grown in LB medium, the guanidino group, rather than its positive charge, at position 31 of SdhC, is critical for succinate-Q reductase activity. The guanidino group of arginine occupies a much larger space than the primary amine of lysine, extends a longer distance, and may provide more chance for hydrogen bond formation (five hydrogen atoms versus two in lysine). Moreover, it may stabilize Q binding through π-π interactions between the guanidino group and the benzoquinone ring.

It should be emphasized that the loss of electron transfer activity in S27A-, S27C-, S27T-, R31A-, R31K-, and R31H-substituted succinate-Q reductases is not due to the failure of association of succinate dehydrogenase to the mutant membrane-anchooring subunits (SdhC-SdhD), because the ratio of FAD to cytochrome \( b_{556} \) and the content of FAD and cytochrome \( b_{556} \) in these mutant succinate-Q reductases are the same as those of complement reductase (Table I). The activity loss is also not due to the mutation effect on the instability of the protein subunit in these mutant succinate-Q reductases, because SDS-PAGE of purified mutant succinate-Q reductases

### Table I

| Mutations | Growth on succinate | Enzymatic activity | Molar ratio of FAD/\( b_{556} \) | \( K_m \) for Q2 |
|-----------|---------------------|--------------------|-------------------------------|----------------|
| Wild type | ++++                | 3.15               | 1.0                           | 10.8           |
| T17A      | ++++                | 2.15               | 1.1                           | 10.2           |
| R19A      | ++++                | 2.67               | 0.9                           | 9.0            |
| F20L      | ++++                | 3.30               | 1.1                           | 10.8           |
| T23A      | +++                 | 1.90               | 0.9                           | —              |
| S27A      | —                   | 0.20               | 0.9                           | —              |
| S27C      | —                   | 0.23               | 1.0                           | —              |
| S27T      | —                   | 0.21               | 1.1                           | —              |
| H30A      | ++++                | 2.33               | 1.1                           | 10.6           |
| R31A      | —                   | 0.17               | 0.9                           | —              |
| R31H      | —                   | 0.17               | 1.1                           | —              |
| R31K      | —                   | 0.21               | 1.1                           | —              |
| S33A      | ++                  | 1.12               | 0.9                           | 11.4           |
| S33C      | ++                  | 1.43               | 1.0                           | 8.9            |
| S33T      | ++                  | 2.81               | 0.9                           | 10.0           |

a, +++, the growth phenotype is essentially the same as the wild type; ++, +, and —, progressively less growth. b, no growth on succinate within 5 days.

The activity is expressed in micromoles of succinate oxidized/min/nmol of \( b_{556} \).

b, Cannot be determined accurately.
show the four subunits in stoichiometric amounts (Fig. 6). Serine 33 of SdhC is also a conserved residue in all type C membrane anchors. To see whether this serine has the same structural importance as serine 27, mutant strains having alanine, cysteine, and threonine replacements at this position were generated and characterized. The S33A, S33C, and S33T mutant cells have retarded aerobic growth rates in succinate/M9 medium (about 20, 20, and 60% of that of the complement strain, respectively), suggesting that succinate-Q reductases in these mutant cells are partially active. The S33A-, S33C-, and S33T-substituted succinate-Q reductases have 35, 44, and 88% of the succinate-Q reductase activity of complement enzyme, suggesting that the hydroxyl moiety, rather than the size of amino acid side chain, at position 33 of SdhC, is important for succinate-Q reductase. The apparent \( K_m \) values of these mutants for \( Q_0 \) are about the same as wild type. The purified mutant proteins have \(^3H\)azido-Q uptakes comparable with that of complement reductase (Fig. 5). Perhaps this serine forms a weak hydrogen bond with quinone and is involved in the protonation of quinone. Thus, the mutation of Ser-33 to alanine may greatly reduce enzyme turnover without affecting the affinity for \( Q \).

Other conserved amino acid residues in SdhC may also affect azido-Q labeling. For example, Arg-90 and His-91 may form the “opposite” side of the labeled Q site if succinate-Q reductase indeed contains two Q-binding sites located at the opposite side.
of the membrane and azido-Q binds only to one of the two sites (14). His-84 has been identified as a ligand for b558 heme (5), and its involvement in Q binding is definitely worth investigation. Work toward better understanding of the Q-cytochrome b558 interactions in succinate-Q reductase is currently in progress in our laboratory.

It should be noted that site-directed mutagenesis studies on E. coli fumarate reductase, a closely related enzyme to succinate-Q reductase, demonstrate that Glu-29 of FrdC is involved in deprotonation of menaquinol, and Ala-32 of FrdC aids in binding and orientation of menaquinol (28). These two residues are located at the N-terminal end of FrdC toward the transmembrane helix I. The positions are similar to those of Ser-27, Arg-31, and Ser-33 of SdhC, according to proposed secondary structures for FrdC and SdhC. So far, no correlation can be made due to the poor sequence similarity between these two proteins.

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