The Distal Heme Center in *Bacillus subtilis* Succinate:Quinone Reductase Is Crucial for Electron Transfer to Menaquinone†

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ABSTRACT: Succinate:quinone reductases are membrane-bound enzymes that catalyze electron transfer from succinate to quinone. Some enzymes in vivo reduce ubiquinone (exergonic reaction) whereas others reduce menaquinone (endergonic reaction). The succinate:menaquinone reductases all contain two heme groups in the membrane anchor of the enzyme: a proximal heme (heme b_P) located close to the negative side of the membrane and a distal heme (heme b_D) located close to the positive side of the membrane. Heme b_D is a distinctive feature of the succinate:menaquinone reductases, but the role of this heme in electron transfer to quinone has not previously been analyzed. His28 and His113 are the axial ligands to heme b_D in *Bacillus subtilis* succinate:menaquinone reductase. We have individually replaced these His residues with Leu and Met, respectively, resulting in assembled membrane-bound enzymes. The H28L mutant enzyme lacks succinate:quinone reductase activity probably due to a defective quinone binding site. The H113M mutant enzyme contains heme b_D with raised midpoint potential and is impaired in electron transfer to menaquinone. Our combined experimental data show that the heme b_D center, into which we include a quinone binding site, is crucial for succinate:menaquinone reductase activity. The results support a model in which menaquinone is reduced on the positive side of the membrane and the transmembrane electrochemical potential provides driving force for electron transfer from succinate via heme b_P and heme b_D to menaquinone.

In aerobic bacteria containing only menaquinone, such as *Bacillus* species, it has been shown that the succinate oxidase activity decreases drastically when the bacteria are disrupted or treated with agents that dissipate the transmembrane electrochemical potential (*1*, *2*). This phenomenon is not seen with bacteria containing ubiquinone, e.g. aerobically grown *Escherichia coli*. On the basis of these and other observations, it has been proposed that the enzymes catalyzing menaquinone reduction by succinate are energy coupled; i.e., they depend on the transmembrane electrochemical potential for function. In this work we have addressed the mechanism for this proposed energy coupling in *Bacillus subtilis*.

Succinate:quinone reductase (SQR)[1] and quinol:fumarate reductase (QFR) are membrane-bound respiratory enzymes (for recent reviews see refs *3* and *4*). SQR is used in aerobic respiration where it couples oxidation of succinate to fumarate in the Krebs cycle with reduction of quinone to quinol in the respiratory chain. QFR catalyzes the reverse reaction in vivo and is used in anaerobic respiration with fumarate as terminal electron acceptor. The crystal structures of *E. coli* QFR and *Wolinella succinogenes* QFR at 3.3 and 2.2 Å resolution, respectively, have recently been determined (*5*, *6*). SQR and QFR are similar in composition and structure. Both enzymes have a membrane-peripheral domain attached to the negative side of the membrane by a membrane anchor (Figure 1A). The membrane-peripheral domain is composed of a flavoprotein (SdhA/FrdA) with one covalently attached FAD (8α-*N*(3)-histidyl-FAD) and an iron−sulfur protein (SdhB/FrdB) with three iron−sulfur clusters, one each of a [2Fe−2S], a [4Fe−4S], and a [3Fe−4S] cluster. The succinate/fumarate binding site is on the flavoprotein. The membrane anchor is composed of one (SdhC/FrdC) or two (SdhC/FrdC and SdhD/FrdD) polypeptides, and depending on the enzyme variant, it contains zero, one, or two protoheme IX (heme B) groups. The heme has bis(histidine) axial ligation (*6*−*9*). The membrane anchor is required for quinone reduction and quinol oxidation. This domain can harbor two quinone/quinol binding sites as shown by the crystal structure of *E. coli* QFR (*5*) and indicated by photoaffinity labeling and mutant data using e.g. bovine heart SQR (*10* and references therein). Electron transfer between succinate/fumarate and quinone/quinol occurs in a linear pathway including FAD, [2Fe−2S], [4Fe−4S], and [3Fe−4S] as electron carriers (*5*, *6*). It has not been established whether heme, when present in the enzyme, is involved as electron carrier in electron transfer between the [3Fe−4S] cluster and quinone (*11*).

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[1] Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; G3P, glycerol-3-phosphate; heme b_D, distal heme *b*; heme b_P, proximal heme *b*; HQNO, 2-*n*-heptyl 4-hydroxyquinoline *N*-oxide; LB, Luria−Bertani medium; PMS, phenazine methosulfate; Q_D, distal quinone; QFR, quinol:fumarate reductase; Q_P, proximal quinone; SQR, succinate:quinone reductase.

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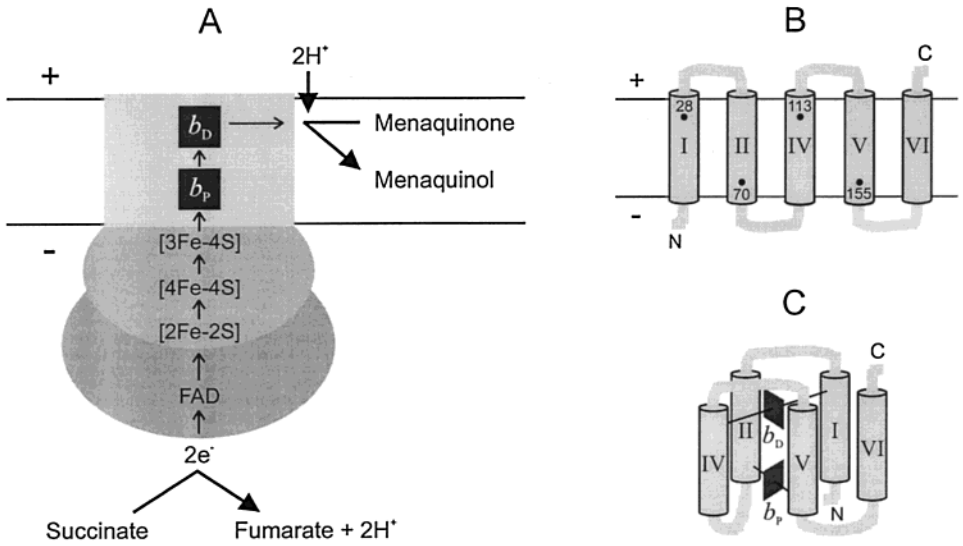

FIGURE 1: (A) *B. subtilis* SQR in the membrane schematically drawn to illustrate the function and subunit composition of the enzyme. The proximal heme (b_P) and the distal heme (b_D) are indicated as black squares. (B) Transmembrane topology of the *B. subtilis* SdhC polypeptide. The histidine residues serving as axial ligands to heme b_P (H70 and H155) and heme b_D (H28 and H113) are indicated. The numbering of transmembrane segments is according to Hägerhäll and Hederstedt (*14*). (C) General 3D-structure of *B. subtilis* SdhC as proposed by Hägerhäll and Hederstedt (*14*) and consistent with the crystal structure of *W. succinogenes* QFR (*6*).

There are two functional classes of SQR enzymes: those that in vivo reduce a high-potential quinone, such as ubiquinone, and those that reduce a low-potential quinone, for example menaquinone. Electron transfer from succinate ($E_{m,7} = +33$ mV) to ubiquinone ($E_{m,7} = +113$ mV) is an exergonic reaction ($\Delta G^{\circ\prime} = -15$ kJ/mol) whereas electron transfer from succinate to menaquinone ($E_{m,7} = -74$ mV) is an endergonic reaction ($\Delta G^{\circ\prime} = +21$ kJ/mol) (*12*). One major difference between the two functional classes of SQR is that the succinate:ubiquinone reductases contain only one heme whereas the succinate:menaquinone reductases contain two. As shown by the crystal structure of the diheme *W. succinogenes* QFR the two hemes of succinate:menaquinone reductases are arranged in such a way that they can promote transmembrane electron transfer (*6*). The proximal heme (heme b_P) is located toward the negative side of the membrane and is close to the [3Fe−4S] cluster (Figure 1A). The distal heme (heme b_D) is located toward the positive side of the membrane with a distance (Fe to Fe) to heme b_P of approximately 16 Å. The single heme of the succinate: ubiquinone reductases corresponds to heme b_P.

The membrane anchor of *B. subtilis* SQR is composed of a single 202 amino acid residues long polypeptide (SdhC) and two hemes (Figure 1A−C) (*13*). Heme b_P has a high redox potential ($E_{m,7.4} = +42$ mV) compared to heme b_D ($E_{m,7.4} = -131$ mV). Hemes b_P and b_D were previously designated heme b_H and b_L, respectively (*14*). Residues His70 and His155 in SdhC are the axial ligands to heme b_P whereas His28 and His113 are the ligands to heme b_D (*13*). To study the role of these His residues for the structure and function of SQR, they were in a previous study individually changed for Tyr (*15*). The resulting mutant SdhC polypeptides did not support assembly of membrane-bound SQR and were therefore not useful for functional studies on the enzyme. The membrane anchor of *W. succinogenes* QFR is similar in composition to that of *B. subtilis* SQR. When the axial ligands to heme (His44, His93, His143, and His182) in FrdC of *W. succinogenes* QFR were individually replaced by Ala, or in the case of His143 also by Lys and Met, no assembled enzyme was obtained (*16*). It has thus proven to be difficult to find amino acids that can replace the His residues which function as axial ligands to heme in the diheme enzymes without preventing enzyme assembly. Here we describe assembled variants of *B. subtilis* SQR being mutated in the axial ligands to heme b_D. These mutant enzymes allowed us to study the role of heme b_D in electron transfer to menaquinone and to test a model which explains the coupling between the succinate:menaquinone reductase activity and the transmembrane electrochemical gradient.

Table 1: Plasmids Used in This Work

plasmid	relevant characteristics[a]	ref
pKIM4	*sdhC*; Ap^r	31
pLEU28	*sdhC6028*; Ap^r (H28L mutation in SdhC)	this work
pLEU113	*sdhC6113*; Ap^r (H113L mutation in SdhC)	this work
pHP13	Cm^{r}Em^r	32
pBSD1400	*sdhCAB*; Cm^{r}Em^r	13
pBSD14H28L	*sdhC6028 sdhAB*; Cm^{r}Em^r (H28L mutation in SdhC)	this work
pBSD14H113L	*sdhC6113 sdhAB*; Cm^{r}Em^r (H113L mutation in SdhC)	this work
pBSD14H113M	*sdhC7113 sdhAB*; Cm^{r}Em^r (H113M mutation in SdhC)	this work

[a] Ap^r, Cm^r, and Em^r indicate resistance to ampicillin, chloramphenicol, and erythromycin, respectively.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Growth Conditions. Plasmids used in this work are presented in Table 1. The *B. subtilis* strain LUH16 (*trpC2* Δ*sdhCAB::ble*) was obtained by transformation of *B. subtilis* strain 168 with chromosomal DNA from *B. subtilis* strain ICD2 (*13*). *B. subtilis* was grown as described before (*17*) in nutrient sporulation medium with phosphate (*18*), or on tryptose blood agar base plates (Difco), or on minimal salts agar plates (*19*). The minimal plates were supplemented with 40 mg/L tryptophan and 0.5% (w/v) glucose or sodium succinate (pH 7.4). Chloramphenicol was

used at 5 mg/L in plates and at 3 mg/L in liquid media. Phleomycin was used at 1.5 mg/L in plates. The *E. coli* strains BMH71-18 (Clontech) and JM83 (*20*) were grown in Luria−Bertani medium (LB) or on LB plates. Ampicillin was used at 50 mg/L.

Mutagenesis of sdhC. The codons for His28 and His113 in *sdhC* were individually changed to a codon for Leu. The mutagenesis was done on the *sdhC* gene in plasmid pKIM4 (Table 1) using the unique restriction site elimination method essentially as described by Zhu (*21*). This method employs two primers: the mutagenic primer which produces the desired mutation and the selection primer which destroys a restricion site that is unique to the parental plasmid. Mutagenic primers His28Leu (CTTTCTTATTCAG**CTG**TTAGTCGTCAAC) and His113Leu (CGTCAGCTGG**CTG**GTGTGGGAAACC) were used to change the codons for His28 (CAT) and His113 (CAC), respectively, to a Leu (CTG) codon. The selection primer (CAAGGAATGGT**CCATGG**AAGGAGATGG) altered the unique *Sph*I site (GCATGC) in pKIM4 into a *Nco*I site (CCATGG). The selection primer and one mutagenic primer were simultaneously annealed to denatured pKIM4, and hybrid plasmids were synthesized using T4 DNA polymerase and T4 DNA ligase. Reannealed pKIM4 was cut with *Sph*I. Hybrid plasmids were transformed into *E. coli* BMH71-18 (*mutS*), and transformants were grown overnight in LB supplemented with ampicillin. Plasmids isolated from the overnight culture, being a mixture of parental type and mutant type plasmids, were incubated with *Sph*I and then used to electrotransform *E. coli* JM83. Transformants were selected on LB plates supplemented with ampicillin. Plasmids were isolated from individual clones (colonies), and a successful mutagenesis was confirmed by dideoxynucleotide sequencing using the Sequenase Version II Kit and [α-^{35}S]dATP (Amersham Pharmacia Biotech). Mutant plasmids containing mutations resulting in His28 → Leu and His113 → Leu replacements were named pLEU28 and pLEU113, respectively.

Construction of Plasmids for Expression of sdhCAB in B. subtilis. The pBSD14 series of plasmids (Table 1) are pHP13 derivatives that were used for expression of wild-type and mutant *sdh* genes in *B. subtilis* strain LUH16. Plasmid pBSD1400 carries the wild-type *sdhCAB* operon. Plasmids pBSD14H28L and pBSD14H113L were obtained by replacing the 0.7 kbp *Mlu*I-*Sca*I fragment (carries *sdhC*) of pBSD1400 with the corresponding fragments from plasmids pLEU28 and pLEU113, respectively. Protoplast transformation (*22*) was used to introduce plasmids into strain LUH16. Successful cloning was confirmed by sequencing the entire *sdhC* gene of plasmids pBSD14H28L and pBSD14H113L isolated from *B. subtilis* transformants.

Chemicals. Stock solutions of phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCPIP) were in water, and those of decylubiquinone and 2-*n*-heptyl 4-hydroxyquinoline *N*-oxide (HQNO), both obtained from Sigma Chemical Co., were in ethanol. The concentrations of PMS and DCPIP were calculated from weight whereas those of decylubiquinone and HQNO were determined spectroscopically using ethanol as solvent and the extinction coefficients 12.5 mM^{-1} cm^{-1} (difference spectrum, air-oxidized minus sodium borohydride-reduced, at 275 nm) and 9.45 mM^{-1} cm^{-1} (absolute spectrum at 340 nm), respectively.

Preparation and Analysis of Membranes. Cell membranes were prepared as described before (*17*). The protein content was determined with the BCA method (Pierce) using bovine serum albumin as the standard. Heme B was determined by the pyridine−hemochromogen difference spectrum (reduced minus oxidized) using the extinction coefficient 20.7 mM^{-1} cm^{-1} (α-band maximum minus minimum between α- and *β*-bands) (*23*). SdhC antigen was detected by Western blot analysis using the ECL system (Amersham Pharmacia Biotech). Proteins were separated by SDS−PAGE according to Schägger and von Jagow (*24*) in 10% acrylamide gels. A semidry blotting apparatus (Semi Dry Blotter II, Kem-En-Tec) with the recommended continuous transfer buffer system was used to blot proteins onto polyvinylidene diflouride membranes (Millipore). The primary antibody, polyclonal rabbit anti-SdhC (*25*), was diluted 1:1000 prior to use. Quantitation of wild-type SdhC antigen in membranes was done using isolated SQR of known concentration (based on the [2Fe−2S] spin content) as the standard.

Enzyme Activity Measurements. Succinate oxidase and NADH oxidase activities were measured as oxygen consumption using a Clark-type oxygen electrode. Assays were done at 30 °C in 50 mM potassium phosphate buffer (pH 7.4). Reactions were started by the addition of 10 mM potassium succinate (pH 7.4) or 1 mM NADH.

Two assays employing artificial electron acceptors were used to measure SQR-dependent activity in membrane preparations from *B. subtilis*. In the succinate:PMS reductase assay electrons are transferred from succinate by way of SQR to the primary electron acceptor PMS. It is not known exactly how PMS interacts with SQR but probably electrons are accepted from several different sites on the enzyme complex. In the succinate:quinone reductase assay electrons are transferred from succinate to quinone. Decylubiquinone was used in this assay. In both types of assay DCPIP was used as terminal electron acceptor. Reduction of DCPIP was registered by the decrease in absorbance at 600 nm (ϵ = 20.7 mM^{-1} cm^{-1}). SQR also catalyzes direct electron transfer from succinate to DCPIP, but this activity is low and it was subtracted from data obtained with the succinate:PMS and the succinate:quinone reductase assays. Assays were done at 30 °C in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 20 mM potassium succinate and 1 mM potassium cyanide. Membranes were preincubated (activated) for 5 min at 30 °C in the potassium phosphate buffer supplemented with succinate and cyanide. Reactions were started by the addition of PMS or decylubiquinone and 0.1 mM DCPIP. Turnover numbers are based on the amount of assembled SQR in the membrane, determined as the [2Fe−2S] spin content.

The effect of HQNO on the succinate:quinone reductase activity was analyzed at 63 μM decylubiquinone. Apparent K_i values were obtained from Dixon plots. The HQNO resistant part of the activity (*26*) was subtracted before construction of the Dixon plots.

Spectroscopy and Redox Titration. EPR spectra were recorded as described before (*17*). Light absorption spectroscopy at room temperature and 77 K was performed with a Shimadzu UV-3000 spectrophotometer, using the 1 nm slit. Cuvettes of 10 and 2 mm light path were used for room temperature and 77 K spectra, respectively. Membranes were suspended in 20 mM Na-MOPS−HCl (pH 7.4), and potas-

Table 2: Enzyme Activities in Isolated Membranes from Different *B. subtilis* Strains

membrane	SdhC variant	growth of strain on succinate	succinate oxidase activity[a]	NADH oxidase activity[a]	succinate:decylubiquinone reductase activity		succinate:PMS reductase activity	
					activity[b]	turnover no.[c]	activity[b]	turnover no.[c]
LUH16/pBSD1400	wild-type	yes	89	48	890	890	4300	4300
LUH16/pHP13		no	<2	93	<5		<50	
LUH16/pBSD14H28L	H28L	no	<2	42	<5		1700	3400
LUH16/pBSD14H113L	H113L	no	<2	40	<5		<50	
LUH16/pBSD14H113M	H113M	yes	10	45	710	710	4700	4700

[a] nmol of O_2 reduced (mg of protein·min)$^{-1}$. [b] Activity at V_{max} expressed as nmol of succinate oxidized (mg of protein·min)$^{-1}$. V_{max} activities were estimated from Lineweaver−Burk plots on the basis of eight initial rate measurements using concentrations of PMS in the range 0.2−2 mM or decylubiquinone in the range 25−250 μM. [c] Turnover numbers (mol of succinate oxidized (mol of SQR·min)$^{-1}$) were calculated in the cases where the activity was higher than the background level found in the membranes of strain LUH16/pHP13.

sium cyanide was added to 1 mM. Spectra at 77 K were recorded after incubation of membranes with either 10 mM potassium succinate (pH 7.4) or a few grains of dithionite for 5 min prior to freezing. Room-temperature spectra were recorded after the membranes had been incubated with 10−60 mM succinate or 10 mM glycerol-3-phosphate (G3P) for 30−40 min. To obtain maximum cytochrome *b* reduction by succinate at room temperature, samples were incubated under a flow of argon gas. After incubation of membranes with succinate or G3P, dithionite was added to register complete cytochrome *b* reduction.

Redox titration of cytochrome *b* in SQR in isolated membranes was done essentially as described before (*17*). Reduction of heme in SQR was recorded spectroscopically as the difference in absorbance between the α-band maximum at 558 nm and the isobestic point at 570 nm.

RESULTS

Phenotype of B. subtilis Strains Containing Mutant SQR. Using site-specific mutagenesis the codons for His28 and His113 in *sdhC* were individually replaced by a codon for Leu. To study the effect of the amino acid substitutions in SdhC on assembly and function of SQR, the *sdhCAB* operon with wild-type or mutant *sdhC* gene was cloned in plasmid pHP13 and expressed in *B. subtilis* strain LUH16 which lacks the *sdhCAB* operon. Plasmid pBSD1400 encodes wild-type SdhCAB. The presence of such a plasmid in a wild-type strain results in 2- to 3-fold overproduction of SQR (*17*). Plasmids pBSD14H28L and pBSD14H113L encode SdhCAB where residues His28 and His113 in SdhC, respectively, are replaced by Leu.

Strain LUH16 containing pBSD1400 grew on minimal agar plates with succinate as sole carbon and energy source whereas LUH16/pHP13 did not grow, as expected. LUH16/pBSD14H28L and LUH16/pBSD14H113L did not grow on minimal-succinate but grew on minimal-glucose plates. These results indicated that active SQR is not formed when the H28L or the H113L mutation is present in SdhC.

Isolation of Suppressor Mutations. Two bases in each of the codons for His28 (CAT) and His113 (CAC) in *sdhC* were replaced in the mutagenesis. The resulting Leu codon (CTG) cannot with a single base change be converted into a His codon. This reduced the risk of reversion and facilitated isolation of suppressor (second site) mutations. Strains LUH16/pBSD14H28L and LUH16/pBSD14H113L were spread on minimal-succinate agar plates. After incubation of the plates at room temperature for several days LUH16/pBSD14H113L, but not LUH16/pBSD14H28L, gave rise to a few colonies (clones) on the plate. Plasmid DNA was extracted from six independent clones and were used to transform LUH16. All six plasmid DNA preparations gave transformants that grew on minimal-succinate agar plates showing that the plasmids encoded functional SQR. The entire *sdhC* gene from one plasmid clone, pBSD14H113M, was sequenced. It contains a transversion in codon 113 (CTG → ATG) causing a Leu to Met replacement in the SdhC polypeptide. The same mutation was found in the other five clones. By a single base change the CTG Leu codon could theoretically also be changed to one for Arg, Gln, Pro, or Val. The fact that such mutations were not isolated indicates that one of these residues at position 28 or 113 in SdhC, or a Met at position 28, results in a nonfunctional SQR.

Strain LUH16/pBSD14H113M grew poorer than the wild-type control, LUH16/pBSD1400, on minimal-succinate agar plates. In broth medium it grew to a higher cell density (optical density) than LUH16/pHP13 but to a lower cell density than the wild-type control.

Enzyme Activities. Isolated membranes from the constructed *B. subtilis* strains were analyzed for four different enzyme activities (Table 2). NADH oxidase activity, indicating the presence of a functional respiratory chain, was normal in all strains. A functional membrane-peripheral domain of SQR is required for succinate:PMS reductase activity whereas the succinate:quinone reductase and the succinate oxidase activities require also a functional anchor domain. Membranes from strains LUH16/pBSD1400, LUH16/pBSD14H28L, and LUH16/pBSD14H113M contained succinate:PMS reductase activity that was higher than the background level found in membranes from strain LUH16/pHP13. Membranes from strain LUH16/pBSD14H113L lacked succinate:PMS reductase activity. Succinate:quinone reductase and succinate oxidase activities were found only in membranes from strains LUH16/pBSD1400 and LUH16/pBSD14H113M. This is in accordance with the fact that these two strains can grow on minimal-succinate agar plates and confirms that the H113M mutant SQR is functional. It is noteworthy that the in vitro succinate:quinone reductase activity in membranes from LUH16/pBSD14H113M was close to normal (80% of wild-type activity) whereas the succinate oxidase activity was only 11% of wild-type activity. The explanation for this discrepancy is probably that the succinate oxidase activity relies on the natural endogenous quinone in the membranes (menaquinone-7) whereas the succinate:quinone reductase activity was measured with a water-soluble ubiquinone analogue (decylubiquinone). It thus

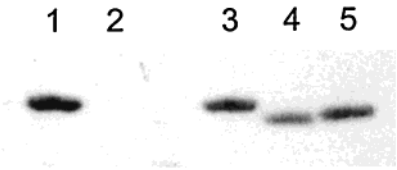

FIGURE 2: Western blot analysis of wild-type and mutant SdhC polypeptides in isolated membranes: lane 1, LUH16/pBSD1400 (wild-type); lane 2, LUH16/pHP13 (lacks SdhC); lane 3, LUH16/pBSD14H28L (H28L mutant); lane 4, LUH16/pBSD14H113L (H113L mutant); lane 5, LUH16/pBSD14H113M (H113M mutant). A 1 μg amount of membrane protein per strain was used in the analysis. The heme B content of the membranes (nmol/mg of total membrane protein) was 4.5, 0.7, 1.9, 1.2, and 4.2, respectively.

seems that the H113M mutant SQR can reduce high-potential quinones more efficiently than low-potential quinones.

SQR, SdhC, and Heme B Content in Membranes from Different B. subtilis Strains. The amount of assembled SQR in membranes was determined by EPR as the concentration of the [2Fe−2S] cluster (*17*, *27*). Membranes from strains LUH16/pBSD1400, LUH16/pBSD14H28L, and LUH16/pBSD14H113M contained assembled SQR (1.0, 0.5, and 1.0 nmol/mg of total membrane protein, respectively) whereas those from strains LUH16/pHP13 and LUH16/pBSD14H113L did not. SdhC polypeptide, detected using Western blot analysis (Figure 2), was found in membranes from all strains except LUH16/pHP13, which does not contain the *sdhC* gene. It is notable that mutant SdhC antigens were present in lower amounts than the wild-type antigen. Differences in apparent molecular mass of mutant SdhC polypeptides as compared to the wild-type polypeptide are due to the single amino acid substitutions. The heme B content in membranes from all strains containing wild-type or mutant SdhC was higher than that found in membranes from LUH16/pHP13 (see legend of Figure 2) showing that the mutant SdhC polypeptides bind heme.

The amount of heme B bound to SdhC in the membrane preparations was estimated by subtracting the amount of heme B found in membranes from strain LUH16/pHP13. Wild-type SdhC is known to contain two bound hemes/polypeptide, and the estimated content, based on the Western blot results and the heme B content, was 1.7. We could, however, not conclude on the number of heme molecules (one or two) bound per mutant SdhC polypeptide. This is mainly because the exact amount of mutant SdhC polypeptide in the membranes could not be determined. Antiserum produced against the wild-type *B. subtilis* SdhC polypeptide was used in the Western blot. From the heme B to SdhC antigen ratios it seemed as if some antibodies in the serum which bind to wild-type SdhC polypeptide did not recognize mutant SdhC leading to an underestimation of the content of mutant SdhC. Furthermore, the membranes might contain a mixture of SdhC polypeptide with and without heme.

The combined results from the activity measurements and the analysis of SQR protein and heme in isolated membranes show that the H28L, the H113L, and the H113M mutant SdhC polypeptides are inserted in the membrane. They can all bind at least one heme molecule, but only the H28L and H113M polypeptides can form a stable complex with the membrane-peripheral domain of SQR. The H28L mutant SQR lacks succinate:quinone reductase activity. The H113M mutant SQR is impaired in this activity.

Mutant SQRs Are Refractory to Purification. To be able to determine the exact chemical composition of SQR isolated enzyme is required. Therefore attempts were made to purify the H28L and the H113M mutant SQR as has been done previously with wild-type (*17*) and one mutant (*13*) *B. subtilis* SQR. However, solubilization of membranes from LUH16/pBSD14H28L and LUH16/pBSD14H113M using the non-ionic detergent Thesit (Roche Molecular Biochemicals) caused a decrease in succinate:PMS reductase activity. Fractionation of the LUH16/pBSD14H28L solubilizate showed that the membrane-peripheral part of SQR was released from the membrane anchor. This is in contrast to the wild-type enzyme which is stable when solubilized in Thesit indicating that the H28L and the H113M mutations in SdhC, respectively, weaken the binding of the membrane-peripheral domain to the membrane anchor. Since it was not possible to isolate the mutant enzymes, they could only be studied in situ in membranes.

Light Absorption Spectra of the Membrane Anchor of SQR. Low-temperature light absorption difference spectra of isolated membranes are shown in Figure 3. The dominating feature in the 550−560 nm region in spectra of all membranes containing the anchor domain of SQR is the α-band absorption peak of this *b*-type cytochrome. Figure 3A shows spectra obtained after incubation of membranes with succinate, and Figure 3B shows those of dithionite reduced (fully reduced) membranes. Reduction of membrane-bound components by succinate is dependent on the succinate dehydrogenase activity of SQR whereas dithionite reduction is nonenzymatic. Cytochrome *b* in membranes containing wild-type, H28L mutant and H113M mutant SQR, respectively, was partially reduced by succinate (compare the intensity of the α-band peaks in Figure 3A,B). Cytochrome *b* in membranes containing the H113L mutant anchor was not reduced by succinate as expected from the fact that the membrane-peripheral domain of SQR is absent. The extent of reduction of cytochrome *b* in the presence of succinate was estimated from the intensity of the α-band peak in spectra recorded at room temperature (spectra not shown). Approximately 50% of cytochrome *b* in membranes containing wild-type SQR was, as expected, reduced by succinate showing complete reduction of heme b_P and little reduction of heme b_D (*17*). The low midpoint potential of heme b_D in wild-type SQR does not allow its reduction by succinate at neutral pH. Approximately 60% of cytochrome *b* in membranes containing H28L mutant SQR and approximately 75% of that in membranes containing H113M mutant SQR was reduced by succinate. Difference spectra of dithionite reduced minus succinate reduced membranes are shown in Figure 3C. Membranes containing wild-type SQR gave a split α-peak corresponding to the α-peak of heme b_D as shown before (*17*). The mutant anchor variants did not show this characteristic split of the α-peak.

The spectra of membranes containing wild-type and H113M mutant SQR exhibited a cytochrome *a* peak at about 600 nm after incubation with succinate showing electron transfer from SQR by way of the menaquinone pool to cytochrome *a* (Figure 3A). Reduction of cytochrome *a* after incubation with succinate was not seen with membranes containing the H28L mutant SQR confirming that this enzyme is blocked in electron transfer from succinate to quinone.

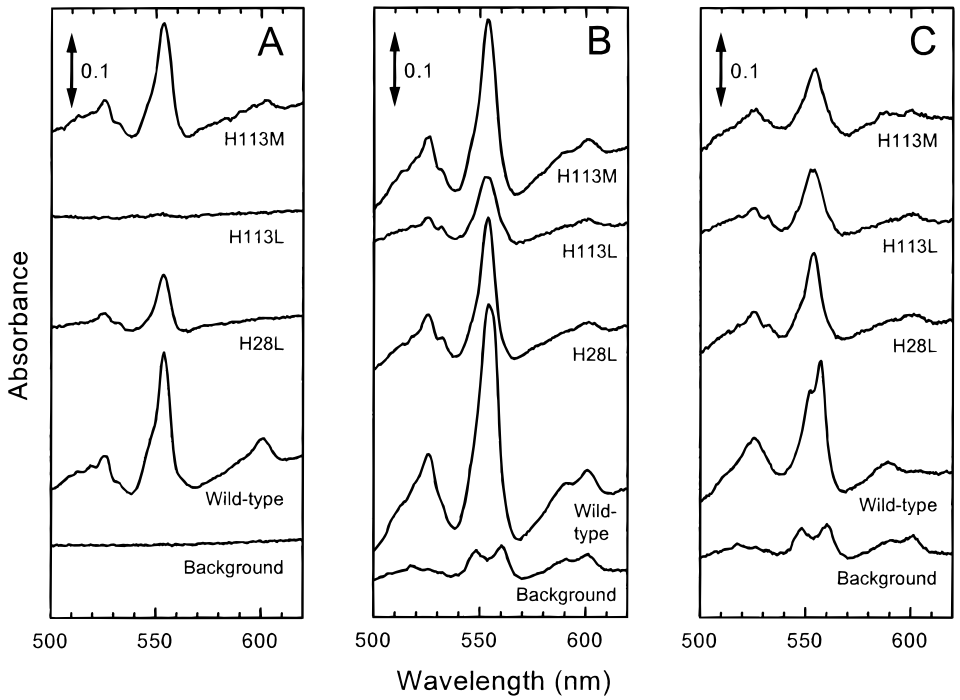

FIGURE 3: Light absorption spectra at 77 K of membranes from *B. subtilis* strains LUH16/pHP13 (background), LUH16/pBSD1400 (wild-type SdhC), LUH16/pBSD14H28L (H28L mutant SdhC), LUH16/pBSD14H113L (H113L mutant SdhC), and LUH16/pBSD14H113M (H113M mutant SdhC). Shown are three types of difference spectra: (A) succinate-reduced minus air-oxidized; (B) dithionite-reduced minus air-oxidized; (C) dithionite-reduced minus succinate-reduced. The difference spectra in panels A and B are as recorded whereas C was obtained by subtracting A from B. The membrane protein concentration was 5 mg/mL. Vertical two-headed arrows indicate the absorption scale.

Reduction of Heme in the Membrane Anchor of SQR Using G3P. *B. subtilis* G3P dehydrogenase is a membrane-associated enzyme catalyzing electron transfer to menaquinone. Addition of G3P to membranes containing G3P dehydrogenase is a way to reduce endogenous quinone which in turn can reduce other components in the membrane. Incubation of membranes in the presence of G3P was found to result in reduction of heme in all variants of membrane anchors (wild-type, H28L, H113L, and H113M). This indicates that all these anchors contain a functional menaquinol oxidation site.

Redox Titration of the Heme in SQR. Since the membrane anchor of SQR was the dominating *b*-type cytochrome in the membrane preparations (Figure 3), it was possible to perform redox titrations of the heme in SQR in membrane suspension with little interference from other cytochromes. Such titrations with membranes containing wild-type, H28L mutant, and H113M mutant SQR, respectively, are shown in Figure 4. Cytochrome *b* in wild-type SQR titrated as two components (Figure 4A) corresponding to heme b_P (E_m = +42 mV) and heme b_D (E_m = −131 mV). Cytochrome *b* in H28L mutant SQR also titrated as two components (Figure 4B). The midpoint potential of one component was close to that of heme b_P in the wild-type enzyme, but the midpoint potential of the other component was more than 50 mV higher than that of heme b_D in the wild type. Cytochrome *b* in H113M mutant SQR showed a complex titration curve (Figure 4C). We interpret the titration data as if heme b_P is present and has a midpoint potential that is increased by approximately 50 mV compared to the wild type. No distinct heme b_D component was apparent, but this heme is present in the H113M mutant SQR as shown by redox titration in the presence of HQNO (see the following section).

Effects of HQNO on Mutant SQR. HQNO, a menasemiquinone analogue, is an inhibitor of *B. subtilis* succinate:quinone reductase activity (*17*). The succinate:quinone reductase activity of membrane-bound H113M mutant SQR was inhibited by HQNO, but the activity was somewhat less sensitive (apparent K_i = 1.4 μM) than that of membranes containing the wild-type enzyme (apparent K_i = 0.5 μM).

Binding of HQNO to isolated *B. subtilis* SQR affects the properties of heme b_D; i.e., it results in a negative shift of the midpoint potential and a shift in the g_{max} value of the EPR spectrum of this heme (*26* and our unpublished data). This indicates that a quinone binding site is located in the vicinity of heme b_D. HQNO caused a −26 mV shift of the midpoint potential of heme b_D in the wild-type membrane-bound SQR (Figure 4A). In contrast, HQNO only caused minor changes in the titration curve of cytochrome *b* in membranes containing the H28L mutant SQR (Figure 4B). Cytochrome *b* in membranes containing the H113M mutant SQR titrated as two distinct components in the presence of HQNO, quite different from the complex titration pattern obtained in the absence of HQNO (Figure 4C). Seemingly the inhibitor stabilizes the heme b_D center in the H113M mutant enzyme.

The redox titration data in the absence and presence of HQNO indicate that both the H28L and the H113M mutant SQR contain two heme groups and that the midpoint potential of heme b_D in these mutant enzymes is more positive than in the wild type. The lack of an effect from HQNO on the heme b_D center in the H28L mutant SQR suggests that this enzyme is defective in quinone binding.

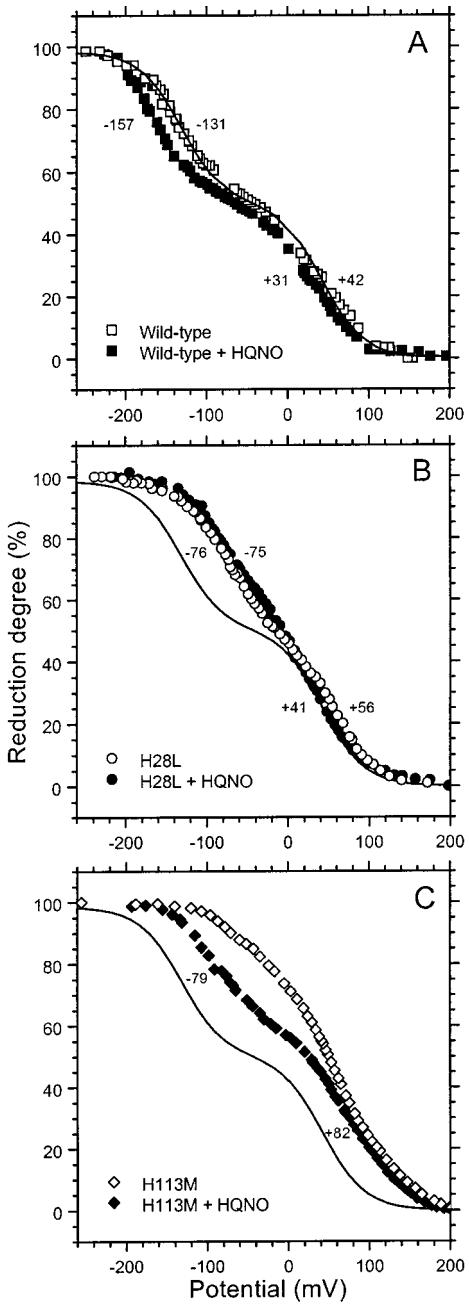

FIGURE 4: Potentiometric analysis of cytochrome *b* in membranes from different *B. subtilis* strains: (A) LUH16/pBSD1400 (wild-type SdhC); (B) LUH16/pBSD14H28L (H28L mutant SdhC); (C) LUH16/pBSD14H113M (H113M mutant SdhC). The titrations were done in the absence (open symbols) and presence (filled symbols) of 40 μM HQNO. The membrane protein concentration was 2−4 mg/mL. Experimental data (except in the case of the H113M mutant in the absence of HQNO) were fitted by Nernst curves for two noninteracting single-electron components (heme b_P and heme b_D). The midpoint potentials (E_m) and the relative absorbances at 558 nm (α) of the two components were optimized in a computer-aided analysis. Optimized parameters are as follows. Wild-type without HQNO: component one, $E_m = +42$ mV, $\alpha = 50\%$; component two, $E_m = -131$ mV, $\alpha = 48\%$. Wild-type with HQNO: component one, $E_m = +31$ mV, $\alpha = 50\%$; component two, $E_m = -157$ mV, $\alpha = 51\%$. H28L mutant without HQNO: component one, $E_m = +56$ mV, $\alpha = 49\%$; component two, $E_m = -76$ mV, $\alpha = 50\%$. H28L mutant with HQNO: component one, $E_m = +41$ mV, $\alpha = 55\%$; component two, $E_m = -75$ mV, $\alpha = 45\%$. H113M mutant with HQNO: component one, $E_m = +82$ mV, $\alpha = 55\%$; component two, $E_m = -79$ mV, $\alpha = 44\%$. The solid curve in the panels corresponds to the fitted curve for the wild type in the absence of HQNO.

DISCUSSION

Heme b_P and heme b_D in the membrane anchor of *B. subtilis* SQR are arranged in such a way that they can mediate efficient electron transfer across the cytoplasmic membrane (Figure 1A). Electron transfer within the enzyme from succinate ($E_{m,7} = +33$ mV) via FAD and the iron−sulfur clusters to heme b_P ($E_{m,7.4} = +42$ mV) is an exergonic reaction. Heme b_P in membrane-bound and isolated *B. subtilis* SQR is reduced by succinate within the same time frame as the turnover of the enzyme (Smirnova, Hägerhäll, Konstantinov, and Hederstedt, unpublished data). Electron transfer from heme b_P via heme b_D ($E_{m,7.4} = -131$ mV) to menaquinone ($E_{m,7} = -74$ mV) is an endergonic reaction. A model has been proposed in which energy to drive the "uphill" electron transfer from heme b_P to heme b_D is supplied by the electrical component of the transmembrane gradient (*2*). This explains the coupling between the succinate:menaquinone reductase activity and the transmembrane electrochemical gradient. The final electron transfer from heme b_D to menaquinone is then thermodynamically "downhill". In the outlined linear electron-transfer pathway both heme b_P and heme b_D play a critical role and menaquinone would be reduced close to the positive (periplasmic) side of the membrane (Figure 1A) (*2*). One turnover of SQR releases two protons from succinate on the negative (cytoplasmic) side of the membrane (Figure 1A). Two protons are consumed upon complete reduction of one quinone molecule. Transmembrane proton gradient will be consumed if the protons used in quinone reduction by SQR are taken from the positive side of the membrane. Thus, in the proposed model succinate:menaquinone reductase activity feeds on the transmembrane proton potential. However, the overall succinate oxidase activity still generates a net transmembrane proton gradient. This is due to the activities of the quinol:cytochrome *c* reductase (respiratory complex III) and the proton pumping terminal oxidases which compensate in excess for the consumption of proton gradient by SQR.

Two tightly bound menaquinone molecules per enzyme were found in the crystal structure of the *E. coli* QFR (*5*). The two quinones are positioned ~27 Å apart on opposite sides of the membrane: the proximal quinone (Q_P) is close to the [3Fe−4S] cluster on the negative side of the membrane, and the distal quinone (Q_D) is close to the positive side of the membrane. Quinone binding sites have not been identified in the diheme *W. succinogenes* QFR crystal structure (*6*), but this enzyme and *B. subtilis* SQR might also contain two menaquinone binding sites. The Q_P site would be located close to heme b_P, and the Q_D site, close to heme b_D (*6*). The presence of a Q_D site in *B. subtilis* SQR is indicated by the effect of HQNO on the properties of heme b_D (*26*). Possibly each quinone binding site is structurally integrated with the respective heme binding site in a heme center; i.e., heme constitutes a part of the quinone binding site as is the case with heme b_H in the mitochondrial quinol:cytochrome *c* reductase (*28*).

In this work we have altered the heme b_D center of *B. subtilis* SQR by substituting the axial ligands (His28 and His113) to heme b_D. Two mutant enzyme variants were analyzed in more detail: the H28L and the H113M SQR. From our experimental data we conclude that heme b_P is present and reduced by succinate in both mutant enzyme

variants. The H113M mutant SQR also contains heme at the heme b_D center, and it most likely has His-Met axial ligation. This type of ligation is common in *c*-type cytochromes (*29*) but also occurs in some *b*-type cytochromes (cf. *30*). An increase in the midpoint potential is expected from changing one heme iron axial ligand from His to Met (*29*). The midpoint potential of heme b_D in the H113M mutant SQR could not be exactly determined in the absence of HQNO, but it is increased by at least 100 mV compared to heme b_D in the wild-type enzyme (Figure 4C). The observed increase in reducibility of cytochrome *b* by succinate from 50% in the wild-type SQR to 75% in the H113M mutant SQR is consistent with an increased midpoint potential of heme b_D.

Redox titration of membranes containing the H28L mutant SQR indicated two hemes with the midpoint potentials +56 and −76 mV (Figure 4B). If the latter heme corresponds to heme b_D, it would have a midpoint potential that is increased by more than 50 mV compared to wild-type heme b_D. Membranes containing the H28L mutant SQR did not show an increased amount of high-spin iron (EPR g_{max} signal at 6.0) compared to membranes containing wild-type SQR (data not shown). This indicates that if present, heme b_D has two axial ligands. A Leu residue can presumably not function as an axial ligand to heme iron. His28 would thus be replaced by an unknown ligand which could be either a residue in the SdhC polypeptide or a small extraneous molecule.

The H28L mutant enzyme is completely blocked in quinone reduction. Probably the Q_D site is destroyed as indicated by the fact that HQNO does not affect the midpoint potential of heme b_D in this enzyme. It is noteworthy that heme in the H28L mutant SQR is reducible by menaquinol (reduced by G3P dehydrogenase activity) indicating the presence of a functional quinol oxidation site on the enzyme. This site would correspond to the Q_P site. The H113M mutant SQR is active but does not reduce menaquinone as efficiently as the wild-type enzyme as indicated by the low succinate oxidase activity (Table 2). The Q_D site is present on the H113M mutant SQR as shown by the negative shift of the midpoint potential of heme b_D in the presence of HQNO. The H113M mutant enzyme is probably defective mainly because of the raised midpoint potential of heme b_D, which makes electron transfer from this heme to menaquinone energetically unfavorable. Thus, a low potential of the heme at the heme b_D center seems important for efficient electron transfer to menaquinone.

In conclusion, we have succeeded in obtaining assembled mutant SQR with altered axial ligation of heme b_D. These types of mutants have not been obtained before, and they allowed us to study the role of heme b_D. Our results with mutant *B. subtilis* SQR show that the heme b_D center is crucial for menaquinone reduction. The functional data obtained with *B. subtilis* SQR combined with the structural data for *W. succinogenes* QFR (*6*) demonstrate that the two hemes in SQR function in transmembrane electron transfer and indicate that menaquinone is reduced and protonated on the positive side of the membrane. This is entirely consistent with the proposed model by Schirawski and Unden (*2*) for the coupling between succinate:menaquinone reductase activity and the transmembrane electrochemical gradient.

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