Two Hemes in *Bacillus subtilis* Succinate:Menaquinone Oxidoreductase (Complex II)†

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ABSTRACT: Succinate:menaquinone-7 oxidoreductase (complex II) of the Gram-positive bacterium *Bacillus subtilis* consists of equimolar amounts of three polypeptides; a 65-kDa FAD-containing polypeptide, a 28-kDa iron–sulfur cluster containing polypeptide, and a 23-kDa membrane-spanning cytochrome b_{558} polypeptide. The enzyme complex was overproduced 2–3-fold in membranes of *B. subtilis* cells containing the *sdhCAB* operon on a low copy number plasmid and was purified in the presence of detergent. The cytochrome b_{558} subunit alone was similarly overexpressed in a complex II deficient mutant and partially purified. Isolated complex II catalyzed the reduction of various quinones and also quinol oxidation. Both activities were efficiently albeit not completely blocked by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. Chemical analysis demonstrated two protoheme IX per complex II. One heme component was found to have an $E_{m,7.4}$ of +65 mV and an EPR g_{max} signal at 3.68, to be fully reducible by succinate, and showed a symmetrical α-band absorption peak at 555 nm at 77 K. The other heme component was found to have an $E_{m,7.4}$ of −95 mV and an EPR g_{max} signal at 3.42, was not reducible by succinate under steady-state conditions, and showed in the reduced state an apparent split α-band absorption peak with maxima at 553 and 558 nm at 77 K. Potentiometric titrations of partially purified cytochrome b_{558} subunit demonstrated that the isolated cytochrome b_{558} also contains two hemes. Some of the properties, i.e., the α-band light absorption peak at 77 K, the line shapes of the EPR g_{max} signals, and reactivity with carbon monoxide were observed to be different in *B. subtilis* cytochrome b_{558} isolated and in complex II. This suggests that the bound flavoprotein and iron–sulfur protein subunits protect or affect the heme environment in the assembled complex.

Succinate:quinone oxidoreductase (complex II; EC 1.3.5.1) of the aerobic, Gram-positive bacterium *Bacillus subtilis* is a membrane-bound enzyme that catalyzes the oxidation of succinate to fumarate in the tricarboxylic acid cycle and donates reducing equivalents to menaquinone-7 in the respiratory chain. The *B. subtilis* complex II consists of three polypeptides in 1:1:1 stoichiometry (Hederstedt et al., 1979) and has several redox components. The largest subunit, the flavoprotein (Fp, 65 kDa),[1] harbors the dicarboxylate active site and carries one FAD covalently bound to a histidine residue (Hederstedt & Hede'n, 1989; Hederstedt, 1987). The iron–sulfur protein (Ip, 28 kDa) contains ligands for three iron–sulfur clusters of [2Fe–2S]$^{(1+,2+)}$, [3Fe–4S]$^{(0,1+)}$, and [4Fe–4S]$^{(1+,2+)}$ type (Hederstedt et al., 1985). These two membrane-extrinsic subunits are anchored to the inner surface of the cytoplasmic membrane by the third subunit, the cytochrome b_{558} (Cyt b_{558}, 23 kDa) (Hederstedt, 1986). The Cyt b_{558}, Fp, and Ip polypeptides are encoded by the *sdhCAB* operon, which has been cloned and sequenced (Magnusson et al., 1986; Phillips et al., 1987).

The primary structure and composition of the Fp and Ip subunits are conserved in complex II from different aerobic organisms, but this is not the case for the membrane anchor (Ackrell et al., 1992). The Cyt b_{558} polypeptide of *B. subtilis* complex II spans the cytoplasmic membrane, probably with five α-helical transmembrane segments, and has the N-terminus in the cytoplasm (Hederstedt & Rutberg, 1983; Hederstedt et al., 1987; Fride'n et al., 1987; Fride'n, 1990). Complex II from beef heart mitochondria (Ackrell et al., 1992) and from the Gram-negative bacteria *Escherichia coli* (Kita et al., 1989) and *Paracoccus denitrificans* (Pennoyer et al., 1988) contains one protoheme IX per FAD and has two[2] membrane-anchor polypeptides. *B. subtilis* complex II immunoprecipitated from Triton X-100 solubilized membranes with anti-Fp antibodies contains 1.6–2 protoheme IX per FAD, and light absorption spectra have indicated that Cyt b_{558} is reduced to about 50% with succinate compared to that with dithionite (Hederstedt, 1980). A possible interpretation of these two observations would be the presence of two heme groups in the *B. subtilis* enzyme, with only one of them being reduced by the substrate. Heme is required for assembly of *B. subtilis* complex II; if heme is lacking, the apocytochrome to Cyt b_{558} is found in the membrane but soluble Fp and Ip subunits accumulate in the cytoplasm (Hederstedt & Rutberg, 1980; Fride'n & Hederstedt, 1990). The previously described complex II enzymes purified from mitochondria and aerobic Gram-negative bacteria use ubiquinone [$E_{m,7}$ = +112 mV (Thauer et al., 1977)] as electron acceptor in the

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[1] Abbreviations: Fp, flavoprotein; Ip, iron–sulfur protein; Cyt b_{558}, cytochrome b_{558}; Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMS, phenazine methosulfate; DCPIP, 2,6-dichlorophenolindophenol; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Q_1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone; Q_2, 2,3-dimethoxy-5-methyl-6-(3,7-dimethyl-2,6-octadienyl)-1,4-benzoquinone; DPB, 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone; NQ, 1,4-naphthoquinone; DMN, 2,3-dimethyl-1,4-naphthoquinone; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; EPR, electron paramagnetic resonance.

[2] Mammalian mitochondrial complex II may contain more than two integral membrane proteins (Capaldi, 1991).

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respiratory chain (Collins & Jones, 1981) whereas *B. subtilis* complex II donates electrons to menaquinone-7 (Lemma et al., 1990) [$E_{m,7} = -74$ mV (Thauer et al., 1977)]. The recently purified complex II from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* contains no heme and in vivo donates electrons to the unusual caldariella quinone (Moll & Schäfer, 1991).

Although *B. subtilis* complex II has been extensively studied, it has not been purified to homogeneity, which is essential for detailed enzymological and structural studies. Knowledge about the differences in the anchor part of complex II enzymes and the functional basis for the differences would help us to better understand electron transfer to quinone and the role of heme in this process. The aim of this work was to determine the chemical composition and to analyze the properties of purified *B. subtilis* complex II, focusing on the redox components of Cyt b_{558}. Using the *sdhC* gene on plasmids, the Cyt b_{558} subunit of *B. subtilis* complex II had previously been produced in large amounts in *E. coli* and partially purified (Fride'n et al., 1990). This strategy cannot be used to produce functional *B. subtilis* complex II since the FAD is not incorporated in *E. coli* (Hederstedt et al., 1987). We have in this work overexpressed and purified the enzyme from *B. subtilis*.

MATERIALS AND METHODS

Bacterial Strains. *B. subtilis* strains 3G18 (*trpC2, met, ade*), 3G18Δ101 (*trpC2, met, ade, ΔsdhCA, ble*) (Fride'n & Hederstedt, 1990), and KA98011 (*trpC2, sdhA11*) (Hederstedt et al., 1982) and *E. coli* strains JM83 [*ara*, Δ(*lac-proAB*), *rpsL, lacZ ΔM15*] (Yannisch-Perron et al., 1985) and 5K/pKIM4 (Ap^R) (Magnusson et al., 1985) were used in this work. *B. subtilis* strains were kept on TBAB plates (Difco Laboratories). *E. coli* strains were kept on LA plates (Sambrook et al., 1989).

General Genetic Techniques. *B. subtilis* strains were grown to competence as described by Arwert and Venema (1973) and kept in 20% (v/v) glycerol at −70 °C until used. *E. coli* was made competent using $CaCl_2$ (Mandel & Higa, 1979). *E. coli* plasmid DNA was isolated as described by Ish-Horowicz and Burke (1981). Agarose gel electrophoresis was performed according to standard procedures (Sambrook et al., 1989). Restriction enzymes and T4 DNA ligase were from Boeringer-Mannheim. Gene Clean (BIO 101 Inc. La Jolla, CA) was used for the isolation of DNA fragments from agarose gels.

Construction of Plasmids for the Overexpression of Complex II and Cyt b_{558}. The plasmid pHP13 (Cm^R, Em^R) was chosen as cloning vector for its high copy number in *E. coli* but low copy number in *B. subtilis* (Haima et al., 1987). The *sdhCAB* operon, coding for the polypeptides of *B. subtilis* complex II, was obtained from plasmid pSH1047 (Km^R, Cm^R, *sdhCAB*) (Hasnain et al., 1985). Plasmid pBSD1300 (Cm^R, Em^R, *sdhC*) was constructed in the following way. The 2.3-kb *Bam*HI–*Eco*RI fragment of pSH1047, containing the *sdhC* gene, was purified and ligated with dito cleaved pHP13. *E. coli* JM83 was transformed with the ligate, and transformants were selected on LA plates containing chloramphenicol (12.5 μg/mL) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/mL). Plasmid pBSD1200 (Cm^R, Em^R, *sdhCAB*) was constructed in two steps. First the purified 4.7-kb *Pst*I fragment of pSH1047, containing the *sdhA* and *sdhB* genes, was ligated with similarly cleaved pHP13 and transformed into *E. coli* JM83. A plasmid obtained, containing the *Pst*I fragment in the desired orientation, was cleaved with *Bam*HI and *Sst*II and ligated with the isolated 2.35-kb *Bam*HI–*Sst*II fragment of pSH1047 containing the *sdhC* gene. *E. coli* JM83 was transformed to chloramphenicol resistance with the final construct. The two plasmids, pBSD1300 and pBSD1200, were isolated from *E. coli* and transformed into *B. subtilis* 3G18. Plasmid pBSD1300 was also transformed into *B. subtilis* KA98011. *B. subtilis* transformants were selected on TBAB plates containing chloramphenicol (5 μg/mL).

Preparation of B. subtilis Membranes. The bacteria were grown in batches of 1 L in NSMP medium (Fortnagel & Freeze, 1968) with chloramphenicol (5 mg/L) in 5-L baffled E-flasks at 37 °C, aerated at 200 rpm, and harvested 1 h after the end of exponential growth phase (Hederstedt, 1986). Cells were washed once in 50 mM potassium phosphate buffer (pH 8.0) and suspended (30 g of wet weight/L) in the buffer containing $MgSO_4$ (10 mM), lysozyme (0.25 g/L, from chicken egg white, Sigma), DNase, and RNase (each 6 mg/L, from bovine pancreas, Sigma). After incubation for 45 min at 37 °C, Na-EDTA (pH 7.4) and 2 min later $MgSO_4$ were added to 15 and 20 mM final concentrations, respectively. The suspension was spun at 4 °C for 30 min at 5000 rpm (Beckman JA-10 rotor) to remove cell debris. Membranes were isolated from the supernatant by centrifugation for 40 min at 20 000 rpm (JA-20 rotor) and washed once in 0.1 M potassium phosphate buffer (pH 6.6). The membranes were finally suspended in 20 mM Na-MOPS (Cl) buffer (pH 7.4), frozen in liquid N_2, and stored at −70 °C.

Purification of Complex II. Membranes from *B. subtilis* 3G18/pBSD1200 were incubated with 6.7 g of Thesit [dodecylpoly(ethylene glycol ether), Boehringer Mannheim] per gram of membrane protein in 20 mM Na-MOPS (Cl) buffer (pH 7.4) for 20 min at 20 °C. Material not solubilized was removed by centrifugation at 4 °C for 45 min (45 000 rpm, Beckman Ti 50.2 rotor). The supernatant was applied on a DEAE-Sephacel (Pharmacia) column (2.6 × 6.5 cm) equilibrated with the MOPS buffer containing 0.1% (w/v) Thesit. The column was then washed with about 200 mL of equilibration buffer. A 210-mL 0–0.6 M NaCl gradient in the MOPS buffer with 0.1% (w/v) Thesit was applied, and complex II eluted at about 0.3 M NaCl. Peak fractions were pooled, dialyzed over night against the MOPS buffer containing 0.1% (w/v) Thesit, and concentrated 5 times using dialysis tubing embedded in poly(ethylene glycol) (PEG 20000, Merck). The concentrate (7 mL) was applied to a Sephacryl S-300 (Pharmacia) gel filtration column (88 × 2.6 cm) equilibrated with the MOPS buffer containing 0.1% (w/v) Thesit. Complex II with bound Thesit eluted at an apparent molecular mass of 440 kDa. Peak fractions were pooled and concentrated 5 times using poly(ethylene glycol) as before and finally to about $^1/_{10}$ the original volume using a Centricon-30 microconcentrator (Amicon). All purification steps were performed at 4 °C. Purified enzyme preparations were stored in an ice bath, protected from light.

Partial Purification of Cyt b_{558} from B. subtilis and E. coli. *B. subtilis* Cyt b_{558} synthesized in *E. coli* 5K/pKIM4 was isolated from solubilized membranes by fractionation on a Sephacryl S-300 column as described before (Fride'n et al., 1990), except that Triton X-100 was replaced by Thesit of corresponding concentrations. Cyt b_{558} expressed in *B. subtilis* KA98011/pBSD1300 was isolated from membranes by the same procedure as that used for *E. coli* 5K/pKIM4.

Complex II Enzyme Activity Measurements. Succinate: acceptor oxidoreductase activity (EC 1.3.99.1) was measured either with phenazine methosulfate (PMS) and 2,6-dichlo-

rophenolindophenol (DCPIP) or with quinone and DCPIP as electron acceptors. In the succinate:acceptor reductase assay, electrons are transferred from succinate via complex II to PMS or quinone and then to the final electron acceptor, DCPIP. Reduction of DCPIP was measured spectroscopically by the decrease in the absorbance at 600 nm (ϵ = 20.7 mM^{-1} cm^{-1}). Before assays, complex II was activated for 5 min at 30 °C with succinate and DCPIP present. The reaction was started by the addition of PMS or quinone. The following final concentrations were used in the assays: 20 μg/mL DCPIP, 20 mM potassium succinate, 0.02–0.05 μM complex II, and 0.5 mg/mL PMS or 50 μM quinone.

Succinate:quinone oxidoreductase activity (EC 1.3.5.1) was determined by measuring the absorbance changes in the UV region caused by the reduction (or oxidation) of the added quinone/quinol, using a dual-wavelength spectrophotometer. The quinones, wavelength pairs, and extinction coefficients used were as follows: 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0), 280/290 nm, 9.6 mM^{-1} cm^{-1}; 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone (Q_1), 280/290 nm, 9.6 mM^{-1} cm^{-1}; 2,3-dimethoxy-5-methyl-6-(3,7-dimethyl-2,6-octadienyl)-1,4-benzoquinone (Q_2), 282/250 nm, 9.7 mM^{-1} cm^{-1}; 1,4-naphthoquinone (NQ), 260/280 nm, 11.9 mM^{-1} cm^{-1}; 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone (DPB), 282/245 nm, 20.8 mM^{-1} cm^{-1}; 2,3-dimethyl-1,4-naphthoquinone (DMN), 270/290 nm, 15.2 mM^{-1} cm^{-1}. The assays were performed in an anaerobic cuvette (l = 0.5 cm) at the following final concentrations; 200 μM quinone, 20 mM potassium succinate (or 10 mM potassium fumarate), and 0.2 μM complex II. DMN was reduced in the cuvette by addition of $NaBH_4$ in water to a final concentration of 0.7 mM. The reaction was started by addition of complex II, activated by preincubation in the presence of 20 mM succinate at 30 °C.

All enzyme assays were done with confirmed excess of substrates in 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.4, at 30 °C, except for the temperature-dependence measurements in which the temperature was varied from 20 to 45 °C.

Light Absorption Spectroscopy. Spectroscopy at room temperature and 77 K was performed with a Shimadzu UV-3000 spectophotometer, using the 1-nm slit. Samples in 20 mM Na-MOPS (Cl) buffer (pH 7.4) were incubated with reductant for 5 min before freezing. Fourth derivatives of spectra were obtained using the data processing program of the spectophotometer and $\Delta\lambda$ set at 4 nm.

Reactivity of Cyt b_{558} with carbon monoxide was analyzed after bubbling the gas through the dithionite-reduced samples for 30 s and monitored as a decrease in the α-band absorption with time at room temperature.

Potentiometric Titrations. Redox titrations were done essentially as described by Wilson (1978). The 3-mL glass cuvette in which the titrations were performed was fitted with a calomel–platinum combination electrode, an argon gas line, and an inlet for additions by means of a 10-μL syringe. Oxygen was excluded from the cuvette by flushing continuously with argon. Before and after each potentiometric titration, the electrode was calibrated by measuring the potential [$E_{m,7}$ = +285 mV (Dawson et al., 1986)] of 10 mM quinhydrone in 50 mM Na-MOPS (Cl) buffer (pH 7.4) at 30 °C. All potentials stated are relative to the standard hydrogen electrode. Titrations were done in 50 mM MOPS buffer (pH 7.4) at 30 °C, on samples containing 6–10 μM protoheme IX. The titrations were carried out in the presence of a mixture of the following electron mediators: quinhydrone ($E_{m,7}$ = +285 mV), 2,3,5,6-tetramethyl-*p*-phenylenediamine ($E_{m,7}$ = +200 mV), phenazine methosulfate ($E_{m,7}$ = +70 mV), phenazine ethosulfate ($E_{m,7}$ = +30 mV), 1,2-naphthoquinone ($E_{m,7}$ = −24 mV), duroquinone ($E_{m,7}$ = −55 mV), 1,4-naphthoquinone ($E_{m,7}$ = −80 mV), and anthraquinone-1,5-disulfonic acid ($E_{m,7}$ = −174 mV). Each mediator was added to a final concentration of 50 μM except for 2,3,5,6-tetramethyl-*p*-phenylenediamine, which was used at 10 μM. Titrations were performed by the stepwise addition of small volumes of anaerobic 50 mM sodium dithionite or 50 mM potassium ferricyanide in the MOPS buffer.

Reduction of Cyt b_{558} was recorded as the difference in absorbance between the α-band maximum at 558 nm and the isosbestic point at 570 nm, using dual-wavelength spectroscopy. Each heme component contributes approximately 50% to the total absorption at 558 nm of the fully reduced cytochrome. The redox midpoint value for the high-potential heme was calculated from data in the interval 0–50% of total Cyt b_{558} reduction, using the Nernst equation. That for the low-potential heme was calculated in the same way, from data in the 50–100% interval.

EPR Spectroscopy. EPR spectra were recorded with a Bruker ER 200D-SRC X-band spectrometer equipped with a standard TE_{102} rectangular cavity and an Oxford Instruments ESR-9 helium flow cryostat. Quantitations of EPR spectra were performed under nonsaturating conditions as described earlier (Aasa & Vänngård, 1975) with Cu(II) in 2 M Na-ClO_2 (pH 2) as reference.

Miscellaneous Methods. Covalently bound FAD was determined essentially as described before (Hederstedt, 1980). Heme was determined from the pyridine hemochromogen difference (reduced minus oxidized) spectrum (Falk, 1964) using the extinction coefficients 23.98 mM^{-1} cm^{-1} (558 nm minus 540 nm) (Berry & Trumpower, 1985) or 29 mM^{-1} cm^{-1} (558 nm minus 570 nm) (Falk, 1964). Protein was determined according to Lowry et al. (1951) in the presence of 1% (w/v) sodium dodecyl sulfate or with the BCA method (Pierce), in both cases bovine serum albumin was used as standard. Quantitation of complex II antigen was done by "rocket" immunoelectrophoresis in the presence of Triton X-100 as described previously (Holmgren et al., 1979) using a complex II sample of known concentration (based on the [2Fe–2S] spin content) as standard. The preparation and specificity of the anti-complex II rabbit serum have been described before (Hederstedt & Rutberg, 1980). Iron contents were determined by atomic absorption with a Varian AA-1475 spectrophotometer at the Department of Analytical Chemistry, University of Lund. Amino acid analysis of complex II samples, hydrolyzed in 6 M HCl for 24 h, was performed at Aminosyraanalyscentralen, BMC, University of Uppsala. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to Schägger and von Jagow (1987) or according to Neville (1971). Molecular mass standards were *E. coli* β-galactosidase (130 kDa), bovine serum albumine (68 kDa), bovine liver catalase (57.5 kDa), pig liver fumarase (48.5 kDa), carbonic anhydrase (29 kDa), horse heart myoglobin (16.95 kDa), and chicken egg white lysozyme (14.3 kDa). Quinone content of complex II preparations was determined as described by Kröger (1978): 2.5 mL of 60% (v/v) methanol in light petroleum was added to purified complex II (200 μL, about 50 μM), the mixture was shaken for 10 min and centrifuged for 2 min (table centrifuge), the upper petroleum layer was saved, the lower layer and precipitate were again shaken with 1 mL of light petroleum, and the combined petroleum layers were evaporated. The

Table I: Composition of Membranes from Different *B. subtilis* Strains

strain	protoheme IX (nmol/mg of protein)	complex II[a] (nmol/mg of protein)	sp act.[b] (μmol/min·mg of protein)	turnover no.[b] (s^{-1})
3G18/pHP13	1.0	0.38	1.48	65
3G18/pBSD1200	2.7	1.2	4.19	58
3G18/pBSD1300	2.0	0.35	0.89	42
3G18Δ101/pHP13	0.2	0	<0.01	

[a] Determined as antigen by "rocket" immunoelectrophoresis. [b] Succinate dehydrogenase activity at 30 °C with PMS/DCPIP as electron acceptors.

resulting residue was dissolved in 1 mL of ethanol and the menaquinone content was estimated from the absorbance at 248 nm and $\epsilon = 18.9\ mM^{-1}\ cm^{-1}$ (Dunphy & Brodie, 1971).

RESULTS

Overproduction of B. subtilis Complex II. To facilitate studies on complex II in membranes as well as the purification of the enzyme, a *B. subtilis* strain, overproducing complex II is desired. Therefore a plasmid, pBSD1200, was constructed from the *B. subtilis/E. coli* shuttle vector pHP13 and a 6.1–kb DNA fragment containing the *B. subtilis sdhCAB* operon. A similar plasmid, pBSD1300, containing only the *sdhC* gene coding for Cyt b_{558} was also constructed. *B. subtilis* wild-type strain 3G18 was transformed with these two plasmids and pHP13. Expression of complex II in *B. subtilis* is influenced by the growth medium and also the growth phase (Hederstedt, 1986). To optimize expression of the enzyme the different strains were grown in a broth medium, containing chloramphenicol for plasmid retainment, and the cells were harvested when in early stationary growth phase. Membranes were isolated and analyzed for the content of protoheme IX and complex II and for succinate dehydrogenase activity (Table I). Complex II was found to be overproduced 2–3-fold in 3G18/pBSD1200 membranes compared to wild-type membranes (3G18/pHP13). The turnover number of the enzyme was approximately the same in membranes of both strains, confirming that the plasmid-encoded complex II was fully functional. To estimate the contribution from Cyt b_{558} to the protoheme IX content in different membranes, the strain 3G18Δ101/pHP13 was also analyzed. This strain has *sdhC* deleted from the chromosome and totally lacks complex II polypeptides in the membrane (Table I). In membranes from the wild-type strain about 80% of the protoheme IX present is in complex II, whereas in 3G18/pBSD1200 membranes about 90% is in complex II. Difference (reduced minus oxidized) light absorption spectra of membranes from 3G18/pHP13, 3G18/pBSD1200, and 3G18Δ101/pHP13 at 77K are shown in Figure 1.

In *B. subtilis* the vector pHP13, and presumably pBSD1200, has a copy number of about 5 (Haima et al., 1987). Despite this, the overproduction of complex II from the *sdhCAB* operon in pBSD1200 together with the chromosomal copy of the operon is at most 3-fold. Thus it appears that factors other than the gene dosage are limiting expression of complex II in 3G18/pBSD1200. Similarly, membrane preparations from strain 3G18/pBSD1300 contained less protoheme IX than that expected if expression was proportional to the *sdhC* gene copy number (Table I). Notably, the apparent turnover number of complex II in membranes isolated from 3G18/pBSD1300, which overexpresses only the Cyt b_{558} subunit, was consistently lower than that of complex II in membranes from 3G18/pHP13 and 3G18/pBSD1200.

Purification of Complex II. B. subtilis Complex II is efficiently solubilized from membranes by Triton X-100 (Hederstedt et al., 1979), but the succinate-reduced complex II was observed to lose succinate dehydrogenase activity in the presence of this detergent. A number of different detergents (Boehringer Mannheim Detergents Set 1124714) were tested for their ability to solubilize complex II in an active form, both in the oxidized and succinate-reduced states. Best results were obtained with the nonionic detergent Thesit.

Complex II (120 kDa) constitutes 10–15% of the total protein in the 3G18/pBSD1200 membrane (Table II). These membranes were extracted with Thesit, and the complex was purified from the solubilizate in two steps, by DEAE-Sephacel and Sephacryl S-300 chromatography. A purification protocol for a preparation of *B. subtilis* complex II from a 12-L culture is given in Table II. The recovery, calculated on complex II as antigen, was about 15%. The apparent succinate dehydrogenase activity of the enzyme increased during the purification process (Table II). Such a phenomenon has also been observed for *E. coli* complex II (Kita et al., 1989). Since the enzyme was activated before assay (see Materials and Methods), the apparent increase in activity seems not to be due to tightly bound oxaloacetate being progressively released from the enzyme.

The polypeptide compositions of samples from the different purification steps are shown in Figure 2. Judging from these gels, stained for protein with Coomassie brilliant blue, the isolated complex II was at least 95% pure. The complex II preparation which eluted from the Sephacryl S-300 column (Figure 2, panel A, lane 5) still contained some impurities (i.e., polypeptides of approximately 40 and 45 kDa), but these disappeared from the preparation during concentration (Figure 2, panel A, lane 6).

Protein was determined using the Lowry and the bicinchoninic acid (Pierce) procedures with bovine serum albumin as standard. By both methods the protein content of *B. subtilis* complex II preparations are overestimated several fold. Trichloroacetic acid precipitation of complex II before protein determination by the Lowry procedure did not affect the result, indicating that the overestimation is not due to interference from non–heme iron or acid-labile sulfide. The error in protein content estimations increases the more pure the enzyme preparation becomes, making the increase in specific activity apparently lower than the actual value (Table II). Due to the difficulties to accurately determine enzyme protein, complex II was routinely quantified as protein antigen by "rocket" immunoelectrophoresis, using a preparation of pure complex II with known concentration (determined by spin quantitation of the [2Fe–2S] cluster in the reduced enzyme) as reference. The specific activity of the purest preparation obtained would be 62 μmol of succinate oxidized per minute and per milligram of protein, calculated from a protein mass of 120 kDa for the complex and the measured turnover number presented in Table II.

Chemical Composition of Purified Complex II. The stoichiometry of Fp:Ip:Cyt b_{558} polypeptides has previously been determined as 1:1:1 by analysis of immunoprecipitated *B. subtilis* complex II labeled with radioactive amino acids (Hederstedt et al., 1979, 1987). Amino acid analysis of the now-purified complex II fully supported these previous results (data not shown). The chemical composition of the purified complex II is presented in Table III. The stoichiometric ratio between covalently bound FAD and the [2Fe–2S] cluster, representing the Fp and Ip subunit respectively, was found to be 1:1 as expected. Protoheme IX was present in about 2:1 stoichi-

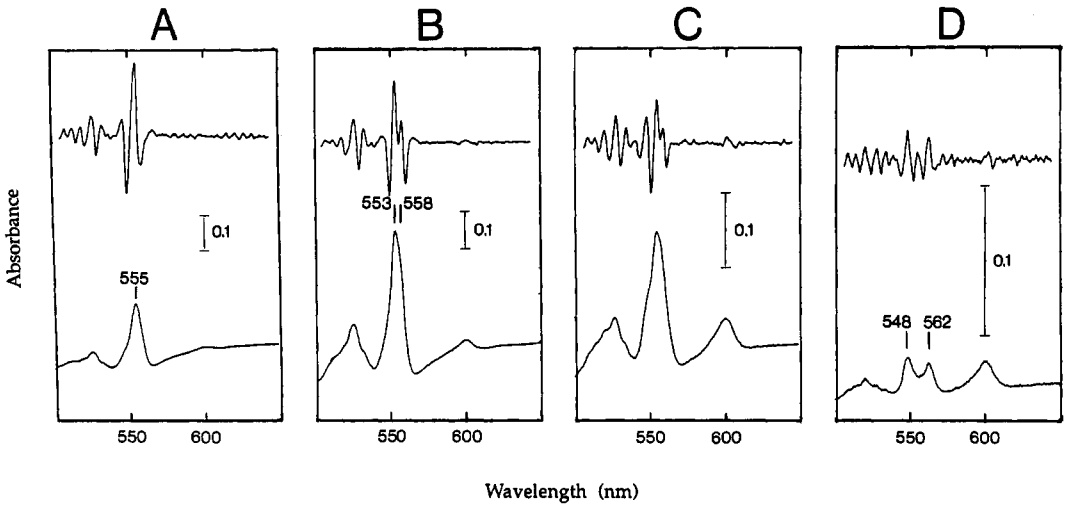

FIGURE 1: Light absorption difference (reduced minus oxidized) spectra at 77 K of membranes from *B. subtilis* strains containing different amounts of complex II: (A) 3G18/pBSD1200 (amplified for complex II) succinate (20 mM) reduced; (B) 3G18/pBSD1200 dithionite reduced; (C) 3G18/pHP13 (wild type) dithionite reduced; (D) 3G18Δ101/pHP13 (lacks complex II) dithionite reduced. Cytochromes *a* are not fully reduced by succinate under steady-state conditions, which explains the relatively low absorption at about 600 nm in (A) as compared to (B). The membrane protein concentration was 6 mg/mL and the cuvette light path length was 4 mm. The fourth derivatives of the spectra are shown in the upper part of the panels. The vertical bar in each panel represent 0.1 absorbance unit; i.e., the gain used for (D) is 4 times higher than that used for (A) and (B).

Table II: Purification Protocol for *B. subtilis* Complex II

purification step	protein (mg)	protoheme IX (nmol)	complex II[a] (nmol)	sp act.[b] (μmol/min·mg of protein)	turnover no.[b] (s^{-1})
membrane	598	1483	723	4.19	58
Thesit extract	361	770	413	7.19	105
DEAE-Sephacel pool	105	419	225	17.1	132
Sephacryl S-300 pool	40	222	120	22.5	125

[a] Determined as antigen by "rocket" immunoelectrophoresis. [b] Succinate dehydrogenase activity at 30 °C with PMS/DCPIP as electron acceptors.

ometry to covalently bound FAD, demonstrating the presence of two hemes per complex II. About 11 iron atoms per complex II were found, which is expected if the enzyme contains one [2Fe–2S] cluster, one [3Fe–4S] cluster, one [4Fe–4S] cluster, and two hemes. From these combined results we conclude that Cyt b_{558} is a diheme cytochrome.

Enzyme Activities. Succinate-dependent quinone reductase activity was measured spectroscopically either directly on the quinone or in a coupled assay where DCPIP was terminal electron acceptor. Activities were compared to that obtained with PMS and DCPIP. The physiological electron acceptor to *B. subtilis* complex II is meaquinone-7 (Lemma et al., 1990). This very hydrophobic, low-potential [E_m = −74 mV (Thauer et al., 1977)] quinone is however not practical to use for in vitro enzyme assays. A variety of water–soluble, high–potential quinones were tested for their ability to accept electrons from *B. subtilis* complex II (Table IV). Significant DCPIP reduction, 15% of that obtained in the presence of PMS, was obtained in the absence of added quinone. This endogenous DCPIP reductase activity is not due to the presence of extractable quinone in the enzyme preparation (Table III). Approximately 75% of the endogenous DCPIP reductase activity was blocked by 10 μM 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO). This concentration of HQNO did not affect succinate dehydrogenase activity when PMS was used as electron acceptor. The ubiquinone analogues Q_0, Q_1, Q_2, and DPB were reduced by the *B. subtilis* complex II at a rate 3–10 times lower than that obtained with PMS. For to us unknown reasons, we could only detect Q_0 reduction in the direct assay and not in the coupled assay using DCPIP. Generally, higher apparent activities were obtained in the coupled assay. HQNO blocked, although not completely, the reduction of all these ubiquinone analogues (Table IV). About 5 mol of HQNO per mole of enzyme blocked the reduction of Q_0 to 70% (not shown). The presence of 150 μM 2-thenoyltrifluoroacetone or 400 μM 3-methylcarboxin did not affect Q_2 reduction (not shown). The menaquinone analogue 1,4-NQ was reduced at a rate similar to the ubiquinone analogues, but the activity was not efficiently blocked by HQNO (Table IV).

The low-potential menaquinone analogue DMN was used to assay quinol oxidation with fumarate as electron acceptor, i.e., fumarate reductase activity. The turnover number observed with reduced DMN was 30% of the succinate dehydrogenase (PMS/DCPIP) activity. The fumarate reductase activity was inhibited to 95% by 10 μM HQNO (not shown). Thus, both quinone reduction and quinone oxidation by complex II can be blocked by HQNO.

Isolated *B. subtilis* complex II in 20 mM Na-MOPS (Cl) buffer (pH 7.4), 1% (w/v) Thesit could be stored on ice for 1 week with less than 5% loss of enzyme activity. Freezing in liquid N_2 and thawing resulted in about a 30% decrease in enzyme activity, measured using PMS/DCPIP and Q_2/DCPIP as electron acceptors. Complex II was found to be active in the examined temperature range 20–45 °C, and a roughly linear increase in turnover number was observed with increasing temperature; the succinate dehydrogenase (PMS/DCPIP) activity of the isolated enzyme at 20 and at 45 °C was 60% and 180%, respectively, compared to that at 30 °C.

Light Absorption Spectroscopy. UV–visible light absorption spectra at room temperature of isolated *B. subtilis* complex II are shown in Figure 3. The oxidized enzyme shows protein absorption at 275 nm and a cytochrome Soret band at 412 nm. Addition of succinate results in partial reduction (about 50%) of the cytochrome, gives a spectrum with absorption maxima at 558 and 528 nm (α- and β-bands), and causes a

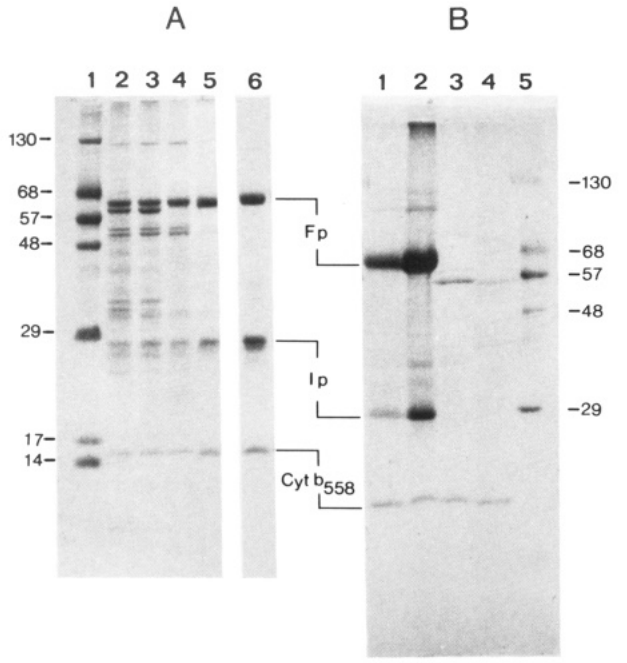

FIGURE 2: Polypeptide composition of samples from different steps of complex II purification, purified *B. subtilis* complex II, and partially purified cytochrome b_{558} subunit: (A) lane 1, protein molecular weight standard; lane 2, *B. subtilis* 3G18/pBSD1200 membranes; lane 3, Thesit extract; lane 4, DEAE-Sephacel pool; lane 5, Sephacryl S-300 pool; lane 6, complex II after concentration (35 pmol of heme). A 7.5-μg sample of protein was loaded in each lane. (B) lane 1, complex II (100 pmol of heme); lane 2, complex II (300 pmol of heme); lane 3, Cyt b_{558} partially purified from *B. subtilis* KA98011/pBSD1300 (100 pmol of heme); lane 4, Cyt b_{558} partially purified from *E. coli* 5K/pKIM4 (100 pmol of heme); lane 5, protein molecular weight standard. The Schägger/von Jagow [10% (w/v) acrylamide] and the Neville [12–15% (w/v) acrylamide gradient] gel systems were used for (A) and (B), respectively. The gels were stained with Coomassie brilliant blue R250.

Table III: Chemical Composition of Purified Complex II

component[a]	content[b] (nmol/mg of protein)	stoichiometry (mol/mol of [2Fe–2S] cluster)
covalently bound FAD	2.5 ± 0.2 (n = 4)	0.9
protoheme IX	5.8 ± 0.4 (n = 6)	2.0
[2Fe–2S]	2.9 (n = 1)	1
iron	32 ± 2 (n = 2)	11
menaquinone-7	<0.001 (n = 2)	

[a] FAD was determined as acid-nonextractable flavin. Protoheme IX was determined as pyridine hemochromogen. The [2Fe–2S] cluster was quantitated from the EPR spectrum of dithionite-reduced complex II. Iron was analyzed by atomic absorption spectroscopy. Menaquinone-7 was determined as petroleum ether extractable quinone. [b] Protein was determined with the Lowry method, which overestimates the protein content of complex II about 3-fold (see text). ± indicates the standard deviation in n determinations.

shift in the Soret peak to 424 nm. The presence of 5 mol of HQNO per mole of complex II had no detectable effect on the rate (full reduction within 10 s) or extent of reduction of Cyt b_{558} by 20 mM succinate. The fully reduced enzyme (dithionite) shows cytochrome α- and β-band absorption maxima at roughly the same wavelengths as the succinate-reduced enzyme, but the Soret peak is more symmetrical. Absorption in the 450-nm region decreases when the enzyme is reduced, due to the reduction of FAD and Fe–S clusters.

Light absorption spectra at 77 K of the isolated complex II are shown in Figure 4. The spectrum of the succinate-reduced enzyme exhibits a single symmetrical α-band absorption peak with maximum at 555 nm. Dithionite-reduced

Table IV: Enzyme Activities of Purified Complex II, with Different Electron Acceptors

additions	succinate:quinone reductase activity[a] DCPIP reductn (s^{-1}) observed	DCPIP reductn (s^{-1}) corrected[b]	observed quinone reductn (s^{-1})
–	14	0	
HQNO	4	0	
PMS	116	102	
PMS + HQNO	110	106	
Q_0	14	0	10
Q_0 + HQNO	4	0	1
Q_1	39	24	18
Q_1 + HQNO	5	1	2
Q_2	49	35	19
Q_2 + HQNO	7	3	2
DPB	27	13	10
DPB + HQNO	6	2	1
1,4-NQ	nd		11
1,4-NQ + HQNO	nd		8

[a] Experimental details are given in Materials and Methods. The enzyme activity measurements were reproducible within 10%. nd indicates that the activity was not determined. The final concentration of HQNO, when used, was 10 μM. [b] The activity obtained with DCPIP as only electron acceptor was subtracted from the activity observed.

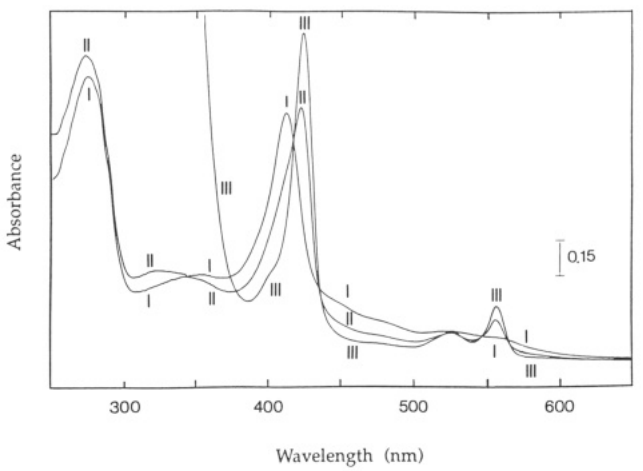

FIGURE 3: UV–visible light absorption spectra of purified complex II at room temperature: Trace I, oxidized state (as isolated); trace II, succinate (20 mM) reduced; trace III, dithionite reduced. The concentration of complex II was 2.5 μM, and a cuvette with 1-cm light path was used. Dithionite absorbs in the UV region (trace III). The vertical bar represents 0.15 absorbance unit.

complex II shows, in contrast, an asymmetrical α-band absorption peak with a shoulder on the "red" side. Absorption spectra of Cyt b_{558} in complex II in intact membranes were the same as in isolated complex II (compare Figure 1, panels A and B with Figure 4, panels A and B). This confirms the integrity of the isolated complex. The presence of 5 mol of HQNO per mole of complex II did not affect the low-temperature absorption spectra of succinate-reduced or dithionite-reduced Cyt b_{558}. The difference (dithionite minus succinate reduced) spectrum of the isolated complex II shows a split α-band, with absorption maxima at about 558 and 553 nm (Figure 4, panel C). These results show that the two hemes in the Cyt b_{558} polypeptide of *B. subtilis* complex II can be differentiated by their light absorption spectra and their reducibility. Evidently there is a succinate-reducible heme component in Cyt b_{558} which shows a single α-absorption peak and a low-potential heme component which shows a split α-absorption peak when reduced by dithionite.

Potentiometric Analysis of Cytochrome b_{558}. Redox titrations of cytochrome b in 3G18/pBSD1200 membranes and isolated complex II corroborated the presence of a high-

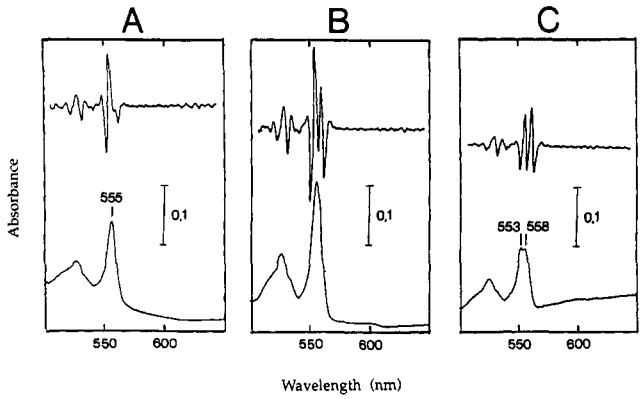

FIGURE 4: Light absorption spectra of purified complex II at 77 K: (A) absolute spectrum of succinate-reduced complex II; (B) absolute spectrum of dithionite-reduced complex II; (C) difference spectrum (B minus A). The fourth derivatives of the spectra are shown in the upper part of the panels. The concentration of complex II was about 5 μM, and the cuvette light path length was 4 mm.

potential and a low-potential heme component in Cyt b_{558} (Figure 5). The apparent midpoint potentials for the two heme components in the membrane-bound complex and in the isolated complex (in Thesit) were found to be different, −132 and +16 mV in the membrane and −95 and +65 mV in the isolated enzyme. Potentials similar to those of the isolated complex were obtained for complex II in 3G18/pBSD1200 membranes in the presence of 1% (w/v) Thesit. The high-potential heme component contributes about 50% to the total absorption at 558 nm of the fully reduced cytochrome (Figure 5). This extent of reduction correlates well with that obtained using succinate as reductant (Figures 3 and 4). We conclude that succinate can fully reduce the high-potential heme component ($E_{m7.4} \geq +16$ mV) but does not reduce the low-potential heme component ($E_{m7.4} \leq -95$ mV) in complex II. The succinate/fumarate couple has an $E_{m,7}$ of +33 mV (Thauer et al., 1977).

EPR Spectroscopy. Previous cryogenic low-resolution EPR spectra of oxidized Cyt b_{558} in immunoprecipitated *B. subtilis* complex II showed a low-spin heme iron signal with g_{max} at about 3.5 (Hederstedt & Andersson, 1986). The EPR spectrum of the here-purified complex II in the oxidized state showed signals at $g = 3.42$ and $g = 3.68$ from two low-spin heme iron atoms (Figure 6, trace A). g_x and g_y could not be detected with certainty for the cytochrome, therefore an accurate quantitation cannot be made (Aasa & Vänngård, 1975), but judging from the shapes and g values of the observed lines, the two hemes seems to be present in about equimolar amounts. The oxidized complex II also showed EPR signals at $g = 4.2$ from adventitious iron in the sample and at $g = 2.02$ from the [3Fe–4S] cluster [Hederstedt et al. (1985) and data not shown]. The $g = 3.68$ heme iron signal disappeared from the spectrum upon reduction with succinate, and both the $g = 3.68$ and $g = 3.42$ signals were absent in the EPR spectrum of the dithionite-reduced complex II (Figure 6, traces B and C). Thus, the high- and the low-potential heme components show different EPR spectra; i.e., the $g = 3.68$ signal originates from the high-potential, succinate-reducible heme iron, and the $g = 3.42$ signal is from the low-potential heme iron. The EPR signals of the two heme irons showed different power saturation curves; i.e., the $g = 3.68$ signal was more difficult to saturate than the $g = 3.42$ signal. The power saturation curve of the $g = 3.42$ signal was the same for oxidized and succinate-reduced complex II. Both the succinate- and dithionite-reduced complex II showed signals at $g = 2.03$, $g = 1.94$, and $g = 1.89$ from the [2Fe–2S] cluster [Hederstedt et al. (1985) and data not shown]. The [2Fe–2S] center was reduced to about 70% by 10 mM succinate.

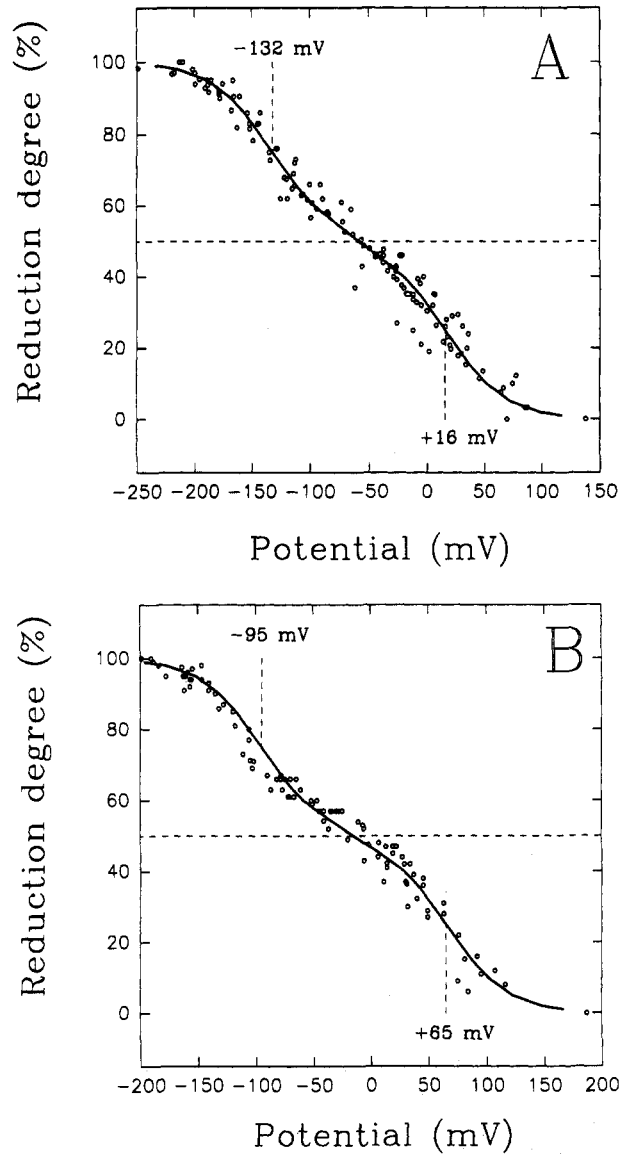

FIGURE 5: Potentiometric analysis of cytochrome b_{558} in complex II: (A) redox titration of *B. subtilis* 3G18/pBSD1200 membranes (containing amplified amounts of complex II); (B) redox titration of purified complex II. The plots shown are the combined results from five and four independent titrations of membranes and complex II, respectively. The midpoint potentials indicated are at pH 7.4. The fitted curve was calculated from the Nernst equation ($n = 1$) and the two indicated midpoint potentials. In this calculation it was assumed that absorption from the two heme components only overlaps in the interval −96 to −20 mV (membranes) and −59 to +29 mV (purified complex II).

Comparison of Cyt b_{558} in Complex II and in Isolated Cytochrome Subunit. Light absorption spectra at 77 K (Fride'n & Hederstedt, 1990) and EPR spectra (Fride'n et al., 1990) of the *B. subtilis* Cyt b_{558} subunit overexpressed in *E. coli* differ from those of complex II in that the asymmetrical α-band absorption peak of the dithionite-reduced cytochrome has the shoulder on the "blue" side instead of on the "red" side and the EPR spectrum of the oxidized cytochrome does not show two resolved signals. Are these differences because the protein has been synthesized in *E. coli*, or are they a result of the absence of bound Fp and Ip? To answer the first part of the question, Cyt b_{558} was overexpressed in *B. subtilis* KA98011/pBSD1300. Strain KA98011 has a mutation in the *sdhA* gene that results in loss of the Fp subunit, while the

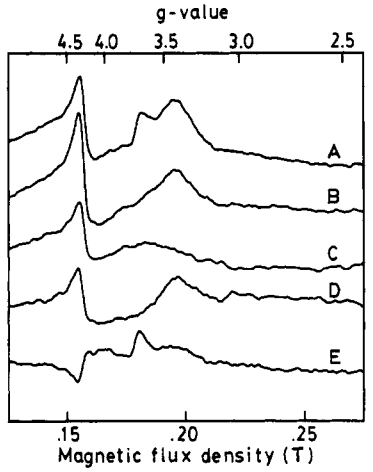

FIGURE 6: EPR spectra of *B. subtilis* complex II showing signals from cytochrome b_{558}: Trace A, oxidized state; trace B, succinate-(10 mM) reduced state; trace C, dithionite-reduced state; trace D, difference spectrum B minus C; trace E, difference spectrum A minus B. Each trace is the average of four scans. Experimental conditions: Microwave frequency, 9.37 GHz; power, 2 mW; modulation amplitude, 2 mT; temperature, 11 K. The complex II concentration was 54 μM.

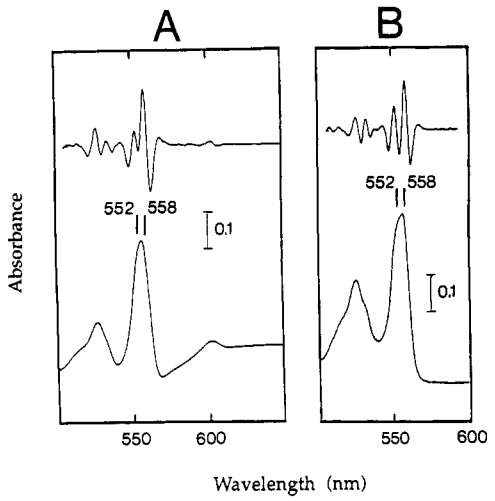

FIGURE 7: Light absorption spectra at 77 K of cytochrome b_{558} from *B. subtilis* KA98011/pBSD1300: (A) Cyt b_{558} in the membrane (dithionite-reduced minus ferricyanide-oxidized difference spectrum); (B) Cyt b_{558} partially purified (absolute spectrum of dithionite-reduced sample). The membrane protein concentration was 6 mg/mL, the heme content of the partially purified cytochrome was 20 μM, and the cuvette light path length was 4 mm. The fourth derivatives of the spectra are shown in the upper part of the panels. The fourth derivative of the spectrum in (A) was recorded with half the gain used for the direct spectrum.

Ip subunit is found in the cytoplasm. The strain contains wild-type Cyt b_{558} in the membrane (Hederstedt & Rutberg, 1980; Hederstedt et al., 1982). A light absorption spectrum at 77 K of membranes from KA98011/pBSD1300 is shown in Figure 7. The bulk of the absorption around 558 nm is due to Cyt b_{558} (compare with Figure 1, panel D). The features of the 558-nm absorption peak of Cyt b_{558} in *B. subtilis* KA98011/pBSD1300 membranes are the same as those for the corresponding peak of the cytochrome in *E. coli* 5K/pKIM4 membranes (not shown). Plasmid pKIM4 carries the *B. subtilis sdhC* gene and about 5% of the total membrane protein in *E. coli* 5K/pKIM4 is *B. subtilis* Cyt b_{558}, which therefore dominates the light absorption spectrum (Magnusson et al., 1985).

Cyt b_{558} was solubilized with Thesit from both *B. subtilis* KA98011/pBSD1300 and *E. coli* 5K/pKIM4 membranes

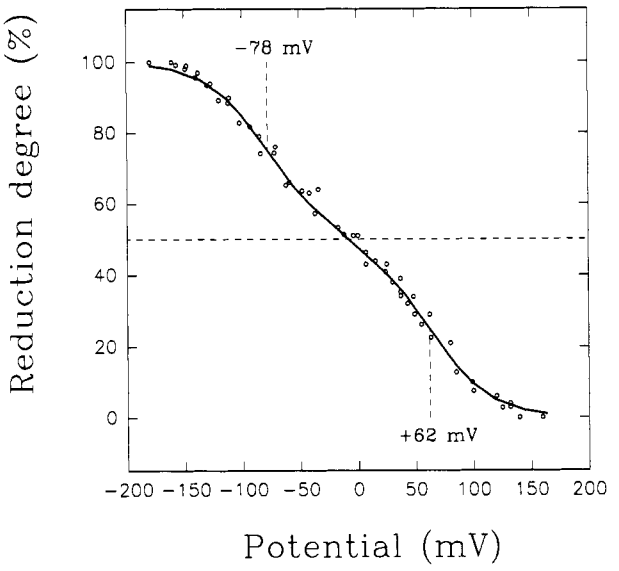

FIGURE 8: Potentiometric analysis of cytochrome b_{558} subunit isolated from *E. coli* 5K/pKIM4. The plot shown is the combined results of two independent titrations. The midpoint potentials indicated are at pH 7.4. The fitted curve was calculated as in Figure 5, and the two heme components were assumed to show overlapping absorption only in the interval −42 to +26 mV.

and partially purified (Figure 2, panel B, lanes 3 and 4). The light absorption spectra of the two preparations were identical, and very similar to that of the Cyt b_{558} subunit in the membrane (Figure 7 and data not shown). From these results we conclude that the light absorption spectrum of Cyt b_{558} is not dependent on whether it was expressed in *B. subtilis* or in *E. coli*. Furthermore, the spectrum was not altered after extraction of Cyt b_{558} from the membrane. It is evident that the spectral properties of the isolated Cyt b_{558} subunit (Figure 7, panel B) differ from those of the cytochrome subunit in complex II (Figure 4, panel B); i.e., the isolated, dithionite-reduced Cyt b_{558} subunit shows a broader α-absorption band and has the "shoulder" on the opposite side compared to complex II. A difference was also observed in the reactivity of the dithionite-reduced cytochrome with carbon monoxide; after 30 min of incubation with carbon monoxide at room temperature, about 10% of Cyt b_{558} in complex II had reacted, whereas in isolated Cyt b_{558} subunit about 60% had reacted.

A redox titration of isolated Cyt b_{558} subunit is shown in Figure 8. Two $n = 1$ redox components were observed with apparent midpoint potentials of −78 and +62 mV. The high-potential component contributed to about 50% of the total absorption at 558 nm. This demonstrates that isolated Cyt b_{558} subunit also contains two heme components with relative extinction coefficients and midpoint potentials similar to those in complex II.

EPR spectra of oxidized Cyt b_{558} subunit isolated from *E. coli* 5K/pKIM4 (Fride'n et al., 1990) and *B. subtilis* KA98011/pBSD1300 (not shown) exhibit two g_{max} signals ($g = 3.39$ and $g = 3.64$), but these are broader than those of *B. subtilis* complex II and are therefore not well resolved. The power saturation curves for the $g = 3.39$ and the $g = 3.64$ signals of the isolated Cyt b_{558} subunit were not significantly different from those of the $g = 3.42$ (low-potential heme) and $g = 3.68$ (high-potential heme) signals, respectively, of the complex II. The light absorption spectra, carbon monoxide reactivity, and EPR spectra together demonstrate that the structure around the heme components in Cyt b_{558} is not identical in complex II and in isolated cytochrome subunit.

DISCUSSION

Succinate:quinone oxidoreductases from both aerobic and anaerobic organisms consist of two membrane-extrinsic subunits, a FAD-containing protein and an iron–sulfur cluster containing protein, and one or two[2] membrane-intrinsic polypeptides which are required for membrane binding of the two other subunits and for interaction with quinone (Ackrell et al., 1992). In all these enzymes studied, except for *S. acidocaldarius* complex II (Moll & Schäfer, 1991) and *E. coli* fumarate reductase complex (Cole et al., 1985), the membrane-anchor part is a cytochrome *b*. We have in this work overproduced *B. subtilis* complex II 2–3-fold in membranes and purified the enzyme in the presence of the nonionic detergent Thesit. The membrane anchor, the 23-kDa Cyt b_{558} subunit, was found to contain two protoheme IX groups as determined by direct chemical analysis as well as by EPR spectroscopy and light absorption spectroscopy in combination with redox titrations. *B. subtilis* Cyt b_{558} can thus be added to the growing list of membrane-bound diheme cytochromes *b* which includes 30-kDa cytochrome *b* of *Wolinella succinogenes* fumarate reductase (Körtner et al., 1990), *E. coli* 20-kDa cytochrome b_{561} of unknown function (encoded by the *cybB* gene) (Nakamura et al., 1988), *E. coli* 26-kDa cytochrome *b* of nitrate reductase (encoded by the *narI* gene) (Blasco et al., 1989), and 38–48-kDa cytochrome *b* of cytochrome bc_1 complexes (Trumpower, 1990).

Purified *B. subtilis* complex II did not contain endogenous menaquinone as analyzed by quinone extraction. The enzyme showed activity with a variety of quinones, both benzo- and naphthoquinones, confirming previous data obtained with a partially purified preparation (Lemma et al., 1991). Quinone reductase activity was sensitive to low concentrations of HQNO. The effective mammalian complex II inhibitors 2-thenoyltrifluoroacetone and 3-methylcarboxin (Ackrell et al., 1992) at >100 μM concentration had no effect on the quinone reductase activity. *B. subtilis* complex II showed significant DCPIP reductase activity without any addition of quinone. Such activity has also been reported for the *S. acidocaldarius* complex II (Moll & Schäfer, 1991) and the mammalian complex II (Tushurashvili et al., 1985). The DCPIP reductase activity of the *B. subtilis* complex was sensitive to HQNO but could not be totally blocked by this inhibitor.

The apparent midpoint potentials of the substrates and redox components in *B. subtilis* complex II moving from the Fp, the Ip, and to the Cyt b_{558} subunit are as follows; succinate/fumarate, +33 mV (Thauer et al., 1977); FAD/$FADH_2$, $E_{m,7}$ not determined but can be assumed to be about −80 mV (Ackrell et al., 1975; Ohnishi et al., 1981); [2Fe–2S], +80 mV; [3Fe–4S], −25 mV; [4Fe–4S], −240 mV (Maguire et al., 1986); high-potential heme component, +65 mV at pH 7.4 (this work); low-potential heme component, −95 mV at pH 7.4 (this work); menaquinone-7/menaquinol-7, −74 mV (Thauer et al., 1977). The high-potential heme component is reducible by succinate and shows in the reduced state a symmetrical α-band light absorption peak at 555 nm at 77 K (558 nm at room temperature) and in the oxidized state an EPR signal at $g = 3.68$. The low-potential heme component shows in the oxidized state an EPR signal at $g = 3.42$, and when reduced by dithionite, it exhibits a split α-band light absorption peak with maxima at 553 and 558 nm at 77 K.

Complex II of mammalian mitochondria, *Ascaris suum* adult worm muscle mitochondria, and *E. coli* contain only one heme group and this heme seems to have the features of either the high- or low-potential heme component of *B. subtilis* complex II. Cytochrome *b* of mammalian complex II is low potential ($E_{m,7} = -185$ mV) (Yu et al., 1987) and not reducible by succinate, but the reduced cytochrome can be oxidized by fumarate (Hatefi & Galante, 1980). It shows an EPR signal at $g = 3.46$ (Orme-Johnson et al., 1971; Yu et al., 1987) and a split α-band light absorption peak when reduced by dithionite (Yu et al., 1987; Davis et al., 1973). The cytochrome in *A. suum* complex II is partly reduced by succinate, has an $E_{m,7}$ of −34 mV and an EPR signal at $g = 3.6$, and shows a split α-band absorption peak (Takamiya et al., 1986, 1990; Kita et al., 1988). *E. coli* complex II cytochrome *b* is reduced by succinate, has an $E_{m,7}$ of +36 mV, and shows a symmetrical α-band light absorption peak at 556 nm at 77 K (Condon et al., 1985; Kita et al., 1989). Thus at least superficially it appears as if the mammalian and the *E. coli* enzymes contain the heme which corresponds in properties to the low- and high-potential hemes, respectively, of *B. subtilis* complex II. The *A. suum* enzyme, which in vivo functions as a fumarate reductase, has a cytochrome *b* with some properties of the high and some properties of the low-potential type of heme components. The potentials of the heme components in *W. succinogenes* fumarate reductase are −20 and −200 mV, and also in this case, the two heme components show different light absorption spectra (Unden et al., 1980). The roles of heme and the [4Fe–4S] cluster in electron transfer between the two catalytic sites of succinate:quinone oxidoreductases (succinate/fumarate and quinone/quinol active sites) remain obscure especially in the light of the occurrence of different functional complexes that contain from zero to two hemes (Ackrell et al., 1992). One important question is whether the low-potential heme component of mammalian and *B. subtilis* complex II and *W. succinogenes* fumarate reductase is ever reducing during catalytic turnover of the enzymes. Both parallel and strictly sequential electron-transfer pathways involving the low-potential tetranuclear iron–sulfur cluster and the low-potential heme have been suggested for the mammalian complex II [for recent overviews, see Ackrell et al. (1992) and Salerno (1991)].

Previous experiments on *B. subtilis* complex II have indicated that the cytochrome *b* has both structural and functional importance, since the complex cannot be assembled in the absence of heme (Holmgren et al., 1979; Hederstedt & Rutberg, 1980; Fride'n & Hederstedt, 1990). Heme in *B. subtilis* Cyt b_{558} subunit isolated and in complex II is not in an identical environment as demonstrated by differences in the EPR spectrum of the oxidized cytochrome and the light absorption spectrum at 77 K of the dithionite-reduced cytochrome. We also found that the isolated dithionite-reduced Cyt b_{558} subunit slowly reacted with carbon monoxide whereas the intact complex II was essentially nonreactive. These observations indicate that the Fp and Ip polypeptides, or redox components in these subunits, protect the heme against solvent or interact with the heme in the assembled complex. We did not detect magnetic interaction between the low-potential heme and the [2Fe–2S] or [3Fe–4S] cluster; i.e., the power saturation curve for the $g_{max} = 3.42$ signal was the same in the oxidized (where the [3Fe–4S] cluster is paramagnetic) and succinate-reduced ([2Fe–2S] cluster paramagnetic) complex II. Furthermore, the power saturation curves for the heme iron EPR signals from isolated Cyt b_{558} subunit and from complex II were not significantly different. More drastic differences have been reported in the properties of the bovine heart cytochrome b_{560} subunit isolated and in complex II: the EPR spectrum of the heme iron shifts from $g_{max} = 3.07$ to $g_{max} = 3.46$, the symmetrical α-band absorption peak changes from symmetrical to asymmetrical, and the re-

dox potential changes from −144 to −185 mV when Fp and Ip are bound to isolated cytochrome b_{560} (Yu et al., 1987).

Results from EPR and near-infrared magnetic circular dichroic spectroscopy on *B. subtilis* Cyt b_{558} produced in *E. coli* strongly suggest bis(histidine) axial ligation of both hemes (Fride'n et al., 1990). There are six histidine residues in the Cyt b_{558} polypeptide. Two, His^{13} and His^{47}, have by site-specific mutagenesis experiments been ruled out as heme ligands [Fride'n and Hederstedt, (1990) and our unpublished data]. The remaining four histidine residues are located in different predicted transmembrane protein segments. Sequence comparisons indicate that these residues, at positions 28, 70, 113, and 155 in *B. subtilis* Cyt b_{558}, are conserved in cytochrome *b* of *W. succinogenes* fumarate reductase (Körtner et al., 1990). Assuming a near-perpendicular orientation of the heme planes relative to the membrane plane, we conclude from the combined experimental findings [Fride'n et al. (1987, 1990), Fride'n (1990), Fride'n and Hederstedt (1990), and our unpublished data] that His^{28} and His^{113} are axial ligands to one heme and His^{70} and His^{155} are axial ligands to the other heme. Degli Esposti et al. (1991) have proposed an alternative model,[3] based on only four transmembrane segments, where His^{28} and His^{70} ligate one (high-potential) heme and His^{113} and His^{155} ligate the other (low-potential) heme.[3]

Cyt b_{558} of *B. subtilis* complex II has several features in common with cytochrome *b* of mammalian and bacterial cytochrome bc_1 complexes (quinol:cytochrome *c* reductase; complex III): bis(histidine) axial ligation of both heme groups; the high- and low-potential hemes show a symmetrical and a split α-band light absorption peak and give EPR spectra with $g_{max} = 3.8$ and $g_{max} = 3.4$, respectively (Ohnishi et al., 1989; Trumpower, 1990). The high-potential heme in the bc_1 complex is located closest to the negative side of the membrane (Trumpower, 1990; Yun et al., 1991). The structural organization of the two cytochromes *b* must however be different because in the bc_1 complex both hemes are ligated "on top" of each other between two transmembrane α-helical protein segments (Link et al., 1986) whereas in *B. subtilis* Cyt b_{558} the histidine residues serving as axial ligands seem to be distributed on four different α-helical segments (Degli Esposti, 1989; Fride'n & Hederstedt, 1990). There is no significant amino acid sequence similarity of *B. subtilis* cytochrome b_{558} to cytochrome *b* of bc_1 complexes except for short sequences around some of the histidine residues (Magnusson et al., 1986; Degli Esposti, 1989; Körtner et al., 1990). The function of the two hemes in the bc_1 complex is transmembrane electron transfer. This role of heme in complex II is not anticipated from the known function and topology of the enzyme, but the question remains to be investigated.

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[3] This model was proposed for the homologous *W. succinogenes* cytochrome *b* where the corresponding histidine residues are at positions 44, 93, 143, and 182.

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