Two Hydrophobic Subunits Are Essential for the Heme b Ligation and Functional Assembly of Complex II (Succinate-Ubiquinone Oxidoreductase) from Escherichia coli*

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Complex II (succinate-ubiquinone oxidoreductase) from Escherichia coli is composed of four nonidentical subunits encoded by the sdhCDAB operon. Gene products of sdhC and sdhD are small hydrophobic subunits that anchor the hydrophobic catalytic subunits (flavoprotein and iron-sulfur protein) to the cytoplasmic membrane and are believed to be the components of cytochrome b₅₆ in E. coli complex II. In the present study, to elucidate the role of two hydrophobic subunits in the heme b ligation and functional assembly of complex II, plasmids carrying portions of the sdh gene were constructed and introduced into E. coli MK3, which lacks succinate dehydrogenase and fumarate reductase activities. The expression of polypeptides with molecular masses of about 19 and 17 kDa was observed when sdhC and sdhD were introduced into MK3, respectively, indicating that sdhC encodes the large subunit (cybL) and sdhD the small subunit (cybS) of cytochrome b₅₆. An increase in cytochrome b content was found in the membrane when sdhD was introduced, while the cytochrome b content did not change when sdhC was introduced. However, the cytochrome b expressed by the plasmid carrying sdhD differed from cytochrome b₅₆ in its CO reactivity and red shift of the absorption peak at 557.5 nm at 77 K. Neither hydrophobic subunit was able to bind the catalytic portion to the membrane, and only succinate dehydrogenase activity, not succinate-ubiquinone oxidoreductase activity, was found in the cytoplasmic fractions of the cells. In contrast, significantly higher amounts of cytochrome b₅₆ were expressed in the membrane when sdhC and sdhD genes were both present, and the catalytic portion was found to be localized in the membrane with succinate-ubiquinone oxidoreductase and succinate oxidase activities. These results strongly suggest that both hydrophobic subunits are required for heme insertion into cytochrome b₅₆ and are essential for the functional assembly of E. coli complex II in the membrane. Accumulation of the catalytic portion in the cytoplasm was found when sdhCDAB was introduced into a heme synthesis mutant, suggesting the importance of heme in the assembly of E. coli complex II.

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Complex II (succinate-ubiquinone oxidoreductase) is a tricarboxylic acid cycle enzyme associated with the inner membrane of mitochondria or the cytoplasmic membrane of bacteria (1, 2). Complex II in E. coli is encoded by the sdhCDAB operon (3, 4) and contains four nonidentical subunits and five prosthetic groups (5, 6). The largest hydrophilic subunit (flavoprotein; Fp), encoded by sdhA, has covalently bound FAD, and the second largest subunit (iron-sulfur protein; Ip), encoded by sdhB, contains three different types of iron-sulfur clusters termed S-1[2Fe-2S], S-2[4Fe-4S], and S-3[3Fe-4S]. Two hydrophobic heme b-containing subunits are encoded by sdhC and sdhD, and at least the sdhC product is a component of cytochrome b₅₆ (7).

Generally, the Fp and Ip subunits comprise the catalytic portion of complex II and catalyze electron transfer from succinate to artificial electron acceptors such as phenazine methosulfate or 2,6-dichlorophenol-indophenol (DCIP) (succinate dehydrogenase; SDH). The amino acid sequences of Fp and Ip are highly conserved among species. For example, the amino acid sequences of Fp and Ip from human liver (8, 9) show 50% similarity to their counterparts in E. coli complex II. The Fp and Ip subunits of succinate-ubiquinone oxidoreductase are also closely related to those of bacterial and mitochondrial fumarate reductases (FRDs), which catalyze the reverse reaction of SDH activity (10–12). In E. coli, FRD encoded by the frdABCD operon is synthesized during anaerobic growth, whereas complex II encoded by the sdh operon is induced in aerobic culture. Studies on the primary structures of different Fps and Ips and the effect of site-directed mutagenesis reveal structural and functional relationships in the binding of prosthetic groups and substrate recognition (1, 2).

It is commonly accepted that the intact four-subunit complex II contains a cytochrome b component composed of hydrophobic large (cybL) and small (cybS) subunits. Exceptions are the enzyme from Bacillus subtilis, which contains two cytochrome b components bound to a single large hydrophobic subunit (13), and FRD from E. coli which does not contain heme b. In contrast to the well characterized Fp and Ip subunits, numerous questions concerning the cytochrome b in complex II remain to be solved due to limited information about the primary structures of the subunits. The nucleotide sequences of both hydrophobic subunits in complex II have only been determined

† The abbreviations used are: Fp, flavoprotein; Ip, iron-sulfur protein; SDH, succinate dehydrogenase; FRD, fumarate reductase; cybL, large subunit of cytochrome b; cybS, small subunit of cytochrome b; PCR, polymerase chain reaction; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; kbp, kilobase pair(s); MT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,4-tetrazodium bromide; DCIP, 2,6-dichlorophenol-indophenol; HPLC, high pressure liquid chromatography.
for E. coli (3) and Saccharomyces cerevisiae (14–16), while that of cybL has been reported in bovine complex II (17, 18). Amino acid sequences of these hydrophobic subunits in complex II from different species show little conservation. The contents and redox properties of heme b in complex II from different organisms vary greatly. B. subtilis complex II contains two hemes b, and the heme with the higher potential (Em = +65 mV) is reduced by succinate, while the heme with the lower potential (Em = −95 mV) is not reduced (19, 20). E. coli and Ascaris suum suum complex II have one heme b in complex II, and the heme b in E. coli complex II (Em = +36 mV) is fully reducible by succinate (6), while the heme b in A. suum complex II (Em = −34 mV) is partially reduced by succinate (21, 22). Bovine cytochrome b556 (Em = −185 mV) is not readily reducible by succinate, and low substoichiometric amounts of heme b are found in this enzyme complex (23). Furthermore, purified complex II from yeast has no heme b (24). Extensive studies on the role of cytochrome b in complex II have been carried out using the B. subtilis enzyme. In B. subtilis complex II, insertion of heme into the apocytochrome b556 subunit is critical for the assembly of the membrane-bound enzyme (25, 26), suggesting a structural role for the cytochrome. Electron paramagnetic resonance (EPR) and near-infrared magnetic circular dichroism (MCD) studies showed that each of the two hemes has bis-histidine ligation (26, 27). Furthermore, site-directed mutagenesis studies have identified the histidine residues that are ligated to heme b (25, 26). However, it is difficult to define accurately the structure and function of the two-subunit cytochrome b complex II, because the B. subtilis complex II has a single large hydrophobic subunit, although this subunit seems to have arisen due to protein fusion between cybL and cybS of the two-subunit cytochrome b (28). Another example of well-characterized hydrophobic subunits in complex II is the frdC and frdD gene products in E. coli FRD (29–31). The role of the two subunits in the synthesis of complex II, and the amino acid residues essential for quinone binding have been analyzed (32–34). However, this enzyme complex does not contain heme b, so the function of heme b in the two-subunit cytochrome b found in most bacterial and mitochondrial complex II has not been analyzed.

Cytochrome b556 from E. coli complex II has been purified and characterized by the present authors during the systematic analysis of cytochromes, including cytochrome bo and bd oxidase, in the membranes of aerobically grown E. coli (35–37). The first 24 residues from the N-terminal amino acid sequence of the cytochrome are identical to residues 4–27 deduced from E. coli sdhC (7). We also have established a one-step protocol to purify complex II by chromatography using an E. coli strain that overproduces the enzyme by 10-fold due to the presence of a multicopy plasmid containing the cloned sdh operon (6). Using this preparation, bis-histidine ligation of the heme in cytochrome b556 was demonstrated by EPR and MCD (38). However, it is not known whether cybL alone or cybL plus cybS provide coordination to the heme.

In the present study, the role of the two hydrophobic subunits in heme ligation and the functional assembly of complex II in the membrane was investigated using plasmids carrying portions of the E. coli sdh operon.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains and Plasmid Construction**—E. coli strain MK3, which is an sdh- and frd- double mutant, was constructed by P1 transduction from strain GVK124 (sdh::Km, recA, SmR) into the recipient strain MK3 (sdhABCD, ΔAmpC, recA, SmR). E. coli strain H500, which lacks the hema gene, was constructed by homologous recombination between λshanA and the chromosome of LE392.2 Plasmids carrying portions of the sdh gene in vectors pACYC184 and pUC19 were derived from a 4.45-kbp BamHI fragment in pGS133 (3). The list of plasmids used in these experiments and the schematic representation of the fragments from the sdh operon are shown in Table I and Fig. 1, respectively. The fragment for the plasmid pSDHC was amplified by PCR using a set of primers and sdhCDAB in pUC19 as a template. Primers were primer A (M13 reverse primer) and primer B (5'-CGTCTGGCATGTAATATCAG-TACGCGATTTTAATCATG-TCTGCGATTTTAATCATG-TCTGCGATTTTAATCATG-TCTGCGATTTTAATCATG-3'). The PCR product was digested with BamHI and NruI, and inserted into the 3.6-kbp fragment of pACYC184 obtained by BamHI and NruI treatment. The plasmid pSDHAB was also constructed by PCR. Amplification of the sdhD'A' fragment was performed with a primer set 5'-AAGGTTTGACATGTAAGCTGGATGATC-3', for sdhD' containing a HindII site (boldface type) and 5'-TGGGTTGAATCCATGGCCACT-3' designed from the sequence downstream of the Nad site in sdhA. The product was digested with HindII and Nael and then inserted into a 2.1-kbp fragment of pSDHAB obtained by treatment with HindII and Nael.

Growth of E. coli—E. coli MK3 and these transformants were grown aerobically on LB medium with kanamycin (final concentration 50 μg/ml), E. coli H500 and the transformants were also grown aerobically on phosphate-buffered LB medium (50 mM, pH 7.0; Ref. 39) with 0.2% (w/v) glucose and 50 μg/ml kanamycin, respectively. E. coli with plasmids carrying portions of the sdh gene were cultured with appropriate antibiotics (ampicillin and chloramphenicol) added to final concentrations of 100 or 50 μg/ml, respectively. If necessary, 5-aminolevulinic acid was added to a final concentration of 100 μM.

**Membrane Preparation**—For enzyme assay, E. coli were suspended in 30 mM Tris-HCl (pH 8.0) and disrupted by sonication. Cell suspensions in 30 mM Tris-HCl (pH 8.0) and 20% (w/v) sucrose were treated with EDTA and lysozyme (final concentration 10 μM and 0.1 mg/ml, respectively) for 30 min at 4°C before sonication for spectrophotometric measurements and HPLC. After removal of unbroken cells by low speed centrifugation, the homogenate was divided into cytosolic fractions by ultracentrifugation at 200,000 × g for 1 h at 4°C. The crude membrane precipitates were suspended in 30 mM Tris-HCl (pH 8.0). Succinate dehydrogenase, succinate-ubiquinone oxidoreductase (with DCIP and ubiquinone-1), and succinate oxidase activities were measured as described (6).

**Spectrophotometric Measurements and Analysis of Cytochromes**—HPLC—Absorption spectra at room temperature or 77 K were recorded by a Shimadzu UV-3000 spectrophotometer. The cytochrome b556 content was calculated using an extinction coefficient of 22.8 cm⁻¹ mM⁻¹ from the dithionite-reduced minus air-oxidized spectrum (6). The content of CO-reactive cytochrome b was determined using an extinction coefficient of 276 cm⁻¹ mM⁻¹ for cytochrome bo from the CO difference spectrum at the wavelength pair 416–430 nm (40). Cytochrome isolation by HPLC (Shimadzu 10AV) on TSK gel-G3000SW (TOYO SODA) was performed as reported previously with slight modification (41). In

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2 Nakayashiki, T., Nishimura, K., Tanaka, R., and Inokuchi, H. (1995) Mol. Gen. Genet., in press.
nucleotide sequences of these PCR products were determined in the following section. Most portions of the sdh operon show the fragments in various plasmids listed in Table I.

RESULTS

Construction of the sdh− and frd− Double Mutant and Plasmids Carrying Portions of the sdh Genes—To define the role of the two hydrophobic subunits of E. coli complex II in heme b ligation and functional assembly, recombinant plasmids carrying portions of the sdh genes were constructed, and their expression was analyzed by in vivo complementation with E. coli lacking SDH activity. As a first step in this study, an sdh−, frd− double mutant was constructed to overcome interference from the frd operon, which is partially expressed even under aerobic condition, and from the wild-type sdh operon. The mutant was obtained by P1 transduction using two E. coli strains, GVK124, with a kanamycin-resistant gene in sdhC, and M11443, which lacks the frd gene. This double mutant, MK3, grew on lactate but not on succinate, and no SDH activity was detected in the cytoplasmic or membrane fractions. Fig. 2 shows Western blotting of cell homogenates from wild-type strain and mutants with an anti-bovine heart Fp antibody. The band from the wild-type strain with the higher intensity and lower molecular mass corresponds to the Fp peptide of complex II; the other band with lower intensity corresponds to that of FRD. The mass lacks the frd gene. This double mutant, MK3, was then transformed with each plasmid listed in Table I. Fragments containing the promoter of the sdh operon, which locates upstream of sdhC, were inserted into plasmid vector pACYC184, and the others were inserted into pUC19. Since these vectors are compatible in E. coli, in vivo complementation from a combination of sdh genes on different vectors could be achieved as shown in the following section. Most portions of the sdh gene were prepared from a 4.45-kbp BamHI fragment (sdhCDAB) by enzymatic digestion (Fig. 1). In the case of pSDHC and pSDHAB, DNA fragments were obtained by PCR using primers with linkers for the construction of the recombinant plasmid. The nucleotide sequences of these PCR products were determined to check for misincorporation during amplification.

Heme b Ligation and Biosynthesis of Cytochrome b_{556}—Our previous work showed that the hydrophobic large subunit encoded by sdhC is a component of cytochrome b_{556} (7), but it was unknown whether the sdhD product is also a component of cytochrome b_{556}. Furthermore, identification of the sdhC and sdhD products as protein bands from purified complex II on SDS-polyacrylamide gel electrophoresis had not been achieved.

Therefore, plasmids carrying sdhC and/or sdhD were introduced into E. coli MK3. Fig. 3 shows the reduced minus oxidized difference spectra of membranes from various transformants, and Table II shows the contents of cytochrome b and CO-reactive cytochrome b in the membranes. MK3 membranes contain less cytochrome b than wild-type NM522 membranes because MK3 lacks cytochrome b_{556} in complex II. MK3/pSDHCDAB membranes have 10-fold more cytochrome b than MK3 membranes, and MK3/pSDHCD membranes also contain more cytochrome b. Cytochrome b from MK3/pSDHCD membranes shows a single peak at the same wavelength, 556.5 nm, as those from NM522 and MK3/pSDHCDAB, MK3/pSDHCD, MK3/pSDHCD, NM522 and MK3 membranes contain almost the same amounts of CO-reactive cytochrome b, suggesting that cytochrome b expressed by pSDHCD does not bind CO. To confirm that cytochrome b expressed by pSDHCD and cytochrome b_{556} are the...
same, the elution profiles of these cytochromes from a gel filtration column were analyzed further by a microanalytical system established for the separation of respiratory complexes from bacterial and mitochondrial membranes. Membranes were solubilized with an anionic detergent, Sarkosyl, and cytochromes were separated on TSK gel-G3000 SW in the presence of Sarkosyl. In this system, the cytochrome b components of E. coli separate into three peaks: peak I, cytochrome bo complex; peak II, cytochrome bd complex; and peak III, cytochrome b_{556} (41). The elution profile of solubilized membranes from wild-type NM522 cells in late log phase is shown in Fig. 4a, and that from MK3 cells is shown in Fig. 4b. Cytochrome b_{556} eluted from the column at 17.8 min. The levels of all cytochromes were lower in the MK3 membranes, and no cytochrome b_{556} elution peak at 17.8 min was observed. The cytochrome of MK3 eluting at 18.4 min appears to be cytochrome b_{556} (44). Cytochrome b_{556} was the main peak when MK3/pSDHCD membranes were subjected to this system (Fig. 4c), and the elution profile was the same as that for MK3/pSDHCDAB membranes. Thus, all of the properties of the b-type cytochrome in MK3/pSDHCD membranes were identical to those of cytochrome b_{556}.

The content of cytochrome b in MK3/pSDHD membranes was 4-fold higher than in MK3 membranes, while MK3/pSDHC membranes (Table II) contained amounts of cytochrome b similar to those in MK3 membranes. However, the properties of the cytochrome b expressed in pSDHDC membranes were different from those of cytochrome b_{556}, although the elution profiles of both cytochromes were similar. The cytochrome b from MK3/pSDHDC membranes showed an absorption peak at 557.5 nm in the reduced minus oxidized difference spectrum at 77 K. In addition, these membranes contained more CO-reactive cytochrome b than membranes from other transformants. Fig. 5 shows the SDS-polyacrylamide gel electrophoresis profile of the fractions eluted from the gel filtration column at 17.8 min (peak III corresponding to cytochrome b_{556} in Fig. 4) visualized by silver staining. The expression of polypeptides with molecular masses of about 19 and 17 kDa was observed when sdhC and sdhD were introduced into MK3, respectively, and these two peptides were highly expressed in MK3/pSDHCAB and MK3/pSDHDCAB. These results indicate that sdhC encodes the large subunit (cybL) and sdhD encodes the small subunit (cybS) of cytochrome b_{556} and that both subunits are essential components of cytochrome b_{556} in E. coli complex II.

Role of Cytochrome b_{556} in the Assembly of Complex II—Cytochrome b in complex II has been shown to play a role as a membrane anchor in enzyme complexes of many species. However, it has not been determined whether cybL or cybS or both are essential for the assembly and localization of complex II. To investigate this point, double transformations were performed that introduced a combination of sdh genes on different vectors into MK3. When pSDHAB was introduced into MK3 cells, SDH activity in the cell homogenates showed almost the same specific activity as that from wild-type strain NM522 cell homoge-

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**Table II**

| Host       | Plasmid | Cytochrome b | CO-reactive cytochrome b | absorption peak at 77 K |
|------------|---------|--------------|--------------------------|------------------------|
| NM522      |         | 0.901        | 59.8                     | 556.5, 559             |
| MK3        |         | 0.397        | 46.8                     | 559                    |
| MK3        | pSDHCD  | 4.92         | 67.7                     | 556.5                  |
| MK3        | pSDHCD  | 3.96         | 66.1                     | 556.5                  |
| MK3        | pSDHC   | 0.424        | 68.9                     | ND*                    |
| MK3        | pSDHD   | 1.88         | 108                      | 557.5                  |

*Not determined.*

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**Fig. 4.** Elution profiles from HPLC gel filtration chromatography. Sarkosyl-solubilized membranes (final protein concentration, 4 mg/ml; 100 μl each) were applied to the column, and the elution of cytochrome b was monitored at 412 nm. The flow rate was 1 ml/min. a, NM522; b, MK3; c, MK3/pSDHCD; d, MK3/pSDHD. Cytochrome b_{556} eluted at 17.8 min. The bar indicates an absorbance of 0.01 (a and b) or 0.02 (c and d).

**Fig. 5.** Silver-stained SDS-polyacrylamide gel of the peak eluted at 17.8 min on HPLC. The proteins in 120 μl of the fractions eluted between 17.3 and 18.3 min (1 ml) were concentrated by treatment with 10% (w/v) trichloroacetic acid. All of the proteins were then dissolved in sample buffer (without boiling) and separated on a 15-25% (w/v) gradient SDS-polyacrylamide gel. Lane 1, purified E. coli complex II (protein concentration, 0.3 μg); lane 2, MK3; lane 3, MK3/pSDHCD; lane 4, MK3/pSDHCDAB; lane 5, MK3/pSDHD; lane 6, MK3/pSDHDCAB; lane 7, purified E. coli Complex II. The closed arrowhead indicates the cybL subunit, and the open arrowhead indicates the cybS subunit.
nates. However, the SDH activity from MK3/pSDHAB cells (more than 99%) was localized in the cytoplasmic fraction (Table III), and the activity in the cytoplasmic fraction was much more unstable than the membrane activity even at 4 °C. Only half of the initial activity was found in the cytoplasmic fraction after 6 h of incubation, while full activity was retained in the membrane. The molecular activity of the catalytic portion in the cytoplasmic fraction was lower than that of the native enzyme because the Fp subunit from pSDHAB showed about 10-fold higher intensity than that of complex II from NM522 in Western blotting when fractions with the same SDH activity were applied (data not shown). It is worth noting that no succinate-ubiquinone oxidoreductase activity was detected in this cytoplasmic fraction. Most of the SDH activity was localized in the membrane when MK3/pSDHAB cells were transformed with pSDHCD, and succinate-ubiquinone oxidoreductase activity was found in the membrane. This membrane showed a level of succinate oxidase activity (315 nmol/min/mg) comparable with that of wild type strain NM522 (336 nmol/min/mg). Double transformations with pSDHC and pSDHAB also resulted in the formation of membrane-bound complex II with succinate-ubiquinone oxidoreductase activity, although the specific activity was lower than that of MK3/pSDHAB+pSDHAB. In contrast, neither the cybL nor cybS hydrophobic subunit was able to bind the catalytic portion to the membrane, and only SDH activity, not succinate-ubiquinone oxidoreductase activity, was found in the cytoplasmic fraction. The activity found in the membrane fraction seems to be contamination from the cytoplasmic fraction because the activity could be removed by extensive washing. Growth of these transformants correlated well with succinate-ubiquinone oxidoreductase activity of the cell.

Table IV shows the effect of deficiency in heme synthesis on the assembly of complex II. No SDH activity was found in the cytoplasmic or membrane fractions of the hemA− mutant, H500, grown on buffered LB medium with glucose. When H500 was transformed with pSDHCDAB, SDH activity was found in the cell homogenates, although the specific activity (80.2 nmol/min/mg) was lower than that from wild-type strain NM522. More than 98% of this activity was localized in the cytoplasmic fraction, suggesting the importance of heme b in the assembly of E. coli complex II. This was confirmed by the association of succinate-ubiquinone oxidoreductase activities in the membrane fractions when H500 and H500 transformed with pSDHCDAB were grown in the presence of 5-aminolevulinic acid. The localization of the Fp peptide, analyzed by Western blotting (data not shown), correlated well with the distribution of enzyme activity.

**DISCUSSION**

The data presented here clearly demonstrate that two hydrophobic subunits, cybL and cybS, encoded, respectively, by sdhC and sdhD in the E. coli sdh operon, are required for heme ligation into cytochrome b556 and are essential for the functional assembly of E. coli complex II in the membrane. The strategy for this study was in vivo complementation by a combination of sdh genes on different vectors, a method that was used successfully to investigate the assembly of E. coli FRD (33). Our recent analysis by EPR and MCD showed that the heme b556 component of E. coli complex II is ligated to the protein by two histidine residues (38). Furthermore, His84 in cybL and His87 in cybS were determined to be possible heme axial ligands in cytochrome b556 as shown using site-directed mutants.3 The data from the present study are consistent with these observations, because cytochrome b556 was expressed only when the sdhC and sdhD genes were both present. Bis-histidine ligation of the heme b between histidine residues

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located in the hydrophobic segments was also found for cytochrome b in B. subtilis complex II, although it contains two cytochromes b in a single hydrophobic peptide (26, 27). The cytochrome b expressed by pSDHD localized in the membrane and eluted from the gel filtration column with the same retention time (17.8 min) as cytochrome b556. However, this cytochrome b differs from cytochrome b556 in that it binds CO and shows a 1-nm red shift of the absorption peak in the low temperature difference spectrum. The main fraction of the sdhC product (cybL) eluted at 18.5 min from the column when membranes from the strain MK3/pSDHC were solubilized and analyzed by HPLC, suggesting that the cytochrome b from pSDHD eluting at 17.8 min is a dimer of the cybS peptide. It is likely that heme b bridges two histidine residues in the cybS dimer. His71 is a candidate for one of these residues because the increase in cytochrome b was not observed when His71 in pSDHD was substituted by glutamine; on the other hand, substitution of His14 did not affect the increase in cytochrome b.4 The cytochrome b content of membranes from MK3/pSDHCDAB was about 5-fold higher than that from wild type strain NM522, whereas the SDH activity of the strains differed only slightly. The reason for this difference remains unclear. However, it is speculated that the amount of cytochrome b556 synthesized from the plasmid is higher than that of the catalytic portion, Fp and Ip, for some reason (e.g. polar effects). The higher ratio of succinat ubiquinone oxidoreductase to SDH in membranes from pSDHCDAB as compared with other membranes (Table III) may be related to the high content of cytochrome b556. The assemblage of heme b in mitochondrial complex II is still unclear except for A. suum complex II (21, 45), even though the genes for SDH3 and SDH4 from S. cerevisiae (14–16) and the cDNA for bovine cybS (17, 18) have been cloned and sequenced. Our present report is the first indication that both hydrophobic subunits are indispensable for heme b ligation into the two subunit cytochrome b in complex II.

The presence of the two hydrophobic subunits, cybL and cybS, is essential for the functional assembly of E. coli complex II and its localization in membranes as well as for heme b ligation. Neither hydrophobic subunit alone is able to bind the catalytic Fp and Ip subunits to the membrane, and the catalytic portion in the cytoplasmic fraction does not transfer reducing equivalents to ubiquinone. This is quite similar to the situation in E. coli FRD (33), including the effects of deficiency on the growth of the strains. In contrast, gene disruption of sdhC and sdhD of E. coli resulted in a growth defect on glycerol-rich medium (16). Membrane attachment of the catalytic portion of this mutant is impaired but not abolished. A tighter association between the catalytic portion and cytochrome b in mitochondrial complex II compared with the bacterial enzyme may explain this difference. Active mitochondrial complex II is eluted from the gel filtration column in the presence of an anionic detergent, Sarkosyl (41, 45), while cytochrome b556 in E. coli complex II is dissociated from the complex and can be purified by the same column (35); this supports the above idea. A difference in the biosynthesis of the enzyme complex was also found between complex II and FRD of E. coli. The introduction of the frdABC and frdD genes on separate plasmids into E. coli M11443, which lacks a chromosomal frd operon, fails to restore anaerobic growth on glycerol and fumarate (33). Moreover, this strain is unable to interact with quinone analogues, although the enzyme complex is completely functional in catalyzing fumarate reduction by reduced benzyl viologen and is localized in the membrane. In this study it was claimed that co-translational association of frdC and frdD products is required for the functional assembly of E. coli FRD (33). In contrast to FRD, we observed active complex II in membranes when both pSDHC and pSDHCDAB were transformed into MK3, and the enzyme complex supported aerobic growth on succinate, indicating differences in the assembly processes of the two enzyme complexes.

An accumulation of the unassociated catalytic portion of complex II in the cytoplasm was observed in heme synthesis mutants, and normal complex II was assembled in the membrane when 5-aminovaleric acid was present in the culture. This result implies that the heme is important for the functional assembly of E. coli complex II. Interestingly, our recent analysis of site-directed mutants showed that complex II in cybL His54 and cybS His27 mutants, which lack heme b in their cytochrome b556, is associated with the membrane and catalyzes succinate-ubiquinone oxidoreductase activity.5 The presence of heme b appears to be essential for the initial step in the assembly of complex II into the membrane and to be unnecessary for electron transfer to ubiquinone once the enzyme complex is assembled.

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Two Hydrophobic Subunits Are Essential for the Heme b Ligation and Functional Assembly of Complex II (Succinate-Ubiquinone Oxidoreductase) from *Escherichia coli*

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