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Cytochrome *b* Reducible by Succinate in an Isolated Succinate Dehydrogenase-Cytochrome *b* Complex from *Bacillus subtilis* Membranes

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In previous work with membranes of *Bacillus subtilis*, the succinate dehydrogenase complex was isolated by immunoprecipitation of Triton X-100-solubilized membranes. The complex included a polypeptide with an apparent molecular weight of 19,000, probably attributable to apocytochrome. This paper reports the further characterization of this cytochrome and its relation to the respiratory chain of *B. subtilis*. The cytochrome was identified as cytochrome *b*, and its difference absorption spectra showed maxima at 426, 529, and 558 nm at room temperature. The oxidized cytochrome had an absorption maximum at 413 nm. The cytochrome was reduced by succinate in the isolated succinate dehydrogenase complex and in Triton X-100-solubilized membranes. In whole membranes cytochromes *b*, *c*, and *a* were reduced by succinate. In membranes from a mutant containing normal cytochromes but lacking succinate dehydrogenase no reduction of cytochrome was seen with succinate. It was concluded that the isolated succinate dehydrogenase-cytochrome *b* complex is a functional unit in the intact *B. subtilis* membrane. An accompanying paper describes cytochrome *b* as a structural unit involved in the membrane binding of succinate dehydrogenase.

The respiratory chain in aerobic bacteria such as *Bacillus subtilis* is located in the cytoplasmic membrane (25). *B. subtilis* cytochromes are spectrophotometrically similar to mammalian mitochondrial cytochromes (17). Two terminal cytochromes have, however, been identified: cytochrome aa_3, which is a part of the major cytochrome oxidase, and cytochrome *o*, which is a *b*-type cytochrome that can interact with oxygen (11, 17, 21). *B. subtilis* contains cytochrome *b* (21) and two different *c* cytochromes, c_{550} and c_{554}, both of which have been purified (18). The succinate-oxidase pathway in *B. subtilis* is proposed to include cytochromes b_{560}, c_{554}, c_{550}, and a_{601} in the order presented (19), and it differs from the NADH-oxidase pathway in which electrons can enter the respiratory chain at two different levels (through cytochrome b_{560} or c_{550}). The amount and relative composition of cytochrome in bacteria are strongly influenced by growth phase and growth conditions (1, 20, 27).

Succinate dehydrogenase [EC 1.3.99.1 succinate:(acceptor)oxidoreductase] is a firmly bound membrane enzyme which transfers reducing equivalents from succinate to the respiratory chain. Succinate dehydrogenase has recently been purified from the *B. subtilis* membrane by immunoprecipitation (8). It was isolated as a complex with cytochrome. The succinate dehydrogenase complex contains three different subunits in equimolar amounts: an M_r 65,000 polypeptide containing acid-nonextractable flavin, an M_r 28,000 polypeptide, and an M_r 19,000 polypeptide. The M_r 65,000 and M_r 28,000 subunits are thought to constitute the succinate dehydrogenase proper and are comparable to succinate dehydrogenase purified from *Rhodospirillum rubrum* (3) and beef heart mitochondria (2).

The M_r 19,000 polypeptide is probably the apocytochrome *b*. The main purpose of the present work is to further characterize this cytochrome.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* BR102 (*hisB trpC2*), used as the wild type, was originally obtained from J. Spizizen. The *B. subtilis* succinate dehydrogenase mutant *citF11* has been described previously (22, 23).

Growth of bacteria. The bacteria were grown in 7 liters of Spizizen minimal medium (26) supplemented with 10 μM $MnCl_2$ and 5 g of Casamino Acids (Difco Laboratories) per liter in a 10-liter fermentor (Biotec FL 110) at 37°C with stirring and aeration (6 liters per min). Bacteria were harvested at the end of the exponential growth phase by centrifugation at 14,000 × *g* for 20 min at 4°C.

Preparation of membranes and solubilization with Triton X-100. Membranes were prepared by the method of Konings and co-workers (14), but with 1 μg of DNase (DNase 1, bovine pancreas; Sigma Chemical Co.) and RNase (RNase A, bovine pancreas; Sigma) per ml. After the last washing the membranes

were suspended in 0.1 M Tris-hydrochloride (pH 6.6)–10 mM EDTA to a concentration of about 10 mg of protein per ml and stored at −20°C until used. On the day of use the membranes were thawed and centrifuged at 27,000 × *g* for 30 min at 4°C; the pellet was suspended in 24 mM Veronal (sodium diethylbarbiturate) buffer (pH 8.6).

Solubilization of the membranes with Triton X-100 was done as recently described (8).

Preparation of antiserum. Succinate dehydrogenase-specific antiserum was prepared by injecting rabbits with succinate dehydrogenase-staining precipitate obtained in crossed immunoelectrophoresis with whole membrane antiserum as described previously (23). Sera from several bleedings were pooled, and the immunoglobulins were purified and stored as described by Harboe and Ingild (7), except that the DEAE-Sephadex chromatography step was excluded.

Absorption spectra. Spectrophotometric measurements were made on an Aminco-Chance double-beam DW spectrophotometer at room temperature. Spectra were recorded between 390 and 640 nm by using a slit of 1 nm and a scan speed of 2 nm/s; 1-ml cuvettes with a 10-mm path length were used.

Membranes, solubilized membranes, and immunoprecipitated succinate dehydrogenase complex were all in 24 mM Veronal buffer (pH 8.6) during recording of the spectra. The base lines in the spectra were obtained by adding the same amount of material to both the sample and the reference cuvettes. Difference absorption spectra were recorded after adding reductant in a small volume of buffer to the sample cuvette and a corresponding volume of buffer to the reference cuvette. 2-Heptyl-4-hydroxy-quinoline *N*-oxide (HQNO) was added as an ethanolic solution. The ethanol did not influence the spectra or the reducibility of the cytochromes. $Na_2S_2O_4$ was added as a solid. Carbon monoxide difference absorption spectra were obtained by bubbling CO for 1 min through a reduced sample and scanning against a reduced reference.

Determination of acid-nonextractable flavin. Acid-nonextractable flavin was determined fluorometrically as described by Wilson and King (31) with an Aminco-Bowman spectrofluorometer. After precipitation and washing with trichloroacetic acid, the immunoprecipitate was incubated for 7 h at 37°C with shaking with subtilisin BPN′ (type VII; Sigma) at 0.4 mg/ml, α-chymotrypsin type I (bovine pancreas; Sigma) at 0.075 mg/ml, and trypsin type III (bovine pancreas; Sigma) at 0.25 mg/ml. A small amount of uniformly ^{14}C-labeled flavoprotein subunit, immunoprecipitated from *B. subtilis* KA11 cytoplasm after 5-aminolevulinic acid starvation (9), was added to the immunoprecipitated succinate dehydrogenase complex. Less than 1% of the radioactive material was lost during washing, and 95% was converted to an acid-soluble form during the proteolytic digestion.

Flavin adenine dinucleotide (disodium salt, grade III; Sigma) was used as the standard. The concentration of the standard was determined with the extinction coefficient given by Koziol (15).

Determination of protoheme. The prosthetic group of the cytochrome in the succinate dehydrogenase complex was identified and quantitated as the reduced pyridine hemochromogen by the method of Falk (5). The immunoprecipitate was completely dissolved in 2.1 M pyridine and 75 mM NaOH.

Other methods. Immunoprecipitation and protein determination by a modification of the Lowry method were done as described previously (8).

RESULTS

Reduction by succinate of the cytochrome in the isolated succinate dehydrogenase complex. Succinate dehydrogenase is solubilized with Triton X-100 from the *B. subtilis* membrane in an enzymatically active form. The succinate dehydrogenase complex purified by precipitation with specific antibody contains cytochrome and retains about 30% of the enzyme activity as measured with the phenazinemethosulfate-dichloroindophenol assay (8). The possibility that succinate could reduce cytochrome in the isolated succinate dehydrogenase complex was investigated. The immunoprecipitate was homogenized in buffer and transferred to two cuvettes, and succinate was added to one of these. The difference absorption spectra showed absorption maxima at 426, 529, and 558 nm at room temperature (Fig. 1). Maximal reduction was obtained in less than 15 s. Sedimentation of the succinate dehydrogenase immunoprecipitate during the recording of spectra made it difficult to study the exact kinetics of reduction and reoxidation. Reduction of the succinate dehydrogenase complex by $Na_2S_2O_4$ is also shown in Fig. 1. The extent of reduction by succinate can be estimated from the 558-nm peak to be about half of that obtained with $Na_2S_2O_4$. The air-oxidized succinate dehydrogenase complex has an absorption maximum at 413 nm (data not shown).

The above results show that the purified succinate dehydrogenase complex contains a cytochrome which is reducible by succinate.

Identity of the cytochrome of the purified succinate dehydrogenase complex. The heme of the succinate dehydrogenase complex was identified by its reduced pyridine hemochromogen spectrum. Absorption maxima at 557, 525, and around 415 nm characteristic for protoheme were recorded. To discriminate between cytochrome *b* and *o*, which both have protoheme as their prosthetic group, a CO spectrum of the succinate dehydrogenase complex was recorded. A CO spectrum characteristic of cytochrome *o* was not exhibited by the complex. The cytochrome of the succinate dehydrogenase complex is thus cytochrome *b*.

Protoheme and acid-nonextractable flavin were quantitated by absorption of the reduced pyridine hemochromogen and by fluorescence of the flavin, respectively. Ratios of 1.9, 1.3, and 2.0 protohemes per acid-nonextractable flavin were

obtained when three different immunoprecipitates were independently analyzed.

Cytochrome composition of *B. subtilis* membrane preparation. The presence of a cytochrome *b* absorbing at 558 nm in the immunoprecipitated succinate dehydrogenase complex may be an artifact of the isolation procedure. Therefore, intact membranes were subsequently investigated.

To determine the cytochrome composition of the membranes, noncovalently bound heme was extracted with acid acetone (5). Extracted heme was then identified as the reduced pyridine hemochromogen by spectral analysis. With this method heme *a* and protoheme were identified, indicating that the membranes contain cytochrome *a* and cytochrome of the *b* type. The residue remaining after extraction gave an absorption spectrum characteristic for heme *c*. These results are in accordance with previous reports on the cytochrome content of *B. subtilis* (17, 21, 27).

Ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylene diamine (TMPD) is commonly used for non-physiological reduction of cytochrome *c* (28). These compounds were used to further characterize the cytochromes in intact *B. subtilis* membranes (Fig. 2). Ascorbate alone reduced cytochrome c_{550}, but not cytochrome *a*, whereas ascorbate plus TMPD reduced cytochromes c_{550}, c_{554}, and *a*. Miki and Okunuki (19) have previously reported that in "electron trans-

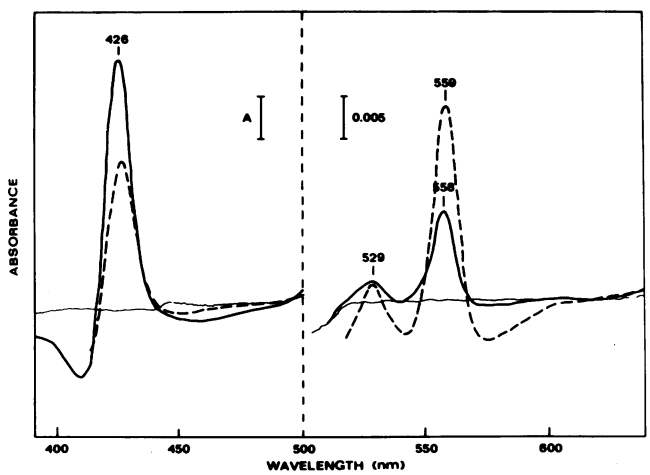

FIG. 1. *Difference absorption spectra of the succinate dehydrogenase complex containing 0.8 nmol of acid-nonextractable flavin and 1.5 nmol of protoheme per ml. Sodium succinate (1 mM)-reduced minus air-oxidized complex* (——) *and* $Na_2S_2O_4$*-reduced minus air-oxidized complex* (-----). *The bar A represents 0.01 and 0.05 absorbance units, respectively, for the succinate- and dithionite-reduced complexes. The horizontal line represents the base line.*

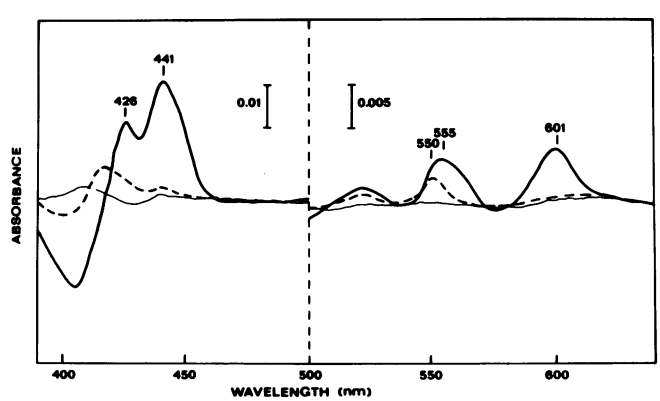

FIG. 2. *Difference absorption spectra of wild-type membranes, 4.3 mg of protein per ml, in the presence of 1 mM KCN. Ascorbate (0.4 mM)-reduced minus air-oxidized membranes* (-----) *and ascorbate (0.4 mM)- plus TMPD (0.2 mM)-reduced minus air-oxidized membranes* (——). *The horizontal line indicates the base line.*

port particles" from *B. subtilis*, cytochromes c_{550} and a_{601} are fully reduced by ascorbate, whereas cytochrome c_{554} is only partially reduced. Cytochrome *a* is not completely reduced by ascorbate-TMPD, as is shown in the spectrum of $Na_2S_2O_4$-reduced intact membranes in Fig. 3 compared with Fig. 2. Air-oxidized membranes were not further oxidized by the addition of ferricyanide. The CO spectrum of our membrane preparation shows the presence of a typical cytochrome a_3 with maxima at 430, 544, and 592 nm and troughs at 444 and 562 nm (Fig. 4). Similar spectra have been reported by others (11). The shoulders at 419 and 572 nm may indicate the presence of some cytochrome *o*.

Reducibility of cytochromes with succinate in intact membranes. A cytochrome b_{560} reducible by succinate in the presence of antimycin A has been reported by Miki and coworkers (21) in *B. subtilis* electron transport particles. To study the reducibility of cytochromes with succinate in our membrane preparations, a difference absorption spectrum was recorded. The spectrum (Fig. 3) shows α-absorption bands of cytochromes *c* and *b* in the 560-nm region and cytochrome *a* absorption at 600 nm. The spectra were recorded 0.5, 2, and 5 min after addition of succinate. During this time the spectra did not change, indicating that a steady-state reduction level had been reached. Difference spectra of $Na_2S_2O_4$-reduced versus air-oxidized membranes (Fig. 3) show that succinate did not reduce all of the cytochromes completely under aerobic conditions with 1 mM cyanide present.

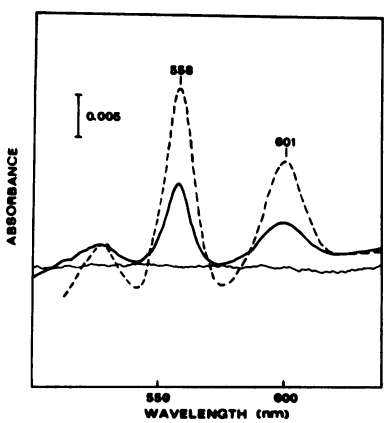

FIG. 3. *Difference absorption spectra of wild-type membranes, 4.2 mg of protein per ml, in the presence of 1 mM KCN. Sodium succinate (1 mM)-reduced minus air-oxidized membranes* (——) *and* $Na_2S_2O_4$*-reduced minus air-oxidized membranes* (-----). *The horizontal line indicates the base line.*

In bacteria, electron transport between cytochromes *b* and *c* is known to be inhibited by HQNO (25). Membranes were reduced by succinate in the presence of this inhibitor, and difference absorption spectra were recorded. HQNO blocked electron transport from succinate to cytochrome *a* and most probably also to cytochrome *c* (Fig. 5). Cytochrome *c* made only a minor contribution to the absorption at 558 nm, as can be seen in ascorbate-TMPD-reduced membranes (Fig. 2) and in HQNO-inhibited, succinate-reduced membranes (Fig. 5). Miki and Okunuki have also found the amount of cytochrome c_{550} to be low in exponentially growing *B. subtilis* (20). The spectra of succinate-reduced membranes show absorption maxima at 558 nm both in the absence and presence of HQNO. It can be concluded that a cytochrome species absorbing at 558 nm and reducible by succinate in the presence of HQNO is present in membranes from *B. subtilis* wild-type cells.

Membranes were prepared from a *B. subtilis* mutant (*citF11*) which has no succinate dehydrogenase enzyme activity and no membrane-bound succinate dehydrogenase antigen (9, 22). The *citF11* membranes contain the same cytochromes as wild-type membranes, but they are

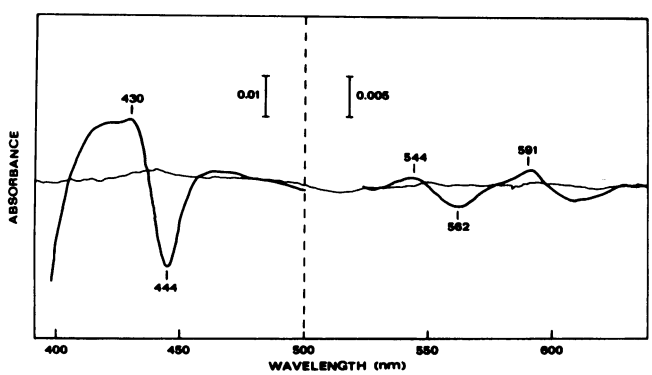

FIG. 4. *CO difference absorption spectrum of wild-type membranes, 2.0 mg of protein per ml. The horizontal line indicates the base line.*

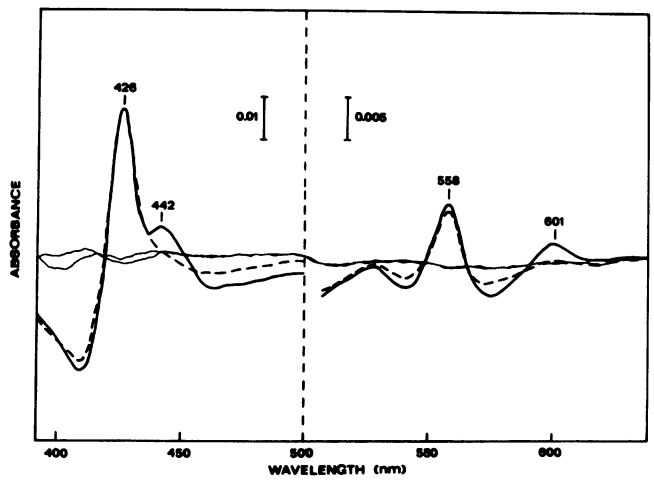

FIG. 5. *Difference absorption spectra of wild-type membranes, 4.2 mg protein per ml, in the presence of 1 mM KCN. Sodium succinate (1 mM)-reduced minus air-oxidized membranes (——) and membranes reduced by sodium succinate (1 mM) in the presence of 0.2 mg of HQNO per ml minus air-oxidized membranes (-----). The horizontal line indicates the base line.*

not reduced by succinate. This shows that succinic dehydrogenase is essential for reduction of cytochrome by succinate.

Cytochrome content in succinate dehydrogenase-depleted Triton X-100-solubilized membranes. Treatment of *B. subtilis* membranes with 4% (vol/vol) Triton X-100 at 10 mg of membrane protein per ml (pH 8.6) and at low ionic strength led to solubilization of about 40% of the protein, about 95% of the succinate dehydrogenase activity, and about 95% of cytochromes *b* and *c*. Only about half of the cytochrome a_{601} was solubilized. The difference absorption spectrum of succinate-reduced solubilized membranes was identical to that of intact membranes reduced by succinate in the presence of HQNO (Fig. 5).

Immunoprecipitation of the succinate dehydrogenase complex from Triton X-100-solubilized membranes with anti-succinate dehydrogenase antiserum removes more than 90% of the succinate dehydrogenase enzyme activity and less than 2% of the total solubilized membrane protein (8). The antiserum only contains precipitating antibody against the M_r 65,000 flavoprotein subunit (9).

The difference absorption spectrum of $Na_2S_2O_4$-reduced versus air-oxidized, solubilized membranes before and after removal of the succinate dehydrogenase complex by immunoprecipitation is shown in Fig. 6. No reduction of cytochrome with succinate was detected in the depleted preparation. The spectrum of the depleted preparation has a strongly diminished α-absorption peak around 558 nm. The bulk of the lost absorption around 558 nm was found in the immunoprecipitated succinate dehydrogenase complex (Fig. 1). The cytochrome in the 554-nm peak that remained after removal of the complex is probably cytochromes c_{550} and c_{554}; unprecipitated cytochrome *b* and/or *o* may account for the shoulder at 559 nm. The height of the cytochrome a_{600} absorption peak was not changed by the immunoprecipitation.

Cytochrome b_{558} was thus quantitatively removed with enzymatically active succinate dehydrogenase by immunoprecipitation with antibodies specific for the M_r 65,000 subunit.

DISCUSSION

The *B. subtilis* succinate dehydrogenase complex isolated from Triton X-100-solubilized membranes by immunoprecipitation contained three polypeptides in equimolar amounts. The M_r 65,000 polypeptide containing acid-nonextractable flavin and the M_r 28,000 polypeptide are the flavoprotein and, probably, the iron-sulfur protein subunits, respectively, of succinate dehydrogenase (8). The third polypeptide, with an apparent molecular weight of 19,000, is most probably apocytochrome *b* (8, 9). The M_r 19,000 polypeptide did not show abnormal migration behavior on sodium dodecyl sulfate-polyacrylamide gel electrophoresis when the relative mobilities of the polypeptides were plotted against the acrylamide concentration (12 to 20%) according to Ferguson (6) (unpublished experiments).

In the present work the cytochrome of the succinate dehydrogenase complex was identified

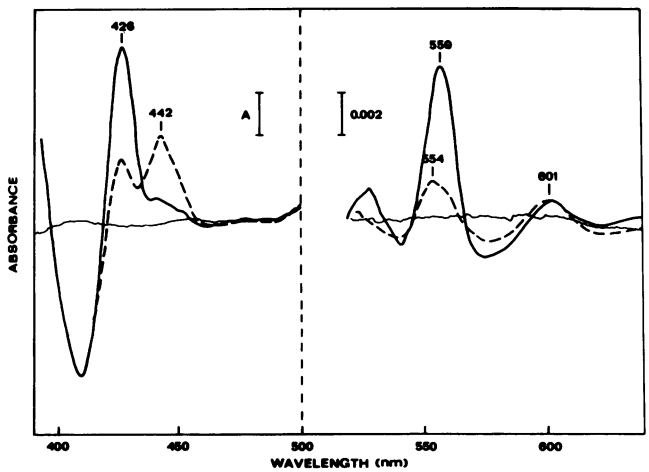

FIG. 6. *Difference absorption spectra of the $Na_2S_2O_4$-reduced minus air-oxidized preparations. Cuvettes contained 1.6 mg of protein per ml, 54 mM NaCl, 5 mM NaN_3 and 1.8% (vol/vol) Triton X-100. Triton X-100-solubilized wild-type membranes (——); the bar in the 390 to 500 nm part of the spectrum represents 0.01 absorbance units. Residue after precipitation of the succinate dehydrogenase complex (99% of the succinate dehydrogenase enzyme activity immunoprecipitated) from Triton X-100-solubilized wild-type membranes (-----); the bar in the 390 to 500 nm part of the spectrum represents 0.005 absorbance units. The horizontal line indicates the base line.*

as cytochrome *b*. It was partially reduced by succinate in the purified complex, and its difference absorption spectrum showed maxima at 426, 529, and 558 nm at room temperature. The determination of an extinction coefficient for cytochrome b_{558} was disturbed by sedimentation of the immunoprecipitate as mentioned above. However, a difference extinction coefficient of 14 $mM^{-1}cm^{-1}$ (558–575 nm) for the $Na_2S_2O_4$-reduced succinate dehydrogenase complex was estimated by suspending the immunoprecipitate in buffer containing sucrose, which minimized sedimentation. The coefficient was calculated on the protoheme content in a preparation containing 2.0 protohemes per acid-nonextractable flavin. The stoichiometry in the succinate dehydrogenase complex of two protohemes per acid-nonextractable flavin and one flavoprotein polypeptide per apocytochrome *b* polypeptide suggests a diheme cytochrome. Considering the low apparent molecular weight of the apocytochrome polypeptide it is unlikely that it would harbor two heme groups. The data could be explained by a systematic error in the determination of the prosthetic groups or in the stoichiometry of the polypeptides. In *Escherichia coli* a cytochrome *b* species has been purified from the cytoplasmic membrane of aerobically grown cells (13). It has an apparent molecular weight of 17,500 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and its difference absorption spectrum has maxima at 427, 529, and 558 nm at room temperature. It has been suggested that this cytochrome contains equimolar amounts of heme and polypeptide. The reported difference extinction coefficient is 22.8 $mM^{-1}cm^{-1}$ (558–575 nm).

The reducibility of cytochrome *b* by succinate in the purified *B. subtilis* succinate dehydrogenase complex is a reflection of the situation that prevails in intact membranes, based on the following observations; (i) cytochrome absorbing at 558 nm was reduced by succinate in the intact membrane in the presence of HQNO; (ii) the respiratory chain was disrupted by treatment with Triton X-100, and only cytochrome b_{558} was then reduced by succinate; and (iii) succinate dehydrogenase and cytochrome b_{558} were completely solubilized with Triton X-100, and by immunoprecipitation of the solubilized succinate dehydrogenase with antibodies specific for the flavoprotein subunit, cytochrome b_{558} was quantitatively removed together with succinate dehydrogenase. The above data strengthen the hypothesis presented before (10) and in an accompanying paper (9) that in *B. subtilis* cytochrome *b* has an important structural function besides its physiological role as an "entrance" to the respiratory chain for electrons from succinate dehydrogenase and other dehydrogenases.

Recently, Weiss and Kolb (30) purified a succinate dehydrogenase-cytochrome complex from *Neurospora crassa* mitochondrial membranes after solubilization with Triton X-100. This complex is very similar to the *B. subtilis* succinate dehydrogenase complex and contains three poly-

peptides of M_r 72,000, 28,000, and 14,000. The *N. crassa* complex also contains cytochrome *b* with difference absorption maxima at 425, 528, and 559 nm at room temperature. The oxidized complex shows a maximum at 413 nm. The *N. crassa* succinate dehydrogenase-cytochrome *b* complex has succinate-ubiquinone reductase activity and contains little, if any, ubiquinone-10. Succinate-cytochrome *b* reductase activity was not reported. The complex contains approximately equimolar amounts of flavin and cytochrome *b*. Quinone reductase activity in the *B. subtilis* succinate dehydrogenase-cytochrome *b* complex has not yet been investigated, and it is not known whether the isolated complex contains quinone.

Membrane-bound succinate dehydrogenase and fumarate reductase have possibly evolved from a common ancestral enzyme. The enzymes catalyze the same reaction, but in different directions, and thus have different K_m values for succinate and fumarate. The strong similarity between succinate dehydrogenase and fumarate reductase is evident when the composition of mammalian (2), *R. rubrum* (3), *N. crassa* (30), and *B. subtilis* succinate dehydrogenases are compared with two bacterial fumarate reductases. Membrane-bound fumarate reductase from *Vibrio succinogenes* consists of two polypeptides of M_r 80,000 and 30,000, and the enzyme is functionally associated with a high-potential M_r 25,000 cytochrome *b* (16). *E. coli* fumarate reductase consists of equimolar amounts of two polypeptides of M_r 70,000 and 24,000 (4). The purified *E. coli* enzyme contains some cytochrome *b* and an M_r 19,000 polypeptide which the authors consider an impurity, possibly the apocytochrome *b*. In both of these fumarate reductases the largest polypeptide contains covalently bound flavin in an 8 α [*N*(3)-histidyl]-flavin adenine dinucleotide (12, 29) analogous to the flavin linkage in mitochondrial succinate dehydrogenase (24). Most probably the *B. subtilis* acid-nonextractable flavin is covalently bound in the same type of linkage to the M_r 65,000 polypeptide.

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