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# Biosynthesis and Membrane Binding of Succinate Dehydrogenase in *Bacillus subtilis*

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Antibodies specific for the $M_r$ 65,000 (flavoprotein) and the $M_r$ 28,000 subunits of the succinic dehydrogenase (SDH) of *Bacillus subtilis* were obtained. By using these antibodies it was shown that both subunits accumulated in the cytoplasm during 5-aminolevulinic acid starvation of a 5-aminolevulinic acid auxotroph. In the cytoplasm the subunits were not associated since they precipitated essentially independently of each other with subunit-specific antibody. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis the cytoplasmic subunits migrated identically with the corresponding subunits from the purified membrane-bound SDH complex. Cytoplasmic subunits were pulse-labeled with L-[$^{35}$S]methionine during 5-aminolevulinic acid starvation. The labeled subunits bound to the membrane when heme synthesis was resumed and also when protein synthesis was blocked by chloramphenicol before readdition of 5-aminolevulinic acid. The experiments thus demonstrated a precursor relationship between cytoplasmic subunits and the subunits of the membrane-bound SDH complex. All SDH-negative mutants isolated so far carry mutations in the *citF* locus. None of the mutants was found to have either the $M_r$ 65,000 or the $M_r$ 28,000 SDH subunits in the membrane. Four *citF* mutants, however, contained both subunits in the cytoplasm. Three of these mutants lacked spectrally detectable cytochrome $b_{558}$. The respective mutations mapped at one end of the *citF* locus. These results strongly support our previous suggestion that cytochrome $b_{558}$ is (part of) a membrane binding site for SDH in *B. subtilis*.

Immunological techniques have proven particularly valuable in studies of membrane-bound proteins and enzymes (2, 23). Due to their hydrophobic character such proteins are difficult to purify by conventional techniques, but they can often be isolated from mixtures of detergent solubilized proteins by precipitation with specific antibody (27). In previous work we have purified a succinate dehydrogenase (SDH) complex from Triton X-100-solubilized *Bacillus subtilis* membranes by precipitation with antibody from rabbits immunized with an SDH-staining precipitate obtained in crossed immunoelectrophoresis of a mixture of membrane proteins (17). The SDH complex contains three subunits of $M_r$ 65,000, 28,000, and 19,000. The largest subunit contains 1 mol of flavin per mol of protein, the $M_r$ 28,000 subunit has been suggested to be an iron-sulfur protein of analogy with the subunit composition of SDH purified from beef heart mitochondria (8) and *Rhodospirillum rubrum* (9), and the smallest subunit is most likely cytochrome $b_{558}$ (16). When heme synthesis is blocked in a 5-aminolevulinic acid mutant of *B. subtilis*, soluble SDH-antigen accumulates in the cytoplasm, suggesting that cytochrome is part of a membrane binding site for SDH (18).

We have now prepared antibody specific for each of the two larger subunits of the SDH complex. These antibodies have been used to study the synthesis and location of the subunits during 5-aminolevulinic acid starvation and the membrane binding of these subunits on resumption of heme synthesis.

All SDH-negative mutants isolated so far carry mutations in the *citF* locus (25; unpublished experiments). In this paper we will show that some of these mutants lack cytochrome $b_{558}$ and are unable to bind the two larger subunits of the SDH complex to the membrane.

## MATERIALS AND METHODS

**Bacteria.** *B. subtilis* BR102 (*hisB trpC2*) was used as the wild type. The strain KA11 (*trpC2 met*, 5-aminolevulinic acid auxotroph) (18) and the nine *citF* mutants (25, 26) have been described previously. Spontaneous *citF* revertants were picked from purification agar plates as described by Carls and Hanson (5). SDH enzyme activity and the presence of SDH antigen in the membrane of the revertants were checked by the methods described previously (17, 26).

**Media and growth of bacteria.** Bacteria were grown in Spizizen minimal medium (28) supplemented with 10 μM $MnCl_2$, 20 mg of required amino acids per liter, and 0.5 g of Casamino Acids (Difco Laboratories) per liter. Medium for the growth of strain KA11 was further supplemented with 5-aminolevulinic acid (2

μg/ml) as indicated in the text. The *citF* mutants and BR102 were also grown in a broth medium (NSMP) described by Fortnagel and Freese (11).

Radioactive labeling of bacteria for use in sodium dodecyl sulfate-polyacrylamide gel electrophoresis-crossed immunoelectrophoresis (SDS-PAGE-CIE) was done by growing the bacteria in medium containing 2 μCi of L-[$^{35}$S]methionine (>400 Ci/mmol; New England Nuclear Corp.) per ml. KA11 was uniformly labeled by supplementing the medium with either 2 μCi of L-$^{3}$H-amino acid mixture (algal protein hydrolysate; New England Nuclear) per ml or 1 μCi of L-$^{14}$C-amino acid mixture (algal protein hydrolysate; New England Nuclear) per ml. In one experiment strain KA11 was pulse-labeled with 2.8 μCi of L-[$^{35}$S]methionine per ml.

General methods for growing the bacteria have been described previously (17, 26). Bacteria were harvested at the end of exponential growth, except when indicated in the text, by centrifugation at 13,700 × *g* for 20 min at 4°C. The cells were washed twice with buffer, and the pellet was stored at −20°C until the next day.

**Preparation of membranes for immunoprecipitation.** Membranes from KA11 cells, used to isolate the SDH complex by immunoprecipitation, were prepared by lysis of the cells in a large volume of buffer as described previously (17).

**Preparation of membranes for cytochrome spectra.** Cells from 600 ml of NSMP at an absorbancy at 600 nm of 0.7 to 0.9 were suspended in 4.4 ml of 0.1 M potassium phosphate buffer (pH 8.0)–0.5 mM phenylmethylsulfonyl fluoride–0.5 mg of lysozyme per ml–18 μg each of DNase and RNase per ml–9 mM $MgSO_4$. Phenylmethylsulfonyl fluoride was dissolved in buffer just before use as the protease inhibitor is not stable in solution (19). After incubation at 37°C for 1 h the particulate fraction (membranes) was spun down at 48,200 × *g* for 30 min at 4°C. The membranes were washed twice in 0.1 M potassium phosphate buffer (pH 8.0) and finally suspended in 1.2 ml of buffer and frozen at −70°C.

**Preparation of membrane and cytoplasm for SDS-PAGE-CIE and "rocket" immunoelectrophoresis.** Cells from a 100-ml culture at an absorbancy at 600 nm of 0.7 to 0.9 were suspended in 0.4 ml of 0.1 M sodium phosphate buffer (pH 8.0)–0.5 mM phenylmethylsulfonyl fluoride–0.7 mg of lysozyme per ml–25 μg each of DNase and RNase per ml–12 mM $MgSO_4$. Cells were lysed by incubation at 37°C for 1 h. The lysate was centrifuged at 48,200 × *g* for 30 min at 4°C. The supernatant was transferred to a new centrifuge tube and centrifuged at 48,200 × *g* for 1 h at 4°C. The top two-thirds of the supernatant, which contained cytoplasm, was withdrawn and stored at −70°C. The pellet obtained after centrifugation of the lysate, which contained membranes, was washed twice in 0.1 M sodium phosphate buffer (pH 8.0) and then homogenized in 0.3 ml of the same buffer and stored at −70°C.

**Preparation of KA11 cytoplasm for immunoprecipitation.** Cells from a 300-ml culture at an absorbancy at 600 nm of 1.6 were suspended in 4.2 ml of 0.1 M potassium phosphate buffer (pH 8.0)–1 mM phenylmethylsulfonyl fluoride–0.2 mg of lysozyme per ml–7 μg each of DNase and RNase per ml–7 mM $MgSO_4$ and incubated at 37°C for 1 h. The lysate was centrifuged at 48,200 × *g* for 30 min at 4°C. The supernatant was centrifuged again at 48,200 × *g* for 2 h at 4°C. The top two-thirds was collected and stored at −70°C until used for the immunoprecipitation of cytoplasmic antigen.

**Preparation of antisera.** Anti-SDH antiserum was prepared as described previously (26). Antibodies against the subunits of the SDH-complex were obtained by boiling immunoprecipitated complex in 2% (wt/vol) SDS–0.1 M DL-dithiothreitol–0.11 M Tris-sulfate buffer (pH 6.1)–10% (vol/vol) glycerol–and 0.01% (wt/vol) bromophenol blue for 3 min. The subunits of the SDH-complex were then separated by SDS-PAGE on a 10 to 15% (wt/vol) acrylamide–0.20 to 0.4% (wt/vol) bisacrylamide gradient gel.

Each polypeptide was located on the gel with the help of a thin slice which was stained for protein. The unfixed gel was covered with Saran Wrap and stored at 4°C during staining and destaining of the gel slice. Gel slices containing each polypeptide were cut out and rinsed in 0.9% (wt/vol) NaCl. As a control of correct slicing the rest of the gel was stained for protein. The polypeptide-containing gel slices were homogenized in phosphate-buffered saline in 4-ml vials with a Sorvall omnimixer. Homogenates were stored at −70°C.

Rabbits were immunized subcutaneously on the back with approximately 0.5 μg of protein. Freund complete adjuvant was mixed with antigen at the first injection. Injections were repeated 7, 17, and 40 days later with the same amount of antigen in Freund incomplete adjuvant. Boosters were then given every 2 months. Rabbits were bled before immunization and then once a month. Sera from several bleedings were pooled, and the immunoglobulins were purified and stored as described by Harboe and Ingild (14), except that the DEAE-Sephadex step was omitted.

The presence and titer of specific antibody were determined by CIE using Triton X-100-solubilized *B. subtilis* BR102 membranes as antigen as described previously (26). Sera from rabbits immunized with SDS-boiled polypeptides gave indistinct immunoprecipitates in several different agaroses tested. However, if the antigen (2 mg of membrane protein per ml) was treated with 1% (wt/vol) SDS–2% (vol/vol) Triton X-100 the immunoprecipitates were considerably sharpened. Preimmune sera did not give any immunoprecipitate in CIE.

**SDS-PAGE-CIE.** SDS-PAGE-CIE was performed by the method of Chua and Blomberg (6). In SDS-PAGE-CIE the polypeptides are separated according to size in an SDS-polyacrylamide gel in the first dimension and then run into an antibody-containing agarose gel in the second dimension.

**Analytical procedures.** Determinations of protein by a modification of the method of Lowry (17, 20), acid-nonextractable flavin (16, 32), and cytochrome absorption spectra (16) were done as described previously. Radioactivity was determined by adding samples of 0.05 to 0.5 ml to 5 to 10 ml of Aquasol (New England Nuclear) and counting in a Nuclear Chicago Isocap/300 scintillation counter.

**Miscellaneous procedures.** Solubilization of membranes with Triton X-100, immunoprecipitation,

SDS-PAGE, and autoradiography was done as recently described (17).

## RESULTS

**Characterization of antisera.** An SDH-cytochrome *b* complex containing three different polypeptides has been purified by immunoprecipitation of Triton X-100-solubilized *B. subtilis* cell membranes with an antiserum prepared by injecting rabbits with an SDH-staining precipitate obtained in CIE by using whole membrane antiserum (17). This antiserum was shown to contain antibody against the largest SDH subunit ($M_r$ 65,000 flavoprotein). Whether the serum also contained antibody against the two smaller subunits was not known; therefore, we have characterized this anti-SDH antiserum further.

Whole membranes were first run in SDS-PAGE-CIE against antisera prepared by immunizing with whole membranes and with purified SDH complex. More than 30 precipitates were easily resolved with the anti-membrane antiserum, whereas only one precipitate was found by using the anti-SDH antiserum. The position of the latter precipitate corresponds to that of the SDH flavoprotein subunit (Fig. 1). Similarly, when the $^{14}C$-labeled SDH complex was analyzed in SDS-PAGE-CIE by using anti-SDH antiserum only the flavoprotein gave a precipitate detectable by autoradiography (Fig. 2). We conclude that rabbits immunized with purified

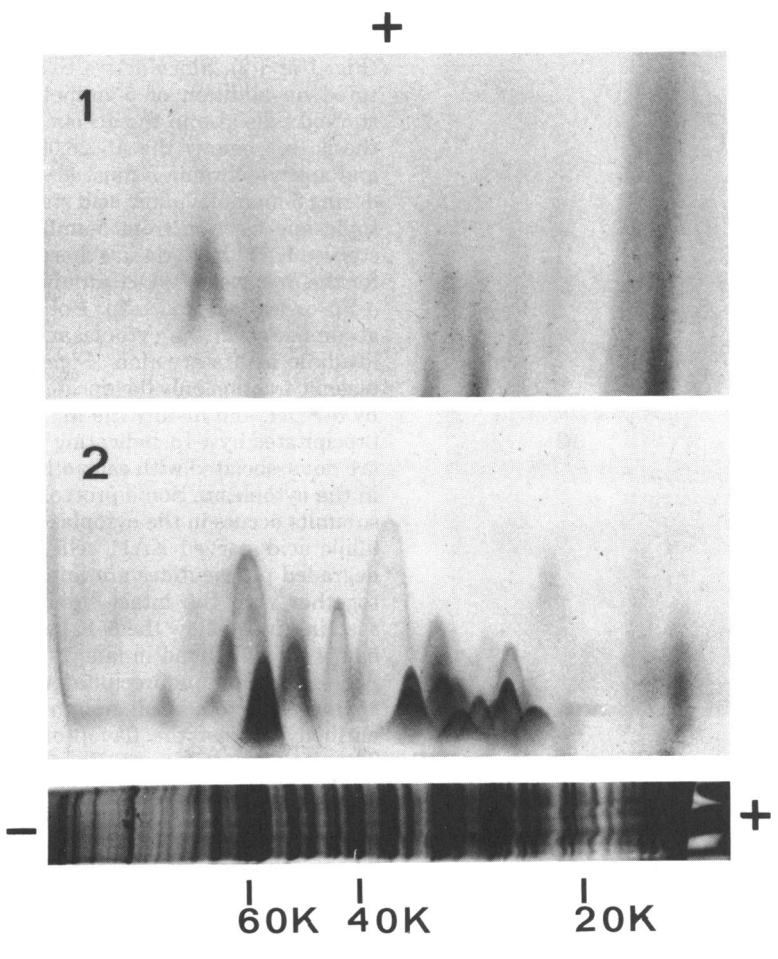


FIG. 1. *Autoradiographs of SDS-PAGE-CIE of membrane proteins from KA11 cells grown in minimal medium supplemented with 5-aminolevulinic acid and $^{14}C$-labeled amino acids. First dimension (horizontal): SDS-PAGE of 25 μg of protein on a 10 to 15% (wt/vol) acrylamide-0.26 to 0.40% (wt/vol) bisacrylamide gradient gel. Second dimension (vertical): immunoelectrophoresis against (1) α-SDH and (2) anti-membrane antisera.*

SDH complex only produce precipitating antibody against the flavoprotein subunit. We next attempted to produce antibody specific for each subunit of the SDH complex by immunizing rabbits with subunits separated by SDS-PAGE. The respective antisera were first analyzed in SDS-PAGE-CIE with purified $^{14}$C-labeled SDH complex as antigen. Antiserum from rabbits immunized with the flavoprotein subunit gave a single precipitate at a position corresponding to that of flavoprotein in the gel (Fig. 2). We will designate this antiserum α-Fp. Similarly, antiserum prepared against the $M_r$ 28,000 subunit gave one precipitate at a position corresponding to that of this subunit in the gel and a faint precipitate at the front of the gel. Moreover, this antiserum did not give a precipitate when run in SDS-PAGE-CIE against purified flavoprotein. We will designate this antiserum α-Ip. Triton X-100-solubilized SDH complex could not be precipitated with α-Ip. However, α-Ip inhibited the enzymatic activity of the complex (data not shown). As yet, we have not obtained precipitating antibody against the smallest subunit ($M_r$ 19,000 apocytochrome).

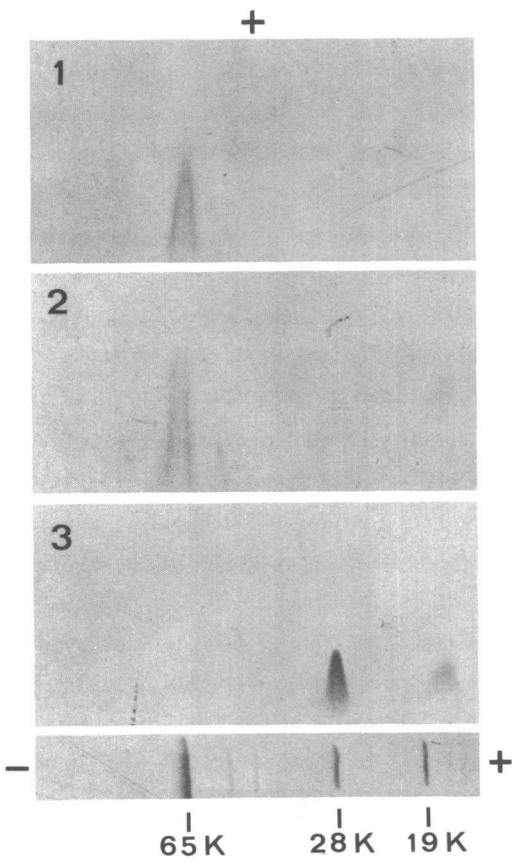


FIG. 2. *Autoradiographs of SDS-PAGE-CIE of SDH complex isolated by immunoprecipitation with α-SDH (17) of Triton X-100-solubilized membranes from B. subtilis wild-type cells grown in minimal medium supplemented with $^{14}$C-labeled amino acids. First dimension (horizontal): SDS-PAGE of 1 μg of SDH complex on a 10 to 15% (wt/vol) acrylamide-0.26 to 0.40% (wt/vol) bisacrylamide gradient gel. Second dimension (vertical): immunoelectrophoresis against (1) α-SDH, (2) α-FP, and (3) α-Ip.*

**Assembly of the SDH complex.** Previous work has shown that the SDH flavoprotein subunit accumulates in the cytoplasm of 5-aminolevulinic acid-starved KA11 cells (18). When 5-aminolevulinic acid is added to the starved bacteria, flavoprotein binds to the membrane with a corresponding increase in SDH enzyme activity. The antibody used in these experiments has now been shown to be specific for flavoprotein (Fig. 1 and 2). Since active SDH was reconstituted on addition of 5-aminolevulinic acid to starved cells also in the absence of protein synthesis (see below) the $M_r$ 28,000 SDH subunit and apocytochrome *b* must also be synthesized during 5-aminolevulinic acid starvation. The cytoplasmic fraction from 5-aminolevulinic acid-starved KA11 bacteria was therefore reexamined for the presence of SDH subunits by using both α-Fp or α-SDH and α-Ip. Both SDH subunits accumulated in the cytoplasm during 5-aminolevulinic acid starvation (Fig. 3). In the cytoplasmic fraction only flavoprotein is precipitated by α-SDH, and mainly the $M_r$ 28,000 subunit is precipitated by α-Ip, indicating that the subunits are not associated with each other when present in the cytoplasm. Some proteolysis of the SDH subunits occurs in the cytoplasm of 5-aminolevulinic acid-starved KA11 cells. These partially degraded polypeptides are immunoprecipitated together with the intact subunits and can be seen in Fig. 3 below the 65K band in lane 1 and below the 28K band in lane 2. Some 65K polypeptide is immunoprecipitated by α-Ip. This could be due to a small amount of precipitating antibody against the flavoprotein in the α-Ip. The titer of possible anti-Fp antibody in α-Ip is too low to be detected in SDS-PAGE-CIE. The amount of 65K polypeptide precipitated by α-Ip is less than 10% of precipitated 28K polypeptide. The cytoplasmic KA11 $M_r$ 65,000 subunit contains 1 mol of acid-nonextractable flavin per mol of subunit (data not shown). Thus, heme synthesis is required for membrane binding of both subunits in *B. subtilis*.

To study further the relationship between the soluble subunits of SDH which appear during 5-aminolevulinic acid starvation of strain KA11 and their membrane binding, the following experiment was done. Strain KA11 was grown in

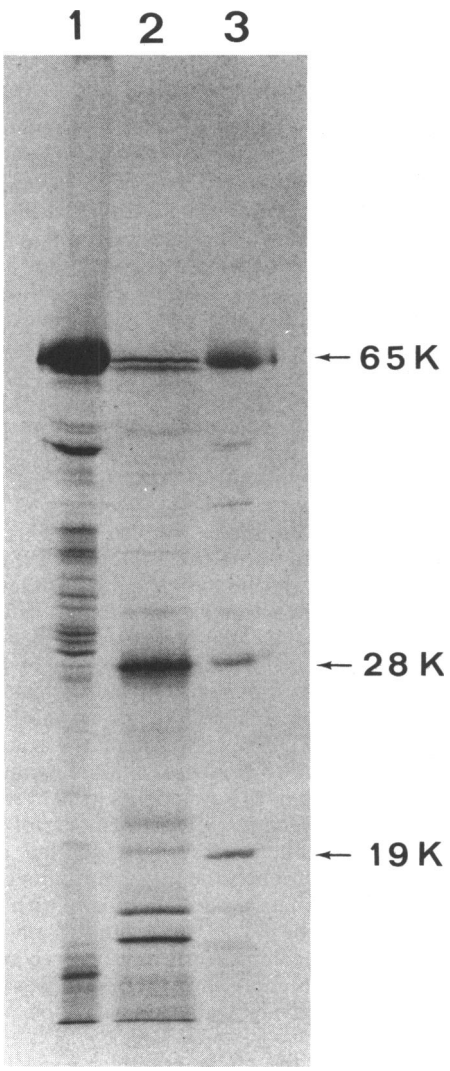


FIG. 3. *Autoradiograph of SDS-PAGE of immunoprecipitated SDH complex from wild-type membranes and of immunoprecipitated cytoplasmic SDH antigen from KA11 cells after 150 min of 5-aminolevulinic acid starvation (18). The bacteria were grown in minimal medium supplemented with $^{14}C$-labeled amino acids. The gradient of the gel was 10 to 15% (wt/vol) acrylamide–0.26 to 0.40% (wt/vol) bisacrylamide. Sample 1, Immunoprecipitate from KA11 cytoplasm incubated with α-SDH (2.5 μg of protein, 3,000 cpm); sample 2, immunoprecipitate from KA11 cytoplasm incubated with α-Ip (1 μg of protein, 1,200 cpm); sample 3, wild-type SDH complex immunoprecipitated with α-SDH (2 μg of protein, 500 cpm).*

minimal medium supplemented with 5-aminolevulinic acid and a mixture of $^{3}H$-labeled amino acids. At an absorbancy at 600 nm of 1, the bacteria were centrifuged and suspended in fresh medium with $^{3}H$-labeled amino acids but without 5-aminolevulinic acid. Part of the culture was then diluted into medium with 5-aminolevulinic acid, and part was diluted into medium without 5-aminolevulinic acid. After 115 minutes of growth L-[$^{35}S$]methionine was added to both cultures. Fifteen minutes later an excess of cold L-methionine was added to each culture together with 50 μg of chloramphenicol per ml to stop protein synthesis. After another 5 min 5-aminolevulinic acid was added to part of the starved culture. After another 25 min the three cultures were harvested, and membrane and cytoplasmic fractions were prepared. An outline of the experiment is shown in Fig. 4. The membrane cytochrome spectrum was completely restored within 30 min when 5-aminolevulinic acid was added to starved bacteria in which protein synthesis was blocked by chloramphenicol (data not shown).

The $^{3}H/^{35}S$ ratio was nearly identical in the corresponding fractions from all three cultures (data not shown). Membranes were solubilized with Triton X-100, and the SDH complex was purified from each culture by immunoprecipitation. The $^{3}H/^{35}S$ ratio was at least fourfold higher in the complex purified from the culture which had not received 5-aminolevulinic acid during the experiment compared with the two other cultures. The respective SDH complexes were then run in SDS-PAGE, the gels were sliced, and the radioactivity of the slices was

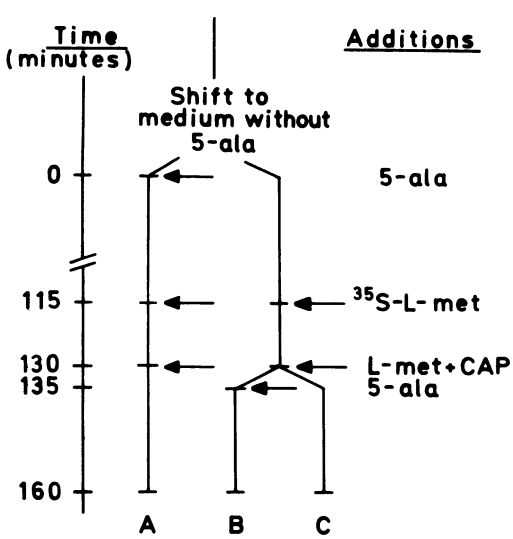


FIG. 4. *Outline of the pulse-chase experiment described in the text. An arrow indicates addition to the culture of 5-aminolevulinic acid (5-ala) (2 μg per ml), L-[$^{35}S$]methionine (met) (2.8 μCi and 0.53 μg per ml), L-met (0.1 mg per ml), chloramphenicol (CAP) (50 μg per ml). $^{3}H$-labeled amino acids were present in the growth medium of all three cultures throughout the experiment.*

determined (Fig. 5). In the control culture roughly equal amounts of $^3H$ and $^{35}S$ label were found in each of the three SDH subunits. Essentially the same result was obtained for the culture which had been incubated with 5-aminolevulinic acid after addition of chloramphenicol. The labeling pattern of the SDH subunits from the bacteria which had been starved for 5-aminolevulinic acid throughout the experiment was distinctly different. The $^3H/^{35}S$ ratio was six to eightfold higher in both flavoprotein and the $M_r$ 28,000 subunit compared with the same subunits from the other two cultures, and $^{35}S$ label was not detected in the apocytochrome band. The results demonstrate that soluble flavoprotein and the $M_r$ 28,000 subunit bind to the membrane when holocytochrome is made. We will discuss the results more fully below.

**Some *citF* mutants lack cytochrome $b_{558}$.** Nine SDH-negative mutants have previously been characterized for the presence of SDH antigen with an antibody which we have here shown to be specific for the flavoprotein subunit (17, 26). Five of these mutants contain soluble flavoprotein. All nine *citF* mutants have now been reexamined for the presence and location of flavoprotein and the $M_r$ 28,000 subunit by using antibody specific for the respective subunit in rocket immunoelectrophoresis (Fig. 6) and SDS-PAGE-CIE. None of the mutants contained membrane-bound antigen, as determined by using both Triton X-100 and SDS to solubilize the membranes. The latter detergent will solubilize essentially all of the membrane proteins (unpublished experiments), which makes it unlikely that our failure to detect SDH antigen in the membranes of *citF* mutants was caused by defective solubilization. Mutants *citF78*, *citF42*, *citF44*, *citF12*, and *citF83* contained soluble flavoprotein, confirming previous results (17). Moreover, the soluble $M_r$ 28,000 subunit was detected in mutants *citF78*, *citF42*, *citF44*, and *citF12*; the respective mutations map at one end of the *citF* locus (25; see also Table 1). In broth cultures most of the soluble $M_r$ 28,000 subunit antigen was found in fragments of lower molecular weight, although phenylmethylsulfonyl fluoride was included in the samples. In bacteria grown in minimal medium there was considerably less fragmentation of the soluble $M_r$ 28,000 subunit. Figure 7 shows an autoradiograph of the SDS-PAGE-CIE of the soluble fraction from *citF44* grown in minimal medium. It is unlikely that the fragments which react with $\alpha$-Ip represent nonsense fragments of the $M_r$ 28,000 subunit. It should be pointed out, however, that although the above mutants contain antigens which migrate identically with flavoprotein and the $M_r$ 28,000 subunit from the wild-type SDH complex, we have no reconstitution system to show that these antigens are functionally equivalent to the wild-type subunits.

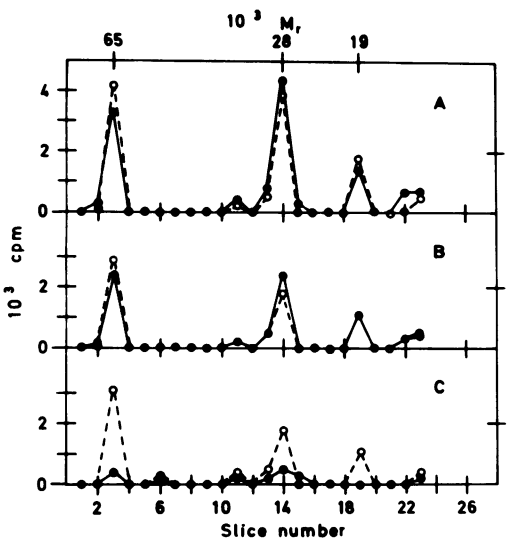


FIG. 5. *Membranes from cultures A, B, and C of Fig. 4 were solubilized with Triton X-100, and the respective SDH complexes were purified by immunoprecipitation with α-SDH. The complexes were then subjected to SDS-PAGE on a 12 to 15% (wt/vol) acrylamide–0.32 to 0.40% (wt/vol) bisacrylamide gradient gel. After a completed run the gel was sliced, and the slices were dissolved by incubation with 0.5 ml of 30% $H_2O_2$ at 45°C overnight. Aquasol (10 ml) was then added, and the radioactivity was determined (●, $^{35}S$; ○, $^3H$). Slices no. 22 and 23 are the front of electrophoresis. Upon prolonged autoradiography of the gel no darkening of the film was found for the $M_r$ 19,000 band of culture C, whereas all other bands gave intense darkening of the film.*

Nevertheless, the above results suggest that mutants *citF78*, *citF42*, *citF44*, and *citF12* are not defective in the synthesis of the two larger subunits of the SDH complex but rather they are unable to bind these subunits to the membrane. There is strong experimental evidence (16; and the present paper) that SDH binds to the membrane via cytochrome $b_{558}$. Consequently, the cytochrome content of the *citF* mutants was determined. Membranes from mutants *citF78*, *citF42*, and *citF44* were found to lack or have very reduced amounts of cytochrome $b_{558}$ (Fig. 8). SDH-positive revertants of *citF78* and *citF44* were isolated. The cytochrome spectrum of one revertant of each mutant was determined and found to be identical with that of wild-type bacteria. The other six *citF* mutants had no detectable alterations in cytochrome content. The phenotypes of the nine *citF* mutants studied are summarized in Table 1.

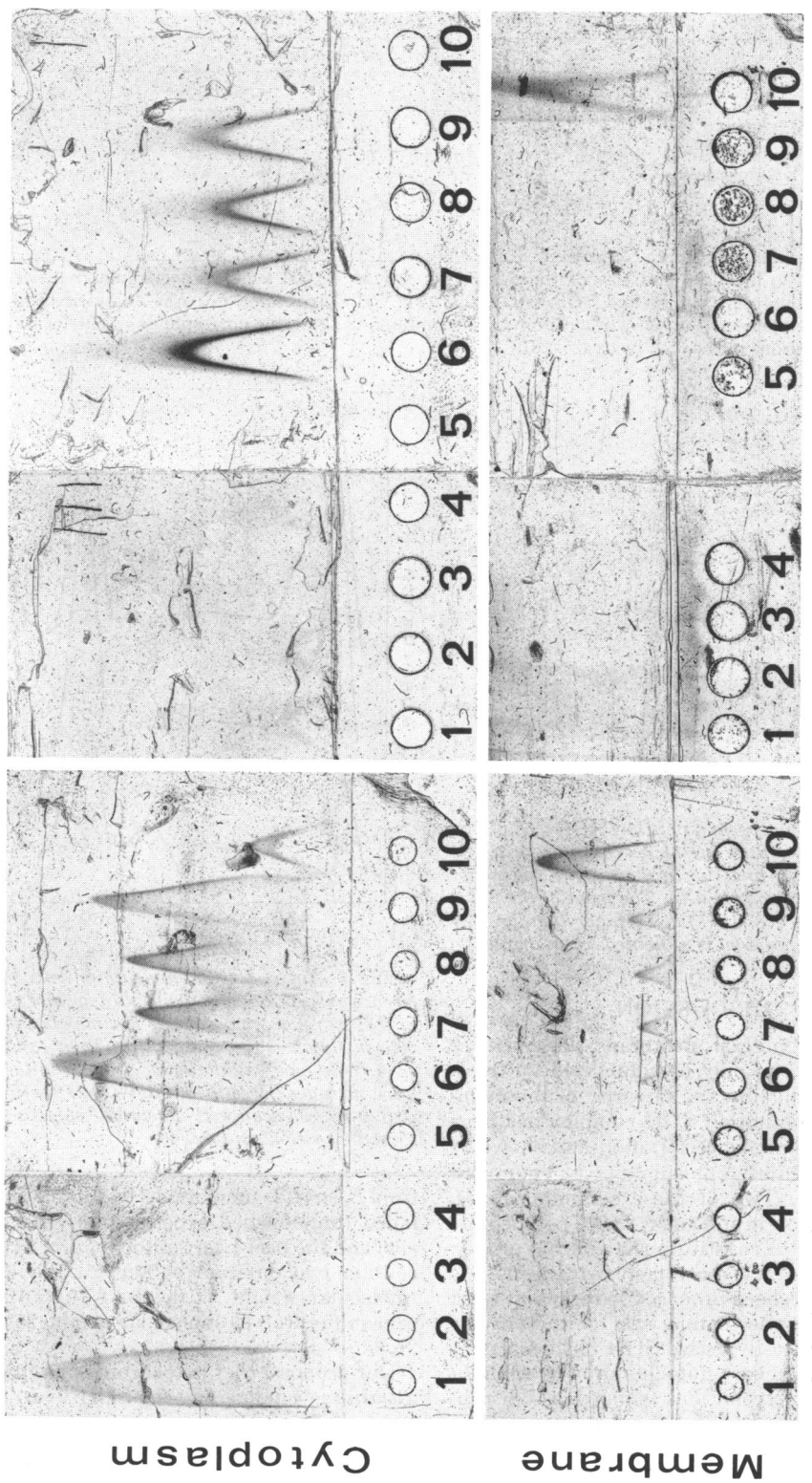


FIG. 6. *Rocket immunoelectrophoresis of cytoplasmic and membrane fractions from wild-type bacteria and from citF mutants by using α-SDH and α-Ip. All samples were first treated with 1% (wt/vol) SDS. To avoid unspecific precipitation, 4% (vol/vol) Triton X-100 was added to the samples before electrophoresis. Lanes: 1, citF83; 2, citF2; 3, citF11; 4, citF8; 5, citF69; 6, citF12; 7, citF44; 8, citF42; 9, citF78; 10, wild type. The small precipitates seen in the membrane fractions from citF12, citF44, citF42, and citF78 in the α-SDH gels are due to trapping of cytoplasmic material as shown previously (26).*

TABLE 1. *Properties of nine citF mutants*

| Location | Subunit[a] | *citF* mutant | | | | | | | | | Wild type |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *78* | *42* | *44* | *12* | *69* | *8* | *11* | *2* | *83* | |
| Membrane | $M_r$ 65,000 | − | − | − | − | − | − | − | − | − | + |
| | $M_r$ 28,000 | − | − | − | − | − | − | − | − | − | + |
| | Cytochrome $b_{558}$ | − | − | − | + | + | + | + | + | + | + |
| Cytoplasm | $M_r$ 65,000 | + | + | + | + | − | − | − | − | + | − |
| | $M_r$ 28,000 | + | + | + | + | − | − | − | − | − | − |

[a] The presence, location, and size of the $M_r$ 65,000 and $M_r$ 28,000 subunits were analyzed with rocket immunoelectrophoresis (Fig. 6) and SDS-PAGE-CIE (Fig. 7). Cytochrome $b_{558}$ was analyzed by difference absorption spectroscopy (Fig. 8). The position of the mutants in the table corresponds to the location of the respective mutations in the *citF* locus (25, 26).

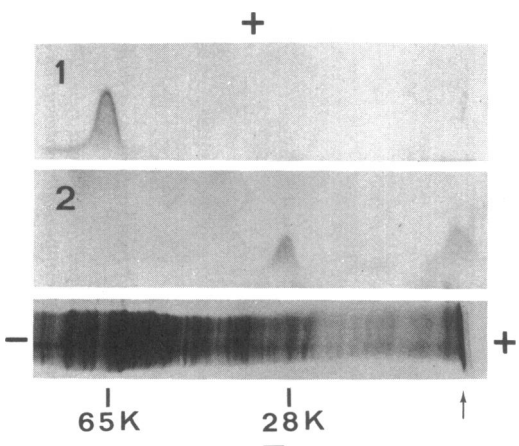


FIG. 7. *Autoradiograph of SDS-PAGE-CIE of $^{35}$S-labeled cytoplasm from citF44; 0.28 mg of protein was loaded on the gel and run in the second dimension against (1) α-Fp and (2) α-Ip. The front of the SDS-polyacrylamide gel (15% [wt/vol] acrylamide–0.4% [wt/vol] bisacrylamide) is indicated by an arrow.*

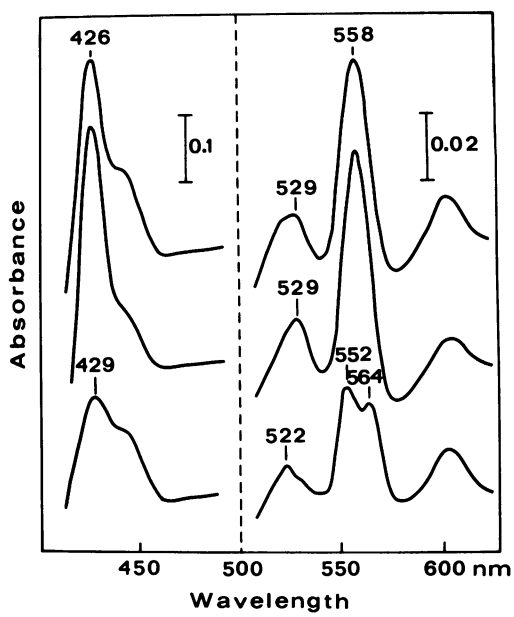


FIG. 8. *Air-oxidized minus dithionite-reduced difference absorption spectra of wild-type and citF membranes at room temperature. The membrane protein concentration was 3.8 mg/ml in each sample. Top scan, Wild type; middle scan, citF83 (similar spectra were obtained for citF12, citF69, citF8, citF11, and citF2); bottom scan, citF78 (similar spectra were obtained for citF42 and citF44).*

## DISCUSSION

SDH is an integral membrane protein in *B. subtilis*, as it can only be solubilized by treatments which disrupt the integrity of the membrane. The solubilized SDH complex has been purified by immunoprecipitation and shown to contain three different subunits in equimolar amounts (17). Two of these subunits, an $M_r$ 65,000 flavoprotein and an $M_r$ 28,000 polypeptide are suggested to constitute the enzyme proper. The third subunit is most likely cytochrome $b_{558}$ (16), which has been proposed to represent (part of) the membrane binding site for the enzyme (18). Enzymatically active SDH has been purified from beef heart mitochondria by solubilization with chaotropic ions, which do not disrupt membrane integrity (8). Chaotropic ions have also been successfully used to solubilize SDH from chromatophores of *R. rubrum* (9), whereas attempts to solubilize (active or inactive) SDH from *B. subtilis* membranes by this method have failed (unpublished experiments). The *R. rubrum* and the beef-heart mitochondria SDH both contain two different subunits, a flavoprotein (molecular weight, 60,000 and 70,000) and an iron-sulfur protein (molecular weight, 25,000 and 27,000).

Our proposal that cytochrome *b* is (part of) a membrane binding site for SDH in *B. subtilis* was originally based on the observation that SDH antigen accumulates in the cytoplasm when heme synthesis is blocked in the bacteria (18). When heme synthesis is resumed the

amount of cytoplasmic SDH antigen rapidly diminishes, with a concomitant rise in membrane-bound antigen and SDH activity. The specificity of the antibody used in these experiments was then only partly known. We have now obtained antibodies specific for each of the two SDH subunits. This makes it possible to examine in more detail the relationship between cytochrome synthesis and synthesis and membrane binding of SDH in *B. subtilis*. From the present results we can first conclude that both SDH subunits accumulate in the cytoplasm when heme synthesis is blocked, indicating that there is no coupling between synthesis and membrane binding of the subunits. In the cytoplasm the two subunits are precipitated essentially independently of each other by subunit-specific antibody, whereas active SDH complex is precipitated with antibody specific for the flavoprotein. The $M_r$ 65,000 subunit which accumulates in the cytoplasm during 5-aminolevulinic acid starvation contains covalently bound flavin, indicating that flavin is bound to the protein before the subunit becomes membrane bound. Possibly, flavin is attached to the protein during translation as has been shown for 6-hydroxy-D-nicotine oxidase in *Arthrobacter oxidans* (13). There is no difference in mobility in SDS-PAGE of the $M_r$ 65,000 or the $M_r$ 28,000 cytoplasmic subunit compared with the membrane-bound subunits. A precursor relationship between cytoplasmic subunits made during 5-aminolevulinic acid starvation and membrane-bound enzyme is clearly shown by the results of pulse-labeling with L-[$^{35}$S]methionine during 5-aminolevulinic acid starvation. The fact that membrane binding of cytoplasmic subunits occurs also when protein synthesis is blocked before heme synthesis is resumed demonstrates that apocytochrome *b* is made during 5-aminolevulinic acid starvation. Although we cannot specifically identify apocytochrome $b_{558}$, it seems reasonable to assume that it is membrane bound. The binding of heme to the apocytochrome could expose a binding site(s) for either or both of the two larger subunits of the SDH complex.

The ratio of L-[$^{35}$S]methionine residues in the three subunits of the SDH complex was 1:1.4:1 both in the control culture and in the culture which was given 5-aminolevulinic acid in the presence of chloramphenicol. Flavoprotein and the $M_r$ 28,000 subunit of the membrane-bound SDH complex from the culture grown continuously without 5-aminolevulinic acid contained only 20% $^{35}$S radioactivity compared with the SDH complex isolated from the other two cultures. No $^{35}$S activity was detected in the apocytochrome in the membrane-bound SDH from the starved culture, however. This shows that there is essentially no escape synthesis of holocytochrome during the L-[$^{35}$S]methionine pulse during 5-aminolevulinic acid starvation.

The results also support our suggestion that cytochrome *b* is required for membrane binding of SDH in *B. subtilis*. However, 5-aminolevulinic acid starvation is not selective in that it prevents synthesis of all holocytochromes as evidenced by the cytochrome spectra of membranes from 5-aminolevulinic acid-starved cells (18). Strong evidence for the specific involvement of cytochrome $b_{558}$ in SDH membrane binding was obtained when the previously described *citF* mutants (25, 26) were studied further. None of these mutants contains membrane-bound antigen which can be detected with subunit-specific antibody. Four of the *citF* mutants, however, contain both flavoprotein and $M_r$ 28,000 subunits in the cytoplasm. Three of these mutants lack membrane-bound cytochrome $b_{558}$, but do contain the other main cytochromes of *B. subtilis*. The remaining six *citF* mutants have normal cytochrome spectra. The respective mutations in the three cytochrome $b_{558}$-deficient *citF* mutants map at one end of the *citF* locus (25). Possibly this region codes for apocytochrome $b_{558}$. The mutant *citF12* contains the two larger subunits of the SDH complex in the cytoplasm, but has a normal cytochrome spectrum. The *citF12* mutation maps next to the three mutations which affect cytochrome $b_{558}$. The basis for the SDH-negative phenotype of *citF12* is unclear. The other five *citF* mutants with normal cytochrome spectra may be mutated in the structural genes for the two larger subunits of the SDH complex.

During fractionation of bacterial respiratory chains dehydrogenases are often found to be firmly bound to cytochromes (12). The cytochromes of these complexes can also often be reduced by the dehydrogenases, indicating a close functional relation between enzyme and cytochrome. Firm evidence for a structural role of cytochrome for membrane binding of enzyme has been obtained in only a few cases, however. In *Escherichia coli* nitrate reductase is induced during anaerobic growth in nitrate-containing medium. The enzyme is bound to the cytoplasmic membrane and may constitute some 15% of the total membrane protein (22). Membrane-bound nitrate reductase contains three subunits; the smallest subunit is cytochrome *b*. During heme starvation increasing amounts of an active complex composed of the two larger subunits accumulate in the cytoplasm. When heme synthesis is resumed, this complex binds to cytochrome *b* in the membrane (21, 22). Thus, nitrate reductase in *E. coli* is synthesized as a cytoplasmic protein which will later bind to a cyto-

chrome *b* site in the membrane. Membrane-bound fumarate reductase has recently been purified from *E. coli* by hydrophobic exchange chromatography of detergent-solubilized membrane proteins (10). This enzyme contains an $M_r$ 70,000 flavoprotein and an $M_r$ 24,000 subunit. However, the purified fumarate reductase has an absorption spectrum characteristic of a *b*-type cytochrome. Also, the published gel scans of the purified enzyme (Fig. 4 of reference 10) indicate the presence of an $M_r$ 19,000 polypeptide in molar amounts comparable to those of the flavoprotein subunit. *E. coli* strains with duplications of the fumarate reductase structural gene(s) have been described by Cole and Guest (7). Some of these strains have a 30-fold-increased fumarate reductase activity. In these overproducing strains most of the enzyme activity is found in the cytoplasm. Two polypeptides, $M_r$ 72,000 and $M_r$ 26,500, probably corresponding to the two polypeptides of fumarate reductase, can be found in the cytoplasm, indicating saturation of a membrane binding site. The cytochrome *b* content is not increased in these strains. A limiting number of membrane binding sites has also been suggested for phosphatidylserine decarboxylase in *E. coli* (29). In contrast, a strain of *E. coli* with 50- to 60-fold-increased levels of NADH dehydrogenase contained all enzyme activity in the membrane fraction (33). It should be pointed out that in *B. subtilis* glycerol auxotrophs the membrane protein/lipid ratio can double during glycerol starvation without any obvious adverse effects on the cells (24).

The simplest fragment of the mammalian mitochondrial membrane which shows succinate: ubiquinone reductase activity is complex II (15). Active SDH purified from this complex by treatment with chaotropic agents is a water-soluble protein consisting of two unequal subunits, flavoprotein and iron-sulfur protein (8). In addition, complex II contains two polypeptides with molecular weights of 13,500 and 7,000 (4). The purified SDH can be used to reconstitute alkali-treated complex II, and the stoichiometry of the reconstitution suggests a limited number of binding sites for the enzyme. However, little is known about the synthesis and membrane binding of mammalian SDH. Bruni and Racker (3) have shown that cytochrome *b* is required for reconstitution of succinate:ubiquinone reductase activity, although the cytochrome was not reduced by succinate. These authors concluded that cytochrome *b* has a structural role in the binding of SDH to the mitochondrial inner membrane. In the alkali-treated complex II used by Baginsky and Hatefi (1) for reconstitution of succinate:ubiquinone reductase activity with purified SDH, cytochrome *b* is a major constituent. It has been suggested that the 13,500-molecular-weight polypeptide of complex II is apocytochrome *b*, and a preliminary report indicates that it constitutes the primary binding site for SDH (4).

Weiss et al. (30, 31) have recently isolated succinate:ubiquinone reductase from mitochondria of *Neurospora crassa* after solubilization with Triton X-100. The reductase contained a flavoprotein subunit ($M_r$ 72,000), an iron-sulfur subunit ($M_r$ 28,000), and possibly an apocytochrome *b* subunit ($M_r$ 14,000).

SDH is an evolutionary conservative enzyme. The subunit composition is very similar in both procaryotic (9, 17) and eucaryotic (8, 30) organisms. The amino acid contents of the SDH subunits from beef heart mitochondria and from *R. rubrum* are quite similar (9). In mammalian mitochondria and in *N. crassa* there is a strong indication that cytochrome *b* plays a structural role in SDH membrane binding. In *B. subtilis* the finding that membrane binding of SDH requires cytochrome and the fact that the purified SDH complex contains an equimolar amount of apocytochrome $b_{558}$ suggest a structural role for cytochrome $b_{558}$ in SDH membrane binding. This suggestion is very strongly supported by our present finding that in three cytochrome $b_{558}$-deficient mutants of *B. subtilis* both subunits of SDH are synthesized but cannot bind to the membrane. The mutations map in the *citF* locus (25). This locus seems rather complex and possibly harbors structural genes for both subunits of SDH as well as for apocytochrome $b_{558}$. We are presently attempting to resolve this locus better by isolating and characterizing more *citF* mutants.

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