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Orientation of Succinate Dehydrogenase and Cytochrome b_{558} in the *Bacillus subtilis* Cytoplasmic Membrane

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The orientation of the three subunits of the membrane-bound succinate dehydrogenase (SDH)-cytochrome b_{558} complex in *Bacillus subtilis* was studied in protoplasts ("right side out") and isolated membranes (random orientation), using immunoadsorption and surface labeling with [^{35}S]diazobenzenesulfonate. Anti-SDH antibodies were adsorbed by isolated membranes but not by protoplasts. The SDH M_r 65,000 flavoprotein subunit was labeled with [^{35}S]diazobenzenesulfonate in isolated membranes but not in protoplasts. The flavoprotein subunit is thus located on the cytoplasmic side of the membrane. The location of the SDH M_r 28,000 iron-protein subunit was not definitely established, but most probably the iron-protein subunit also is located on the cytoplasmic side of the membrane. Antibodies were not obtained to the hydrophobic cytochrome b_{558}. The cytochrome was strongly labeled with [^{35}S]diazobenzenesulfonate in protoplasts, and labeling was also obtained with isolated membranes. Cytochrome b_{558} is thus exposed on the outside of the membrane. In *B. subtilis* SDH binds specifically to cytochrome b_{558}, which suggests that the cytochrome is exposed also on the cytoplasmic side of the membrane. The results obtained suggest that the *B. subtilis* SDH is exclusively located on the cytoplasmic side of the membrane where it is bound to cytochrome b_{558}, which spans the membrane.

Bacterial respiratory systems show great diversity (21). Even within a particular bacterial species the amounts and types of respiratory systems may vary depending on the growth conditions. In comparison to mitochondria (39), our knowledge about the structural components of most bacterial respiratory systems, especially in aerobic cells, is rather fragmentary.

In *Bacillus subtilis*, as in other aerobic cells, the respiratory enzyme succinate dehydrogenase [SDH; EC 1.3.99.1, succinate:(acceptor)oxidoreductase] is tightly bound to the membrane. *B. subtilis* SDH is solubilized as a monodisperse SDH-cytochrome b_{558} complex by extraction of the cytoplasmic membrane with the nonionic detergent Triton X-100 (14). Cytochrome b_{558} is a hydrophobic protein (15) that is required for membrane binding of SDH (16). The cytochrome also interacts functionally with SDH; about 50% of the dithionite-reducible cytochrome b_{558} in the complex is reduced by succinate (13). The Triton X-100-solubilized SDH-cytochrome b_{558} complex has been purified by immunoprecipitation with SDH-specific antibody. It contains equimolar amounts of three different polypeptides with M_r of 65,000, 28,000, and 19,000, respectively. The complex also contains 1 mol of acid-nonextractable flavin per mol of complex and one to two protohemes per acid-nonextractable flavin (13, 14, 16). The M_r 65,000 subunit contains the flavin and is designated Fp. SDH purified from beef heart mitochondria (5) and from *Rhodospirillum rubrum* (6) consists of two unequal subunits. The larger subunit (Fp with M_r of 70,000 and 60,000, respectively) contains a covalently bound flavin adenine dinucleotide group and two iron-sulfur centers, and the smaller subunit (Ip with M_r of 27,000 and 25,000, respectively) contains one iron-sulfur center. In analogy with the beef heart and the *R. rubrum* enzymes, the *B. subtilis* SDH M_r 28,000 subunit is tentatively called Ip. The M_r 19,000 subunit of the *B. subtilis* SDH-cytochrome b_{558} complex is the cytochrome apoprotein. In the present work we have determined the orientation of the SDH and cytochrome b_{558} proteins in the *B. subtilis* cytoplasmic membrane. By using immunoadsorption and by surface labeling with diazobenzene-[^{35}S]sulfonate ([^{35}S]DABS) of both protoplasts and isolated membranes, we confirm (2, 17) that SDH is located on the inside of the cytoplasmic membrane. In contrast, cytochrome b_{558} is found to be exposed on both sides of the membrane and is thus a transmembrane protein.

MATERIALS AND METHODS

Bacteria. *B. subtilis* BR102 (*hisB trpC2*) was used in all experiments.

Antisera. Antimembrane antiserum was produced

by injecting rabbits with membranes prepared from BR102 grown in minimal medium (33). Anti-Fp-specific antibodies were produced by immunizing rabbits with SDH-staining precipitates excised from crossed immunoelectrophoresis (CIE plates) (33). The specificity of this serum was determined in sodium dodecyl sulfate-polyacrylamide gel electrophoresis-CIE (SDS-PAGE-CIE) with both BR102 membranes and purified SDH-cytochrome b_{558} complex (16). The immunization scheme and the purification and storage of the antisera were recently described (16, 33).

Immunoadsorption with protoplasts and membranes. Protoplasts were prepared from BR102 grown in 2× 400 ml of NSMP (7) in 1,600-ml indentated Fernbach flasks at 37°C with shaking at 200 rpm. When the cultures reached an absorbancy at 650 nm of 0.7, the bacteria were harvested by centrifugation at 10,000 × *g* for 10 min at 15°C. The pellet was suspended in 80 ml of 0.8 M sucrose–10 mM $MgCl_2$–70 mM NaCl–50 mM Tris-hydrochloride, pH 7.5 (TSNM), containing 2 mg of lysozyme per ml. The cells were incubated with gentle agitation at 37°C for 30 min, at which time more than 95% of the bacteria were converted to osmotically sensitive protoplasts. The protoplasts were centrifuged at 5,500 × *g* for 10 min at 20°C, washed once with 80 ml of TSNM, and finally suspended in 14 ml of TSNM.

Membranes were prepared by suspending the protoplast pellet in 200 ml of 50 mM potassium phosphate, pH 8.0, with 9 mM $MgSO_4$ and containing 1 mg each of DNase and RNase. After 30 min of incubation at 37°C the lysate was centrifuged at 48,000 × *g* for 30 min at 4°C. The pellet, containing the membrane fraction, was washed once with 50 mM Tris-hydrochloride, pH 7.5, and suspended in 14 ml of the same buffer.

One milliliter of antimembrane antiserum at 80 mg of protein per ml was mixed with various amounts of protoplasts or membranes in a final volume of 8 ml. The samples were incubated at room temperature for 1 h. Samples containing protoplasts were then centrifuged at 4,000 × *g* for 10 min at 20°C. The supernatant was dialyzed against distilled water for 24 h to remove the sucrose, followed by centrifugation at 48,000 × *g* for 30 min at 4°C to remove membrane fragments from lysed protoplasts. Samples containing membranes were centrifuged at 48,000 × *g* for 30 min at 4°C. The unadsorbed antibodies in the protoplast and membrane samples were precipitated with 31.3% (wt/vol) $AmSO_4$. The precipitate was collected by centrifugation at 12,000 × *g* for 10 min at 4°C. The $AmSO_4$ precipitate was dissolved in 0.5 ml of distilled water and dialyzed against 0.1 M NaCl containing 15 mM NaN_3.

Unadsorbed antibody was analyzed by CIE, using a constant amount of Triton X-100-solubilized BR102 membranes as antigen. All CIE plates were run in duplicate. The resulting precipitates were projected on paper. Two projections of each precipitate were cut out and weighed, which gives a relative measure of the peak area.

Preparation of protoplasts and membranes for DABS labeling. BR102 was grown in 6× 400 ml of NSMP (pH 7.2) in 1,600-ml indentated Fernbach flasks at 32°C with shaking at 200 rpm. When the cultures reached an absorbancy at 600 nm of 0.9, the bacteria were centrifuged at 10,000 × *g* for 15 min at 15°C. The pellets were washed once with 200 ml of 0.8 M sucrose–10 mM $MgCl_2$–70 mM NaCl–25 mM sodium phosphate, pH 7.8 (PSNM), and then suspended in 60 ml of PSNM containing 9.6 mg of lysozyme. After incubation at 42°C for 30 min with gentle agitation, more than 95% of the cells were converted to osmotically sensitive protoplasts.

Membranes were prepared by centrifuging 60 ml of protoplast suspension at 8,000 × *g* for 15 min at 15°C. The pellet was suspended in 30 ml of 10 mM $MgCl_2$–70 mM NaCl–25 mM sodium phosphate, pH 7.8, containing 0.3 mg each of DNase and RNase. The suspension was incubated at 37°C for 15 min. Whole cells and protoplasts were then removed by centrifugation at 5,000 × *g* for 15 min at 4°C. The resulting supernatant was centrifuged at 48,000 × *g* for 30 min at 4°C, and the pellet (membrane fraction) was suspended in 60 ml of PSNM.

DABS labeling. [^{35}S]DABS (20 to 30 Ci/mol) was synthesized from sulfanilinic acid (E. Merck AG) and [^{35}S]sulfanilinic acid (The Radiochemical Centre) as described by Tinberg and Packer (37). A 25 mM stock solution was prepared in PSNM and used within a few hours after synthesis. The DABS concentrations given in the text are calculated on the amount of sulfanilinic acid used, assuming a 100% yield of DABS. The same batch of [^{35}S]DABS was used for each set of experiments. Different amounts of [^{35}S]DABS stock solution were added to tubes containing protoplasts or membranes, and the volumes were adjusted with PSNM. The tubes were incubated for 20 min at room temperature protected from light. The reaction was stopped by adding an equal volume of 10 mM histidine–20 mM Tris-hydrochloride, pH 7.8, in PSNM to the protoplasts and histidine–Tris-hydrochloride buffer to the membranes. Membranes were then prepared from the protoplasts as described above, but using a lysis buffer containing 10 mM histidine–20 mM Tris-hydrochloride (pH 7.8)–10 mM $MgSO_4$.

Labeled membranes and membranes isolated from labeled protoplasts were washed twice with 10 mM histidine–20 mM Tris-hydrochloride, pH 7.8, and once in 24 mM sodium diethylbarbiturate (pH 8.6) by centrifugation at 48,000 × *g* for 30 min at 4°C. The membranes were finally suspended to about 8 mg of protein per ml in 24 mM sodium diethylbarbiturate, pH 8.6. SDH activity, radioactivity, and protein content were determined, and the membranes were stored at −80°C. The amount of DABS bound did not interfere with protein determinations.

Electrophoresis. CIE was done with ME agarose (Miles Laboratories) in the presence of Triton X-100 as recently described (33). SDH and NADH zymogram staining was done as described by Owen and Salton (31). The SDH staining could be intensified by adding 50 μg of 5-*N*-methylphenazonium sulfate (PMS) per ml to the reaction mixture. The method of Neville was used for SDS-PAGE (21), and SDS-PAGE-CIE was done as described by Chua and Blomberg (4). Fluorography of radioactive SDS-PAGE gels with sodium salicylate was done as described by Chamberlain (3). The gels were exposed at −80°C with Kodak XAR-5 film.

Determination of SDH activity. SDH activity was measured as the succinate-dependent reduction of 2,6-dichlorophenol-indophenol in the presence of various concentrations (0.15 to 0.75 mM) of PMS. Enzyme activity at infinite PMS concentration was calculated

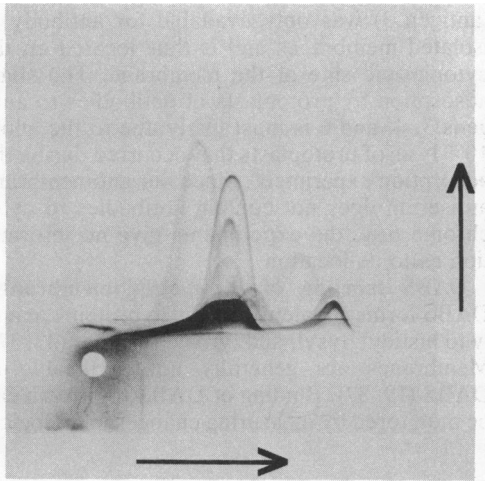

FIG. 1. CIE of Triton X-100-solubilized *B. subtilis* membranes, 46 μg of protein, against antimembrane antiserum. The plate was stained for protein with Coomassie brilliant blue.

by extrapolation from a double-reciprocal plot as described by Singer (34). The enzyme reaction mixture contained, in addition to PMS, 69 μM 2,6-dichlorophenol-indophenol, 9 mM sodium succinate, 1 mM KCN, and 11 mM potassium phosphate, pH 7.4. All measurements were done at 20°C. The apparent K_m of SDH for succinate was estimated at infinite PMS concentration at four different succinate concentrations. An absorption coefficient for 2,6-dichlophenol-indophenol at 600 nm of 16.1×10^3 cm M^{-1} was used to calculate enzyme activity.

Other methods. Solubilization of membranes with Triton X-100 and immunoprecipitation of the SDH-cytochrome b_{558} complex was done as described before (14). The degree of lysis of protoplasts was estimated by the release of the cytoplasmically located enzyme malate dehydrogenase, as recently described (15). Protein was determined by the method of Lowry et al. (27). Radioactivity was determined by scintillation counting as recently described (16).

RESULTS

CIE. About 40% of total membrane proteins and 95% of the SDH enzyme activity is solubilized from the *B. subtilis* cytoplasmic membrane with 4% (vol/vol) Triton X-100 at 10 mg of membrane protein per ml (13). Solubilized membrane proteins were run in CIE against antimembrane antiserum. The immunoprecipitate pattern observed is shown in Fig. 1, and a schematic drawing of the major immunoprecipitates is shown in Fig. 2. Immunoprecipitate 3 stains for SDH activity. This precipitate is symmetric and gives no indication of antigenic heterogeneity. Its position on the plate is similar to that previously reported by us using another batch of antiserum (33). Immunoprecipitates 7 and 8 stain for NADH dehydrogenase activity. The shape and position of these precipitates are similar to the results reported by Bergsma et al. for *B. subtilis* W23 membranes (2).

Most likely the SDH-staining immunoprecipitate in Fig. 1 only contains Fp, Ip, and cytochrome b_{558}. The *B. subtilis* SDH-cytochrome b_{558} complex solubilized with Triton X-100 is monodisperse in detergent (15), and only the above three polypeptides are precipitated by anti-Fp antibody in test tube immunoprecipitation. The SDH-staining immunoprecipitate contains acid-nonextractable flavin (manuscript in preparation) and heme (14). Furthermore, when excised from the CIE plate and injected into rabbits the SDH-staining precipitate does not induce antibodies to any membrane antigens other than the SDH Fp and Ip polypeptides (16). The specificity of the anti-SDH antibodies in the antimembrane antiserum was determined by SDS-PAGE-CIE, using purified SDH-cytochrome b_{558} complex as antigen (Fig. 3). The antimembrane antiserum contains precipitating antibodies against Fp and Ip but not cytochrome b_{558}. The lack of precipitating antibodies against the cytochrome is in agreement with our previous findings that this hydrophobic protein is a poor immunogen (16).

Immunoadsorption. Immunoadsorption followed by quantitative analysis of unadsorbed antibody in CIE is an excellent method for determining the orientation of membrane proteins in bacteria (30). Antibodies cannot pass an intact membrane, and the technique thus requires sealed membrane preparations with known orientation. *B. subtilis* membranes with

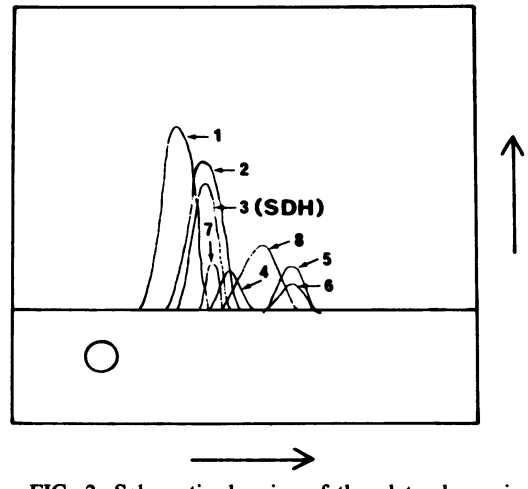

FIG. 2. Schematic drawing of the plate shown in Fig. 1 presenting the immunoprecipitates considered in the immunoadsorption experiments.

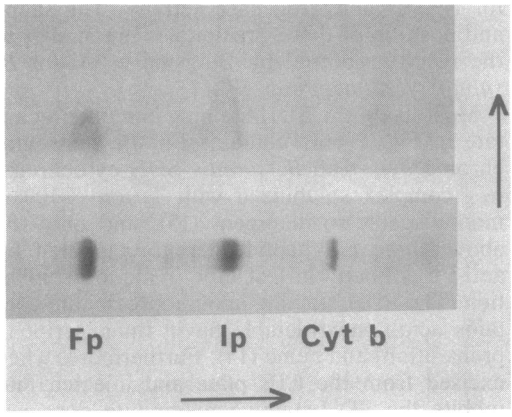

FIG. 3. Autoradiograph of SDS-PAGE-CIE. First dimension (horizontal): SDS-PAGE of L-[^{35}S]methionine-labeled *B. subtilis* SDH-cytochrome b_{558} complex on a 15% (wt/vol) acrylamide gel. Second dimension (vertical): Immunoelectrophoresis against antimembrane antiserum.

"right side out" orientation are easily obtained in the form of protoplasts. No method which produces exclusively or predominantly "inside out" membranes from *B. subtilis* is known to us.

In immunoelectrophoresis the peak area under the immunoprecipitate is inversely proportional to the amount of antibody at constant antigen load (30). Control experiments with dilutions of the antiserum showed this relation to be valid also in our system (data not shown). A constant amount of antimembrane antiserum was adsorbed with various amounts of protoplasts or membranes prepared from lysed protoplasts. Unadsorbed antibody was analyzed in CIE with Triton X-100-solubilized *B. subtilis* membranes. The inverse of the relative peak area of the immunoprecipitates was plotted against the relative amount of antigen used for adsorption (Fig. 4A and B).

Antibodies to antigens 2, 5, 7, and 8 are adsorbed by both protoplasts and isolated membranes. These antigens are thus exposed on the outside of the cytoplasmic membrane. Antibodies to antigens 3 (SDH), 4, and 6 are only adsorbed by isolated membranes. Antibodies to antigen 1 were not adsorbed by either protoplasts or isolated membranes. However, if the antiserum was incubated with Triton X-100-solubilized membranes also, antibodies to antigen 1 were adsorbed, indicating that this antigen is largely buried in the membrane. From the above results we conclude that both sides of the cytoplasmic membrane are exposed to antibody in isolated membranes. Antigens exposed only at the outside of the membrane therefore cannot be identified as such in the experiments. SDH (antigen 3) was only available for antibody in isolated membranes and is thus located on the cytoplasmic side of the membrane. The slight adsorption by protoplasts of antibodies to antigens 3, 4, and 6 is most likely due to the about 10% lysis of protoplasts that occurred during the adsorption experiment. Since our antimembrane antiserum does not contain antibodies to cytochrome b_{558}, the experiments give no information as to its location.

DABS labeling of *B. subtilis* membranes. DABS forms covalent adducts to proteins, mainly to histidyl, lysyl, and tyrosyl residues (32, 37). Membranes are generally not permeable to DABS (19, 37). Binding of DABS to protein can be monitored by measuring changes in biological

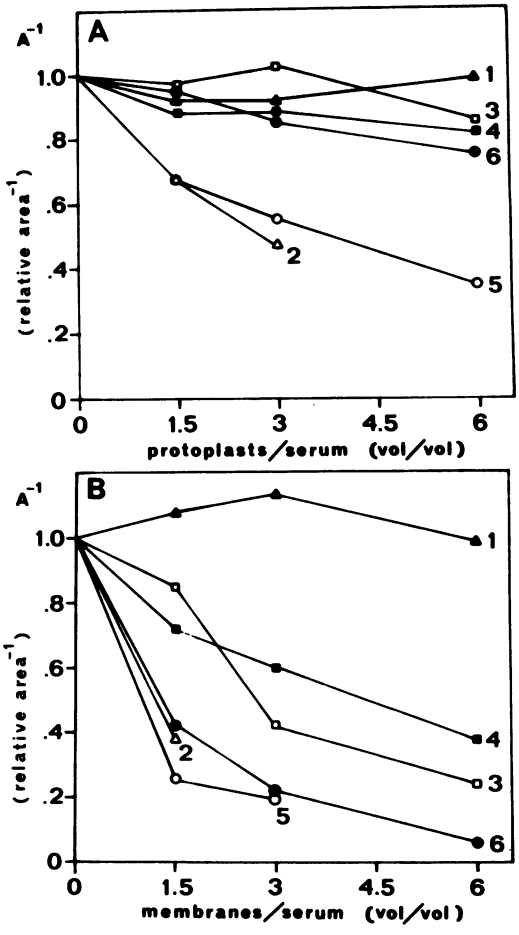

FIG. 4. Immunoadsorption of antimembrane antiserum to protoplasts (A) and isolated membranes (B). Numbers refer to the different immunoprecipitates shown in Fig. 2. Data on immunoprecipitates 7 and 8 are not shown. Antibodies to these two antigens were adsorbed away at the lowest amount of both protoplasts and isolated membranes added.

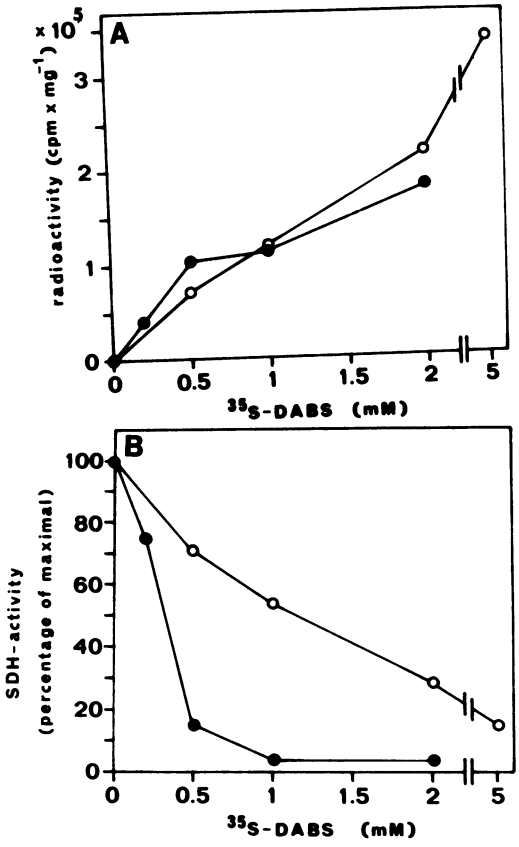

FIG. 5. DABS labeling of a constant amount of protoplasts (●) and membranes prepared from lysed protoplasts (○) with increasing amounts of [^{35}S]DABS as described in the text. (A) Radioactivity bound to membranes. (B) SDH activity remaining in membranes. Maximal enzyme activity was 420 nmol of succinate oxidized mg of membrane protein^{-1} min^{-1}.

activity and by incorporation of radioactivity, using [^{35}S]DABS. *B. subtilis* protoplasts and membranes prepared from lysed protoplasts were reacted with various concentrations of [^{35}S]DABS as described in Materials and Methods. Membranes were then isolated and their SDH activity and radioactivity were determined. The amount of labeling was approximately the same in both preparations and proportional to the amount of [^{35}S]DABS added (Fig. 5A). SDH enzyme activity was inactivated at lower DABS concentrations in protoplasts than in isolated membranes (Fig. 5B). In either preparation the apparent K_m for succinate (0.3 mM at 20°C) was not affected by DABS. SDH enzyme activity was not affected when DABS was reacted with an excess of histidine and Tris before addition to protoplasts or membranes.

The pattern of polypeptides labeled with [^{35}S]DABS in protoplasts and isolated membranes is shown in Fig. 6. Covalent binding of DABS does not alter the mobility of polypeptides in SDS-PAGE (36). The labeling patterns observed for the two preparations are not identical, which confirms that the membrane proteins exposed are not all the same in protoplasts and in isolated membranes.

DABS labeling of the SDH-cytochrome b_{558}

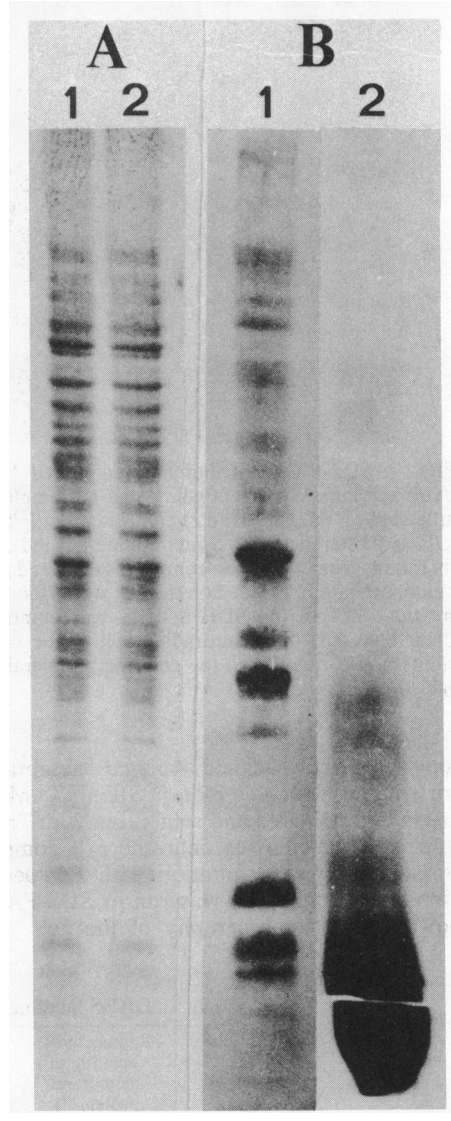

FIG. 6. SDS-PAGE of [^{35}S]DABS-labeled membranes on a 10 to 15% (wt/vol) acrylamide gradient gel. Protoplasts and isolated membranes labeled with 0.7 mM [^{35}S]DABS. (See data in Table 1.) (Lane 1) Labeled isolated membranes, 33 μg of protein loaded on the gel; (lane 2) membranes, 36 μg of protein loaded on the gel, prepared from labeled protoplasts. (A) Gel stained with Coomassie brilliant blue; (B) fluoroautoradiograph of a parallel gel.

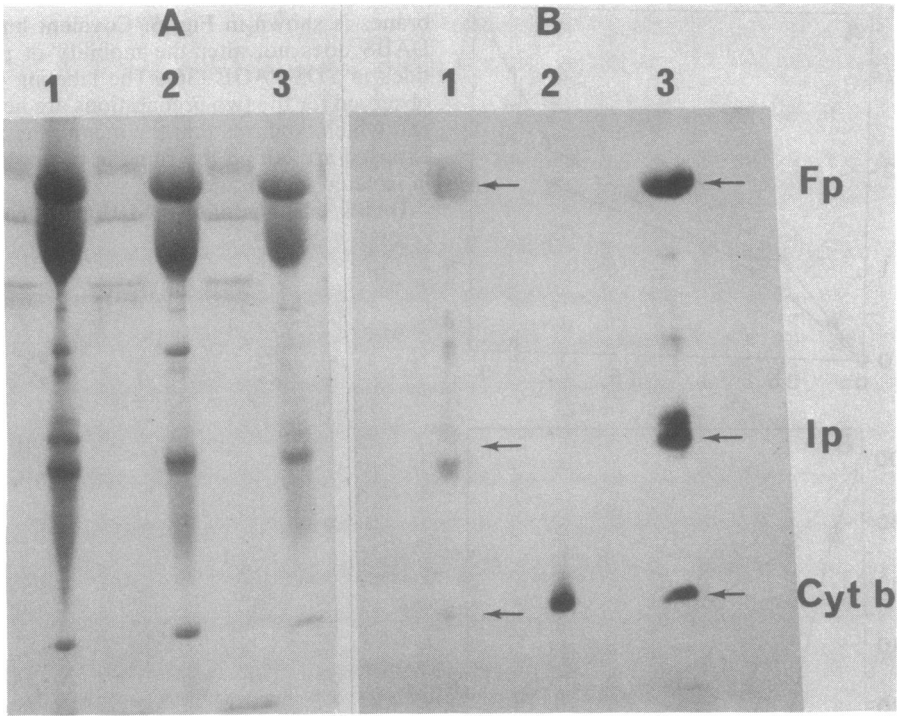

FIG. 7. SDS-PAGE of immunoprecipitated ^{35}S-labeled SDH-cytochrome b_{558} complexes on a 10 to 15% (wt/vol) acrylamide gradient gel. (Lane 1) Complex from [^{35}S]DABS (0.7 mM)-labeled isolated membranes, 960 cpm loaded on the gel; (lane 2) complex from [^{35}S]DABS (0.7 mM)-labeled protoplasts, 770 cpm loaded on the gel; (lane 3) reference, L-[^{35}S]methionine-labeled complex, 3,750 cpm loaded on the gel. Quantitative data, on the preparations from which the complexes analyzed in lanes 1 and 2 were isolated, are shown in Table 1. The whole immunoprecipitates, loaded on the gel, were isolated from 1.8 mg of Triton X-100-solubilized membrane protein. More than 80% of the SDH activity was immunoprecipitated. (A) SDS-PAGE gel stained with Coomassie brilliant blue; (B) fluoroautoradiograph of the same gel. Arrows indicate the positions of each of the three subunits of the complex in the gel. The polypeptide bands seen in (A) between lanes 1, 2, and 3 are molecular weight markers.

complex. Protoplasts and isolated membranes were treated with 0.7 mM [^{35}S]DABS. Membranes were prepared and solubilized with Triton X-100, and the SDH-cytochrome b_{558} complex was isolated by precipitation with Fp-specific antibody. The complex was run in SDS-PAGE followed by autoradiography of the gels. The results of this experiment are shown in Fig. 7A and B and Table 1. Cytochrome b_{558} is the major polypeptide labeled in protoplasts. Slight labeling of the Fp polypeptide was also found in protoplasts, whereas no labeling of the Ip polypeptide was detected (Fig. 7B, lane 2). In isolated membranes the Fp polypeptide was strongly

TABLE 1. DABS labeling of the SDH-cytochrome b_{558} complex[a]

Membrane prepn	Radioactivity (cpm mg of membrane protein^{-1})	% Lysis[b]	SDH activity[c]	% Triton X-100-solubilized SDH activity	% Triton X-100-solubilized membrane protein
[^{35}S]DABS-labeled protoplasts	14×10^4	14	98	98	56
[^{35}S]DABS-labeled isolated membranes	9×10^4	100	172	98	55
Unlabeled protoplasts		10	420	98	40

[a] Labeling with 0.7 mM [^{35}S]DABS and preparation of membranes was done as described in the text. About 17% of the radioactivity in both types of preparations was found to be extractable (1) by methanol-chloroform.
[b] Measured as release of malate dehydrogenase.
[c] Nanomoles of succinate oxidized per milligram of membrane protein per minute.

labeled and weaker labeling was found in the cytochrome b_{558} polypeptide, whereas no labeling of the Ip polypeptide was found (Fig. 7B, lane 1). The polypeptide labeling patterns of the SDH-cytochrome b_{558} complex was the same at the following [^{35}S]DABS concentrations: 0.2, 0.5, 1, and 5 mM (isolated membranes) and 1 mM (protoplasts). With increasing DABS labeling both the Fp and the cytochrome b_{558} polypeptide become more susceptible to proteolysis. The labeled polypeptides observed between Fp and cytochrome b_{558} in Fig. 7B (lane 1) are proteolytic fragments of Fp.

From the results of the [^{35}S]DABS labeling experiments we conclude that both the Fp and the cytochrome b_{558} polypeptides are free to react with DABS in isolated membranes, whereas only cytochrome b_{558} is exposed to DABS in protoplasts. The weak labeling of Fp observed in protoplasts is most likely due to protoplast lysis during the 20-min labeling period (Table 1).

DISCUSSION

The membrane orientation of the *B. subtilis* SDH-cytochrome b_{558} complex has been studied in this work by immunoadsorption and by [^{35}S]DABS labeling, using protoplasts (right side out membranes) and isolated membranes (randomly oriented membranes). Anti-SDH antibodies were adsorbed by isolated membranes but not by protoplasts. The antiserum contained antibodies to both the Fp and the Ip subunits. However, the anti-Fp titer is higher and anti-Fp antibodies are probably the major SDH-precipitating antibodies in the antiserum. The Ip subunit was not labeled with [^{35}S]DABS in protoplasts or isolated membranes, whereas heavy labeling of the Fp subunit was found in isolated membranes. Taken together, the results show that the Fp subunit is located on the cytoplasmic side of the *B. subtilis* membrane. Although the orientation of the Ip subunit has not been clearly established, the following facts indicate that this subunit also is located on the cytoplasmic side of the membrane in close contact with the Fp subunit. In *B. subtilis* both SDH subunits are synthesized as soluble precursors with an apparent molecular weight indistinguishable from the subunits of the membrane-bound enzyme (16). Membrane binding of only one subunit does not occur; i.e., Fp and Ip subunits can only stably interact with the membrane as a complex. Furthermore, in Triton X-100-solubilized membranes the Fp and Ip subunits can be covalently cross-linked with dimethylsuberimidate (unpublished data). An alternative interpretation, which we consider less likely, is that the Ip subunit is exposed on both sides of the membrane but is unable to react with DABS or antibody on either side. A localization of the Ip polypeptide exclusively on the outside of the cytoplasmic membrane in *B. subtilis* is not probable in view of the findings listed above.

Cytochrome b_{558} is the major subunit of the SDH-cytochrome b_{558} complex which is labeled by [^{35}S]DABS in protoplasts. This shows that the cytochrome is exposed on the outside of the cytoplasmic membrane. Relatively weak labeling of cytochrome b_{558} is found in isolated membranes compared with the amount of label in the Fp subunit. From the fact that the cytochrome is a specific binding site for SDH in the *B. subtilis* membrane (16) we conclude that cytochrome b_{558} must also be exposed on the cytoplasmic side of the membrane and thus qualifies as a transmembrane protein.

The topology of SDH in beef heart mitochondrial inner membranes has been studied with SDH-specific antibodies (28), by labeling with [^{35}S]DABS (28), and by photoaffinity labeling with phospholipid analogs (8). Mitochondrial SDH is inactivated by DABS and by specific antibody in submitochondrial particles (inside out) but not in mitoplasts (28, 36). SDH has been found exclusively located on the matrix side of the inner mitochondrial membrane, and the Ip subunit seems to penetrate into the lipid bilayer (8).

SDH has been located to the inner surface of the cytoplasmic membrane in several bacterial species, mainly by immunoadsorption (17, 30). When immunoadsorption is used to study the orientation of membrane-bound proteins it is important to establish the specificity of the antibodies. The detergent-protein micelles obtained after solubilization of the membrane may contain protein complexes which are not necessarily functionally related (18). Antibody, which gives an enzyme-staining precipitate in CIE, may thus be directed against an unrelated antigen. In most studies that use immunoadsorption to determine the orientation of SDH, the specificity of the antibody has not been established.

By using artificial electron acceptors with different abilities to penetrate the membrane, Konings (22) has suggested that SDH is exposed on the outside of the cytoplasmic membrane in *B. subtilis*. In later work from the same laboratory results from immunoadsorption experiments were taken to indicate that SDH is located exclusively on the cytoplasmic side of the membrane (2). These latter experiments were performed with only one type of membrane preparation, i.e., membrane vesicles from *B. subtilis* W23, which are claimed to be at least 80% right side out (23). Although the specificity of the antiserum was not established, we suspect that it contained mainly anti-Fp antibodies. These seemingly conflicting results can be resolved by our present experiments. Modification of cyto-

chrome b_{558} by DABS in protoplasts inactivates SDH enzyme activity as measured with the artificial electron acceptors PMS and 2,6-dichlorophenol-indophenol. In this type of assay one thus measures the activity of the SDH-cytochrome b_{558} complex, with the cytochrome being a site of interaction with the electron acceptors. Since our experiments show that cytochrome b_{558} is exposed on the outside of the membrane, this type of electron acceptors cannot be used to study the orientation of the SDH protein in *B. subtilis*.

Cytochromes of the *b* type which span the membrane have been found in *Escherichia coli* (35) and in mitochondria (11, 26, 40) as components of electrochemical potential-generating devices. *E. coli* nitrate reductase is bound on the inside of the cytoplasmic membrane to a dimeric, transmembranous, cytochrome b_{556}^{NO-3} (9, 10, 12). Analogous to what seems to be the case in the *B. subtilis* SDH-cytochrome b_{558} complex, the *E. coli* cytochrome b_{556}^{NO-3} can interact with PMS and transport-reducing equivalents across the membrane to nitrate reductase and ultimately to nitrate (20).

Vibrio succinogenes fumarate reductase is located on the cytoplasmic side of the membrane, where it catalyzes the reduction of fumarate to succinate. The enzyme is part of the anaerobic energy-transducing electron transport chain (24). Triton X-100-solubilized fumarate reductase contains three different polypeptides: an M_r 79,000 iron-sulfur flavoprotein, an M_r 31,000 iron-sulfur protein, and two M_r 25,000 cytochromes *b* (24). The two cytochromes have different midpoint potentials. The cytochrome with the higher potential is required for succinate-quinone reductase activity and is reduced by succinate (24, 38). The topology of the cytochrome in the *V. succinogenes* membrane is not known. DABS inactivates fumarate reductase activity when added to the cytoplasmic side, but not to the outside, of the cytoplasmic membrane (25).

The composition of the *B. subtilis* SDH complex is very similar to that of the *V. succinogenes* fumarate reductase complex. In *B. subtilis* about 50% of the dithionite-reducible cytochrome *b* is reducible with succinate (13). It is not clear, however, whether cytochrome b_{558} exists as a monomer or a dimer in the *B. subtilis* membrane. Cytochrome b_{558} is not essential for growth of the strictly aerobic *B. subtilis* since mutants that specifically lack this cytochrome (and SDH enzyme activity) can be isolated (15, 16).

The functional significance of the transmembrane orientation of cytochrome b_{558} in the *B. subtilis* membrane is uncertain. Possibly the cytochrome is directly involved in the generation of an electrochemical potential across the membrane during succinate oxidation.

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