Succinate Dehydrogenase in *Rhodopseudomonas sphaeroides*: Subunit Composition and Immunocross-Reactivity with Other Related Bacteria

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Antibodies were raised against the succinate dehydrogenase (SDH) present in the chromatophores of phototrophically grown *Rhodopseudomonas sphaeroides*. Crossed immunoelectrophoresis experiments indicated that the SDH present in the cytoplasmic membranes of heterotrophically grown *R. sphaeroides* is probably the same enzyme observed in the chromatophores. The enzyme was extracted by Triton X-100 in a form which consisted of only two subunits (molecular weight, 68,000 and 30,000) and was not associated with a cytochrome b. The antibodies directed against SDH from *R. sphaeroides* showed no immunocross-reactivity with SDH from phylogenetically related bacterial species, including *Rhodopseudomonas capsulata*, *Paracoccus denitrificans*, *Rhodopseudomonas palustris*, *Rhodospirillum rubrum*, and *Rhodospirillum fulvum*.

Succinate dehydrogenase [SDH; EC 1.3.99.1 succinate: (acceptor) oxidoreductase] is an enzyme which has been examined from a variety of eucaryotic and procaryotic sources (25). Most of the biochemical studies have been performed with the enzyme from beef heart mitochondria (11, 16, 28), but studies have also been reported on SDH from plant mitochondria (21, 33), *Neurospora crassa* (45), *Bacillus subtilis* (22–24), *Escherichia coli* (8, 34), *Micrococcus luteus* (9, 10), *Rhodospirillum rubrum* (17, 18), and *Rhodopseudomonas sphaeroides* (26). SDH from beef heart mitochondria and from *Rhodospirillum rubrum* can be removed from the membrane without the use of detergents by exposing the membranes either to alkaline conditions or to a chaotrope salt (25). This form of the enzyme contains two subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and has covalently bound flavin and nonheme iron. However, this form of the enzyme lacks succinate:ubiquinone oxidoreductase activity (25). A large body of work suggests that additional membrane-bound subunits are required for ubiquinone reductase activity. SDH from *N. crassa* mitochondrial membranes has been solubilized and purified from Triton X-100 and shown to contain a third subunit which is a low-molecular-weight cytochrome b (45). This enzyme preparation does have ubiquinone reductase activity. Similarly, SDH from *B. subtilis* has been isolated with Triton X-100 and also contains a low-molecular-weight cytochrome b (42, 43), which is clearly implicated in binding SDH to the bacterial membranes (24). A form of the beef heart enzyme ([i.e., complex II] has also been isolated with bile salts in which the two SDH subunits are associated with a cytochrome b and additional low-molecular-weight subunits (1, 5, 19, 20). These additional subunits are required for ubiquinone reductase activity, and a structural and functional role for the cytochrome b in succinate:ubiquinone oxidoreductase activity of this form of the enzyme is indicated (1, 19).

A major objective of this work was to examine the SDH from *R. sphaeroides* and, in particular, to see whether the enzyme extracted with Triton X-100 is associated with a cytochrome b and low-molecular-weight subunits. This is of particular interest since one branch of the respiratory system of this bacterium (14, 15, 40, 41, 46) is similar to the eucaryotic mitochondrial chain (27).

**Bacterial strains and growth conditions.** *R. sphaeroides* (strain Ga and wild type), *Rhodopseudomonas palustris*, *Rhodospirillum rubrum*, *Rhodospirillum fulvum*, and *Rhodopseudomonas capsulata*, and *Paracoccus denitrificans* were used. All strains were obtained locally. *R. sphaeroides* was grown either anaerobically in the light or aerobically in the dark in medium A of Sistrom (38). *R. capsulata* and *P. denitrificans* were grown aerobically in the dark in the same medium and conditions as for *R. sphaeroides*. *Rhodospirillum fulvum* and *R. palustris* required 250 µg of p-aminobenzoate per liter (43) in the medium. *Rhodospirillum rubrum* required 0.01% yeast extract for optimal growth in the medium A of Sistrom.

35S labeling of *R. sphaeroides* membranes was carried out during aerobic growth by the procedure described by Shephard and Kaplan (37).

**Chromatophore isolation.** Chromatophores were generously provided by A. Crofts (University of Illinois, Urbana). They were obtained from freshly harvested *R. sphaeroides* Ga cells by the procedure described by Bowyer et al. (4).

**Membrane preparations.** *R. sphaeroides* 2.4.1 cells were aerobically grown in the dark. After being harvested and washed, cells were passed through a French press and treated with DNase and RNase (about 10 µg/ml). Whole cells were discarded by 20 min of centrifugation at 3,000 × g. A crude membrane pellet was obtained by centrifugation of the supernatant at 135,000 × g for 1.5 h. After being washed, the membrane pellet was suspended and layered onto 40% (wt/vol) sucrose in 0.1 M phosphate buffer (pH 7.0). The inner membrane preparation was collected at the interphase after 16 h of centrifugation at 63,000 × g. Sucrose was removed by dialysis.

In experiments involving 35S labeling, the French press procedure was replaced by sonication. The resulting mem-
brane pellet (inner and outer membrane together) was used for the analyses.

**SDH extraction and assay.** SDH was extracted from chromatophores by the method described by Ingledew and Prince (26). In experiments with aerobically grown cells, SDH was detergent extracted either from the envelope fraction (whole membrane) or from isolated inner membranes. Membrane pellets were suspended in a glass homogenizer in 0.1 M phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride, 1% Zwittergent 3-12 (CalBiochem-Behring), and 1% Triton X-100 and allowed to stand on ice for 15 min. Insoluble material was removed by ultracentrifugation at 160,000 × g for 1 h.

SDH activity was determined by the method of Michels and Konings (32) with a molar extinction coefficient at 600 nm of 18,800 M⁻¹ cm⁻¹ for dichlorofenolindophenol (3).

**Immunization of rabbits and preparation of antibodies.** Two rabbits (A and B) were each injected with 5 mg of inner membrane protein from aerobically grown *R. sphaeroides*. Membranes were first suspended in 4% Triton X-100 and emulsified with an equal volume of complete Freund adjuvant. One month later, the same rabbits were each injected with boosters containing 3 mg of membrane protein in incomplete Freund adjuvant. Sera were collected weekly. One month later, the same operation was repeated, and all the serum was pooled in one batch.

A third rabbit (rabbit C) was initially injected with arcs obtained from crossed immunoelectrophoresis (CIE) between crude SDH preparations from chromatophores of *R. sphaeroides* and immunoglobulins from rabbits A and B. One booster injection with a similar preparation was given to rabbit C 1 month later. After 1 week and during the subsequent 3 weeks, rabbit C was bled, and the serum was pooled. This antisera was used in line immunoelectrophoresis with detergent-solubilized SDH from aerobic inner membranes to obtain bands for the second booster. This enhanced the production of anti-SDH immunoglobulin G. The antisera obtained after the second booster had the same CIE characteristics as did the previous antisera. All antisera, including those obtained after the third booster, were pooled to make up anti-SDH serum.

Both the anti-inner membrane serum and the anti-SDH serum were purified by the procedure described by Kranz and Gennis (29).

CIE and rocket immunoelectrophoresis were carried out under the conditions described by Kranz and Gennis (29) for CIEs except that saline washings were avoided to allow better expression of SDH antigens (8). SDS-PAGE was performed with 12.5% polyacrylamide by the Laemmli method (30). In the ³⁵S labeling experiments, the stained SDS-PAGE plates were first soaked in 8% sodium salicylate-0.5% glycerol (6). The dried gels were put in close contact with XS-1 Kodak X-ray films inside intensifier screens and stored at −70°C during exposure (31).

SDH and protein staining were done for all CIE and rocket immunoelectrophoresis experiments. Fumarate reductase, heme, lactate dehydrogenase, and NADH dehydrogenase stains were performed on CIEs obtained with detergent-solubilized aerobic membranes with anti-SDH immunoglobulin G. Fumarate reductase was visualized by using methyl viologen as the electron acceptor (44). Heme staining was carried out with tetrathymethyl benzidine (42). Lactate dehydrogenase staining was performed by the method of Smyth et al. (39). NADH dehydrogenase and SDH were assayed on the plates by the method of Owen and Salton (35). SDH staining was enhanced by the addition of 200 μM phenazine methosulfate to the solution. Protein staining was performed with Coomassie brilliant blue dissolved either in water or in acetic acid–ethanol for preparative or permanent stains, respectively.

**Characterization of the SDH antigen in *R. sphaeroides*.** Antisera were raised against the Triton X-100 solubilized cytoplasmic membrane proteins of *R. sphaeroides* grown aerobically. This anti-inner membrane preparation was examined by CIE against crude SDH preparations either from chromatophores of anaerobic photosynthetically grown cells or from membranes isolated from aerobic heterotrophically grown cells. In each case a large number of immunoprecipitin arcs were apparent by protein staining, but only a single arc exhibited SDH activity (Fig. 1). The data show immunocross-reactivity of SDH from the aerobic inner membranes and from chromatophores of *R. sphaeroides*. This suggests that heterotrophically and phototrophically grown *R. sphaeroides* utilize the same SDH. A similar conclusion was reached recently for SDH from *Rhodospirillum rubrum* (36), an organism which is related to *R. sphaeroides* (13) but which has distinctly different cytochromes making up its respiratory chain (2).

CIE plates obtained with chromatophore and aerobic membranes were examined with a variety of different stains. Specific staining procedures for heme and for fumarate

![FIG. 1. CIE analysis of membrane extracts containing SDH challenged with antibodies against *R. sphaeroides* cytoplasmic membrane protein. (A) Sample contained 80 μg of crude extract from chromatophores from cells grown phototrophically. (B) Sample contained 240 μg of protein extracted from cytoplasmic membranes from aerobic heterotrophically grown cells. Antiserum (350 μL) was used for each plate. The plates were first stained for SDH activity and then for protein. The arrow indicates the arcs which stained for SDH.](http://jb.asm.org//download/779)
reductase activity both gave negative results. Lactate dehydrogenase activity was only faintly visible in one arc on the CIE plates. NADH dehydrogenase activity was apparent in one arc but only with membranes obtained from aerobically grown cells. Neither the lactate dehydrogenase nor NADH dehydrogenase arcs were coincident with the arc exhibiting SDH activity (data not shown).

Membrane proteins of aerobically grown *R. sphaeroides* were radiochemically labeled by growing cells in the presence of [35S]sulfate. Membranes from these cells were prepared, and a Triton X-100 extract containing SDH activity was obtained. CIE was carried out with the anti-SDH serum and this radiochemically labeled protein preparation. The arc exhibiting SDH activity was excised and examined by SDS-PAGE followed by autoradiography. This arc contained only two subunits, with molecular weights of 68,000 and 30,000 (Fig. 2).

One major objective of this work was to examine the form of SDH solubilized in Triton X-100 for the presence of an associated cytochrome b and associated low-molecular-weight subunits. The arc staining for SDH activity did not stain for the presence of heme, suggesting that no cytochrome was present. Also, the subunit analysis indicated only the two expected subunits, with molecular weights of 68,000 and 30,000. No third subunit analogous to those associated with SDH from either *N. crassa* (45) or *B. subtilis* (22, 23) was found. It is possible that Triton X-100 dissociates the two-subunit form of SDH from subunits with which it may be associated in the membrane. Additional studies with other detergents will be required to investigate this possibility.

**Cross-reactivity with SDH from other species.** A secondary objective of this work was to examine immunocross-reactivity with SDH from phylogenetically related bacterial strains. SDH was recently shown to be a major cross-reacting antigen in members of the family *Enterobacteriaceae* (7). The anti-SDH serum was used to examine immunocross-reactivity with SDH extracted from membrane preparations from a number of phylogenetically related bacteria. Detergent extracts containing SDH activity were
obtained from *R. sphaeroides* and related species (2, 12, 13, 27) *R. palustris*, *P. denitrificans*, *Rhodospirillum fulvum*, *Rhodospirillum rubrum*, and *R. capsulata*. Previous studies indicate that *R. sphaeroides* is most closely related to *P. denitrificans* (13). Rocket immunoelectrophoresis was performed with equal units of SDH activity from each extract and with the anti-serum prepared towards SDH from *R. sphaeroides* chromatophores. The results (not shown) indicated an inability of this antiserum to precipitate SDH from sources other than *R. sphaeroides*. It is possible that evidence for immunocross-reactivity would be manifest by a less stringent test, such as immunoblotting. However, the antibody preparation did not blot well. Nevertheless, it is apparent that there has been significant phylogenetic divergence between *R. sphaeroides* and other strains examined in this work.

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LITERATURE CITED


