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Molecular Properties of Succinate Dehydrogenase Isolated from *Micrococcus luteus* (*lysodeikticus*)

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Succinate dehydrogenase (EC 1.3.99.1) of *Micrococcus luteus* was selectively precipitated from Triton X-100-solubilized membranes by using specific antiserum. The precipitated enzyme contained equimolar amounts of four polypeptides with apparent molecular weights of 72,000, 30,000, 17,000, and 15,000. The 72,000 polypeptide possessed a covalently bound flavin prosthetic group and appeared to be strongly antigenic as judged by immunoprinting experiments. Low-temperature absorption spectroscopy revealed the presence of cytochrome b_{556} in the antigen complex. By analogy with succinate dehydrogenase purified from other sources, the 72,000 and 30,000 polypeptides were considered to represent subunits of the succinate dehydrogenase enzyme, whereas one (or both) of the low-molecular-weight polypeptides was attributed to the apoprotein of the *b*-type cytochrome. A succinate dehydrogenase antigen cross-reacting with the *M. luteus* enzyme complex could be demonstrated in membranes of *Micrococcus roseus*, *Micrococcus flavus*, and *Sarcina lutea*, but not in the membranes isolated from a wide variety of other gram-positive and gram-negative bacteria.

Succinate dehydrogenase (EC 1.3.99.1) is an integral component of the respiratory electron transfer chain of both eucaryotic and procaryotic cells. The enzyme catalyzes the oxidation of succinate to fumarate and transfers reducing equivalents to the cytochrome chain (1, 14). Enzymes and enzyme complexes of various complexities have been isolated from a number of diverse sources, including mammalian and yeast mitochondria (7, 35), photosynthetic bacteria (2, 8), and more recently from *Bacillus subtilis* (13) and *Escherichia coli* (21). The enzyme has consistently been resolved as a metalo-flavoprotein consisting of two subunits, the larger of which has a molecular weight of 60,000 to 70,000 and possesses a flavin prosthetic group in covalent linkage. The smaller subunit has a molecular weight of 25,000 to 30,000. Both subunits are thought to possess iron-sulfur centers (12, 14). Other low-molecular-weight components have often been observed in association with succinate dehydrogenase, and in several instances these have been shown to correspond to *b*-type cytochromes (11, 12, 35). In addition, the mammalian enzyme complex is known to contain a ubiquinone-binding protein, which is thought to be essential for succinate-ubiquinone reductase activity (36, 37).

For a number of years we have been interested in the succinate dehydrogenase found within the plasma membranes of the model gram-positive aerobic bacterium *Micrococcus luteus*. Early investigations on this topic dealt primarily with the properties of the membrane-bound or partially purified enzyme but failed to resolve it in molecular detail (17, 23, 29). More recently, crossed immunoelectrophoresis (CIE) of appropriately labeled membranes has made it possible to identify succinate dehydrogenase as a major enzyme-active immunogen possessing covalently bound flavin and iron (4). Moreover, the results of progressive immunoadsorption experiments conducted with protoplasts and isolated membranes (27, 28) have indicated that the major determinants of this enzyme antigen are expressed on the cytoplasmic face of the membrane, as suggested by Mitchell (20).

In this communication we extend our immunological approach to the analysis of *M. luteus* succinate dehydrogenase. Specific immunoglobulins were used to precipitate the enzyme selectively from Triton X-100-solubilized membranes, thereby facilitating an examination of the molecular properties of this important membrane-bound immunogen.

(A preliminary account of this work has been presented at the 94th Ordinary Meeting, Society for General Microbiology, Cambridge, England, 1982, p. 4.)

MATERIALS AND METHODS

Growth of cells. Bacteria were grown aerobically in an orbital incubator at 200 rpm, except where indicated. Details of growth conditions are as follows: *M.*

luteus (*lysodeikticus*) NCTC 2665, *Micrococcus flavus* NCTC 7743, and *Sarcina lutea* (laboratory strain) in peptone-water-yeast extract medium at 30°C; *Micrococcus roseus* NCTC 7523, *Planococcus citreus* CCM 316, *B. subtilis* NCTC 3610, *Staphylococcus aureus* NCTC 8532, and laboratory strains of *Serratia marcescens*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* in nutrient broth at either 30°C (*M. roseus* and *P. citreus*) or 37°C (others); *Streptococcus pyogenes* NCTC 8198 on Todd-Hewitt broth (Oxoid Ltd.) at 37°C without shaking; *Leuconostoc mesenteroides* (laboratory strain) on a glucose-tryptone-yeast extract-based medium at 20°C; *Lactobacillus casei* NCTC 10302 on de Man-Rogosa-Sharpe broth (Oxoid Ltd.) at 30°C; and *E. coli* ML308-225 on succinate minimal medium at 37°C (6). Both *Clostridium perfringens* (laboratory strain) and *Peptococcus indolicus* PcR14 were grown anaerobically at 37°C on PRAS peptone-yeast extract-glucose medium (32). Cells were harvested in the late exponential phase of growth and were washed twice in 50 mM Tris-hydrochloride buffer (pH 7.5). To obtain cells of *M. luteus* uniformly labeled with ^{14}C and ^{35}S, bacteria were grown in defined media (26) devoid of either unlabeled amino acids or unlabeled sulfate but supplemented with uniformly ^{14}C-labeled protein hydrolyzate (3.3 μCi/ml) or $H_2{}^{35}SO_4$ (2.8 to 5.7 μCi/ml) as appropriate. ^{59}Fe-labeled and D-[2-^{14}C]riboflavin-labeled cells of *M. luteus* were obtained as previously described (4).

Preparation of membranes and membrane extracts. Cells of *M. luteus*, *P. citreus*, *B. subtilis*, and *Staphylococcus aureus* were lysed osmotically with the aid of either lysozyme (100 μg/ml) or lysostaphin (15 μg/ml) as appropriate, and membranes were prepared by standard procedures (24). The remaining gram-positive bacteria were lysed by several passages through a French press (32,000 lb/in^2) in the presence of lysozyme (100 μg/ml) and DNase (50 μg/ml). The membranes of these organisms were subsequently isolated by centrifugation (48,000 × *g*) after the removal of whole cells at 6,000 × *g* (24). Membrane vesicles of *E. coli* were prepared as previously described (15), and envelopes of the other gram-negative bacteria were obtained by French pressing (20,000 lb/in^2) cell suspensions in the presence of lysozyme and EDTA as described elsewhere (3).

Methods for preparing Triton X-100 extracts of gram-positive membranes and for obtaining Triton X-100–EDTA extracts of gram-negative envelopes and for the mild dispersal of Triton X-100-insoluble membrane residues in sodium dodecyl sulfate (SDS) have been described (4, 22, 25, 27).

Preparation of antisera. Antiserum to plasma membranes of *M. luteus* was raised in rabbits as outlined previously (4, 27). Serum specific for succinate dehydrogenase was obtained by procedures involving precipitate excision (5). Succinate dehydrogenase-active immunoprecipitates from a total of 60 wet CIE immunoplates were used to immunize six rabbits by intradermal and subcutaneous routes. Two weeks later, each rabbit was boosted with immunoprecipitates from four more immunoplates. This process was repeated at monthly intervals. The rabbits were bled every 10 to 14 days, and immunoglobulins were fractionated and concentrated to about 1/10 serum volume as described previously (4, 27).

Immunoprecipitation. Before immunoprecipitation, Triton X-100-solubilized membrane extracts were centrifuged at 48,000 × *g* for 20 min to remove any sedimentable material which might impair analysis. Extracts (normally 20 to 50 μl) were then mixed with appropriate volumes (see below) of anti-succinate dehydrogenase serum and were incubated at 30°C for 1 to 2 h. Immune complexes were pelleted by centrifugation at 25,000 × *g* for 10 min and were washed twice with a large excess of 0.1 M NaCl containing 0.5% (vol/vol) Triton X-100. Washed immunoprecipitates were finally suspended by gentle sonication in a small volume of Laemmli sample buffer (16). If large quantities of immunoprecipitated material were required (e.g., for absorption spectroscopy), the reaction was scaled up 50-fold, and the incubation period was extended to 48 h at 4°C. In these instances, immunoprecipitates were finally suspended by sonication in 50 mM Tris-hydrochloride buffer (pH 7.5) containing 0.5% (vol/vol) Triton X-100. These modifications did not alter the polypeptide profile as judged by SDS-polyacrylamide gel electrophoresis.

Immunoprinting. A modification of the sensitive immunoprint procedure (1) was employed to determine which membrane polypeptides reacted with the anti-succinate dehydrogenase serum. ^{35}S-labeled membrane polypeptides were initially resolved by SDS-polyacrylamide gel electrophoresis. To remove the SDS, unfixed gels were washed twice (20 min) in distilled water and subsequently in barbital-hydrochloride buffer (I = 0.02, pH 8.6) containing 1% (vol/vol) Triton X-100. The washed gels were then overlaid with 1% (wt/vol) agarose gels (1.2 mm thick) containing either specific serum or preimmune serum and buffered with barbital-hydrochloride (I = 0.02, pH 8.6) containing 1% (vol/vol) Triton X-100 and 15 mM NaN_3. After incubation in a humidified chamber at 30°C for 16 h, overlay gels were removed and were washed, pressed, and dried (34) before autoradiography.

Electrophoretic techniques. Procedures for performing CIE and related techniques; for washing, drying, and staining immunoplates with Coomassie brilliant blue; and for performing succinate dehydrogenase zymograms have been described in detail elsewhere (4, 28).

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (16) with 12.5% (wt/vol) acrylamide separating gels and 4% (wt/vol) acrylamide stacking gels. Radioactive polypeptides were visualized by autoradiography, and their molecular weights were calculated in the standard fashion with the following molecular weight markers: phosphorylase A (94,000), bovine serum albumin (66,000), catalase (61,000), glutamate dehydrogenase (55,400), aldolase (40,000), *E. coli* matrix protein (37,200), carbonic anhydrase (30,000), chymotrypsinogen A (25,500), trypsin inhibitor (20,100), myoglobin (17,200), and lysozyme (14,300).

Difference spectroscopy. Difference spectra at a temperature of 77°K were obtained between 500 and 620 nm with an Aminco DW-2 spectrophotometer fitted with a liquid nitrogen dewar. For membranes and Triton X-100 extracts, ferricyanide-oxidized (1.25 mM) versus dithionite-reduced (1.25 mM) spectra were recorded at a scan speed of 0.75 nm/s. In the case of the succinate dehydrogenase immunoprecipitate, a base line was initially obtained with two buffer blanks.

Ferricyanide-oxidized (1.25 mM), succinate-reduced (12.5 mM), and dithionite-reduced (1.25 mM) spectra were then recorded consecutively against a buffer blank. Reduced minus oxidized spectra were obtained by computer subtraction.

Analytical methods and radiochemicals. Succinate dehydrogenase and NADH dehydrogenase (EC 1.6.99.3) activities were determined spectrophotometrically as described by Owen and Freer (23) and by Gel'man et al. (10), respectively. Protein was estimated by the method of Lowry et al. (18), modified (9) to eliminate interference by Triton X-100. Bovine serum albumin was used as the standard. $^{59}FeCl_3$ (9.6 mCi/mg of iron), D-[2-^{14}C]riboflavin (60.6 mCi/mmol), $H_2{}^{35}SO_4$ (4.33 mCi/mmol) and uniformly ^{14}C-labeled protein hydrolysate (59 mCi per milligram-atomic weight of carbon) were obtained from the Radiochemical Centre, Amersham.

RESULTS

Preparation and specificity of anti-succinate dehydrogenase serum. To obtain specific serum of good quality by precipitate excision and immunization (5), it is desirable to work from CIE profiles in which the immunoprecipitate in question is well resolved and free of other intersecting immunoprecipitates. We have reported previously that such a situation occurs for the *M. luteus* succinate dehydrogenase antigen after the CIE analysis of Triton X-100-insoluble membrane residues which have been dispersed in subdenaturing concentrations of SDS (22). A typical CIE immunoplate illustrating this point and indicating the regions of the succinate dehydrogenase-active immunoprecipitate which were chosen for excision is shown in Fig. 1A. A pool of over 60 such excised arcs provided the antigen source for raising the anti-succinate dehydrogenase serum (see above).

The specificity of the resultant serum was assessed by CIE, by immunodiffusion, and by enzyme inhibition studies. A single immunoprecipitate which possessed succinate dehydrogenase activity was resolved when *M. luteus* membranes were tested by either CIE or immunodiffusion against the anti-succinate dehydrogenase serum (Fig. 1B; see also Fig. 7). Moreover, no additional antigens reacting weakly with the serum could be discerned by the sensitive procedure of CIE with intermediate gel (Fig. 1C and D), even when this was coupled by autoradiography with the use of ^{35}S-labeled membranes (data not shown) or with the use of radioisotopes such as ^{59}Fe (Fig. 1D and F) or [^{14}C]riboflavin (data not shown) designed to selectively label prosthetic groups of iron-containing and flavoprotein antigens. In contrast to the single immunoprecipitate observed in tests against the specific serum, up to 32 immunoprecipitates could be resolved for the homologous (membrane–anti-membrane) reaction (Fig. 1C; ref. 4).

The effect of anti-succinate dehydrogenase serum on the succinate dehydrogenase activity expressed in Triton X-100 extracts of *M. luteus* membranes is illustrated in Fig. 2. About 75% of the total succinate dehydrogenase activity could be inhibited and precipitated by the specific serum. As anticipated, the immunoprecipitated material expressed very low levels of succinate dehydrogenase activity. For reasons that are unclear at the present time, the residual succinate dehydrogenase activity in the supernatant fraction (25% of the total) was not inhibited at serum excess. This could reflect, among other things, the presence of (i) a related form of the enzyme which, by virtue of altered antigenicity or formation of soluble immune complexes, is not inhibited or precipitated by antibody to the parent molecule, or (ii) a second unrelated succinate dehydrogenase-active enzyme which has escaped detection by CIE so far (4, 27, 28).

The specificity of the succinate dehydrogenase–anti-succinate dehydrogenase reaction was further confirmed by the facts that (i) preimmune serum had very little neutralizing activity, and (ii) NADH dehydrogenase activity of the membrane extract was virtually unaffected by concentrations of anti-succinate dehydrogenase serum that produced maximal inhibition of homologous enzyme activity (Fig. 2). On the basis of these and other similar experiments, the specific anti-succinate dehydrogenase serum was judged acceptable for use in further studies of the biochemical and biophysical properties of the enzyme complex.

The concentration of serum for optimum immunoprecipitation was determined by experiments similar to that displayed in Fig. 2, in which the following parameters were monitored as a function of antibody concentration: (i) inhibition of succinate dehydrogenase activity, (ii) the amount of ^{59}Fe precipitated from Triton X-100 extracts of ^{59}Fe-labeled membranes, and (iii) the amount of residual succinate dehydrogenase antigen left after immune complexes were removed by centrifugation. This final parameter was measured by CIE of supernatant fractions and involved estimates of peak area. It was observed that the complete removal of succinate dehydrogenase antigen occurred at a point (Fig. 2, arrow) which coincided with the maximum precipitation of radiolabeled iron and maximal inhibition of enzyme activity (data not shown).

Analysis of the succinate dehydrogenase-antibody complex by SDS-polyacrylamide gel electrophoresis. The polypeptide composition of the immunoprecipitated succinate dehydrogenase complex was ascertained by SDS-polyacrylamide gel electrophoresis. To facilitate the resolution of polypeptides of bacterial origin (as opposed to those derived from the antiserum)

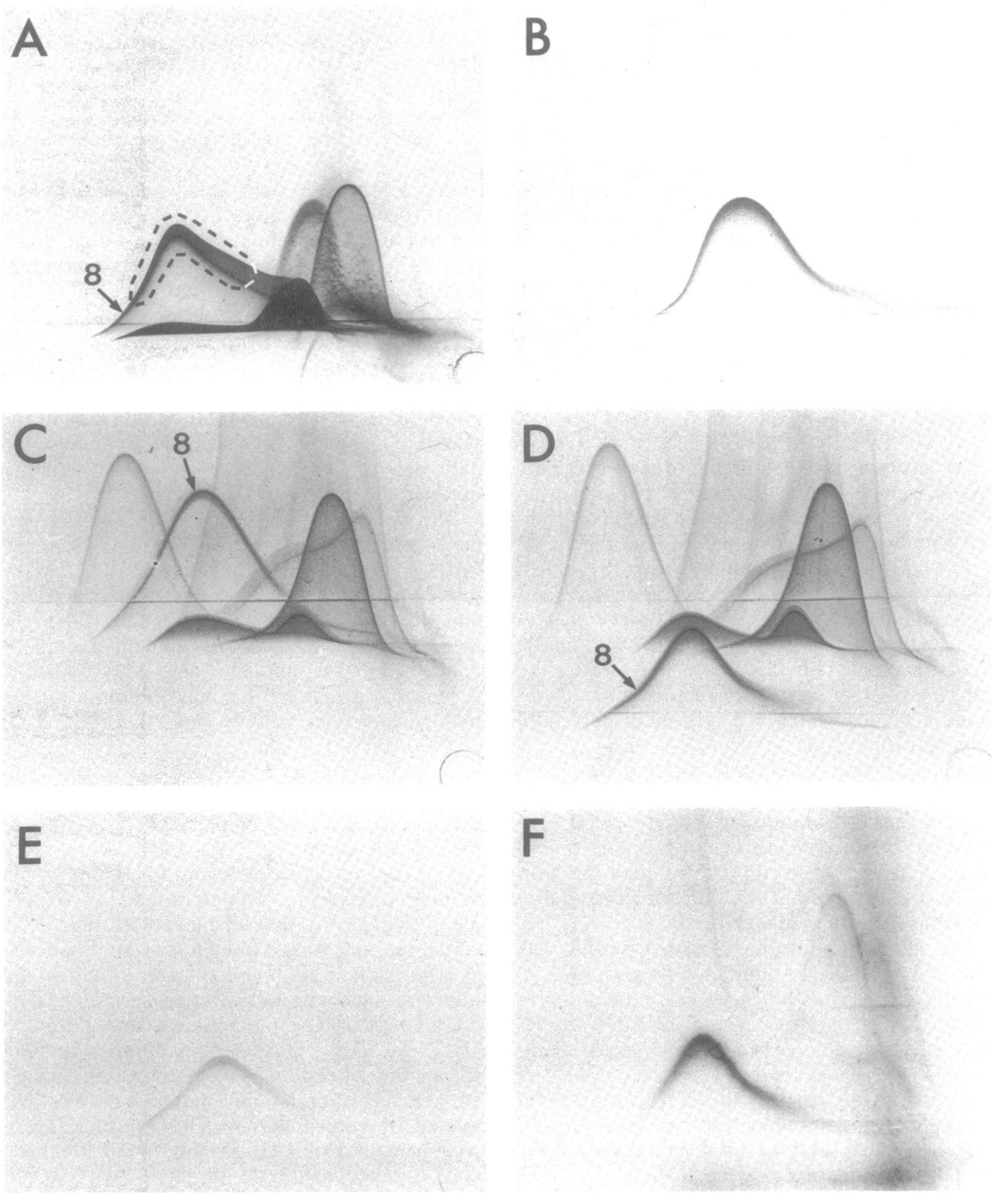

FIG. 1. Preparation and specificity of anti-succinate dehydrogenase serum. Succinate dehydrogenase-active immunoprecipitates were excised (A, dotted region) from CIE immunoplates in which a 0.15% (wt/vol) SDS extract (46 μg of protein) of Triton X-100-insoluble membrane residues had been analyzed against anti-membrane serum. After immunization, the specificity of the resultant serum was tested by CIE (B) and by CIE with intermediate gel (C through F) by using Triton X-100 extracts of unlabeled (B, 74 μg of protein) and ^{59}Fe-labeled (C through F, 42 μg of protein) membranes. The main antibody gels of A and C through F contained anti-membrane serum (4.5 mg of protein per ml), and the main gel of B together with intermediate gels of D through F contained anti-succinate dehydrogenase immunoglobulins (3.6 mg of protein per ml). Immunoprecipitates were visualized by protein staining (A through D), enzyme staining for succinate dehydrogenase activity (E), and autoradiography (F). Note that only a single antigen (no. 8 of the CIE reference immunoprecipitate profile; ref. 27) appears to react with the specific serum (B, D, and F) and that this component possesses succinate dehydrogenase activity (E) and iron (F). Anode is to the left and top of all gels.

and to allow a determination of the stoichiometry of the individual subunits, uniformly ^{14}C-labeled membrane extracts were employed. Fig. 3 shows the protein-stained profiles (tracks 1 and 2) and corresponding autoradiograms (tracks 3 and 4) of a Triton X-100 extract of ^{14}C-labeled membranes (tracks 1 and 3) and ^{14}C-labeled immunoprecipitated material (tracks 2 and 4). It is clear from these results that the addition of monospecific serum to Triton X-100-solubilized extracts of *M. luteus* membranes results in the selective precipitation of four polypeptides with molecular weights of 72,000, 30,000, 17,000, and 15,000. The subunit of highest molecular weight could often be resolved as a doublet. Radioactive measurements made on excised polypeptide bands indicate a stoichiometry of 1:1:1:1 for the four polypeptides (Fig. 3). The apparent stoichiometry was unaffected by either incorporation into the Triton X-100 extraction buffers of, or pretreatment of antiserum for 1 h with, a cocktail of protease inhibitors present in the following final concentrations: phenylmethylsulfonyl fluoride (1 mM); *N*-α-*p*-tosyl-1-lysine chloromethyl ketone (1 mM), EDTA (5 mM), trypsin inhibitor (0.5 mg/ml), and Aprotinin (0.6 trypsin inhibitor units (TIU)/ml).

We have previously shown that the *M. luteus* succinate dehydrogenase antigen possesses covalently bound flavin (4), a feature shared by similar enzymes from other sources (12, 14). It was therefore of interest to establish to which of the resolved subunits this prosthetic group was bound. Accordingly, D-[2-^{14}C]riboflavin-labeled membranes and material precipitated therefrom by anti-succinate dehydrogenase serum were analyzed by SDS-polyacrylamide gel electrophoresis in conjunction with autoradiography (Fig. 4). A single radiolabeled polypeptide with a molecular weight of 72,000 was observed for both preparations (Fig. 4, tracks 3 and 4), establishing the highest molecular weight subunit of the succinate dehydrogenase complex as the major polypeptide possessing covalently bound flavin. In instances in which this polypeptide

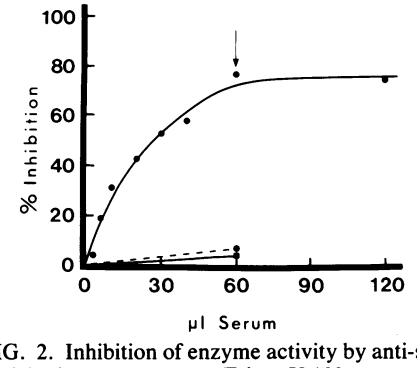

FIG. 2. Inhibition of enzyme activity by anti-succinate dehydrogenase serum. Triton X-100 extracts (120 μg of protein) of *M. luteus* membranes were incubated with 0 to 120 μl of anti-succinate dehydrogenase serum (120 mg of protein per ml, ——) or equivalent amounts preimmune serum (-----) in a total volume of 140 μl. After 1 h at room temperature, samples (17 to 34 μg of membrane protein) were assayed for succinate dehydrogenase (●) or NADH dehydrogenase (■) activities. Arrow indicates point at which complete precipitation of succinate dehydrogenase antigen and maximal precipitation of bound iron were observed (see text for further details).

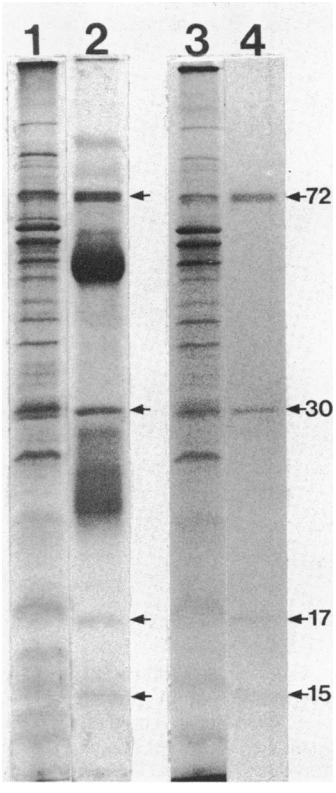

FIG. 3. Polypeptide composition of the *M. luteus* succinate dehydrogenase antigen complex. A Triton X-100 extract (23 μg of protein) of ^{14}C-labeled membranes (track 1) and succinate dehydrogenase complex precipitated from twice the amount of Triton X-100 extract used in track 1 (track 2) were analyzed by SDS-polyacrylamide gel electrophoresis, and the resolved polypeptides were stained for protein. Direct autoradiograms of tracks 1 and 2 are displayed in tracks 3 and 4. The positions and apparent molecular masses (in kilodaltons) of the four polypeptides resolved for the enzyme complex are indicated. For a typical preparation, excision of individual polypeptides from pairs of tracks similar to track 2 followed by peroxide treatment and scintillation counting yielded (over a background of 35 cpm) the following counts for the four subunits; 599 cpm (72,000 molecular weight subunit), 251 cpm (30,000 molecular weight subunit), 143 cpm (17,000 molecular weight subunit), and 129 cpm (15,000 molecular weight subunit).

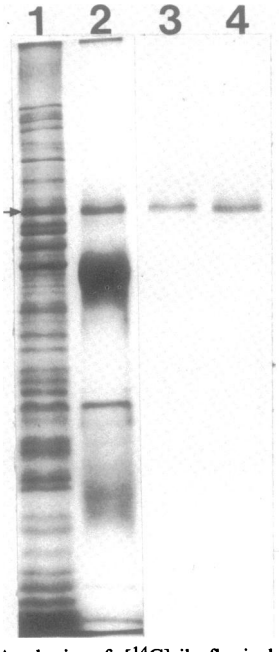

FIG. 4. Analysis of [^{14}C]riboflavin-labeled membrane polypeptides from *M. luteus* by SDS-polyacrylamide gel electrophoresis. The figure shows the polypeptides resolved by protein staining (tracks 1 and 2) and by autoradiography (tracks 3 and 4) for [^{14}C]riboflavin-labeled membranes (58 μg of protein, tracks 1 and 3) and for the succinate dehydrogenase antigen complex (track 2 and 4) immunoprecipitated from a Triton X-100 extract (110 μg of protein) of [^{14}C]riboflavin-labeled membranes. The identity of the single radiolabeled band seen in tracks 3 and 4 is indicated by an arrow in the full spectrum of membrane polypeptides (track 1).

was resolved as two closely migrating bands, both components of the doublet possessed flavin.

Presence of cytochrome b_{556}. Preliminary results based upon CIE analysis of membranes prepared from cells grown in the presence of the heme precursor [^{14}C]aminolevulinic acid have indicated that the succinate dehydrogenase antigen of *M. luteus* may be associated with cytochrome (4). To confirm this and to ascertain the nature of any cytochrome species, an analysis of *M. luteus* membranes, Triton X-100 membrane extracts, and immunoprecipitated succinate dehydrogenase was carried out by low-temperature difference spectroscopy (Fig. 5). Of the four cytochromes known (10, 19) to be present in *M. luteus* membranes (cytochromes c_{550}, b_{556}, b_{560}, and a_{601}; Fig. 5A), only cytochrome b_{556} partitioned to a significant extent into the Triton X-100-soluble fraction (Fig. 5B). It is notable that cytochrome b_{556} could also be detected in the dithionite-reduced versus ferricyanide-oxidized difference spectrum of the immunoprecipitated succinate dehydrogenase complex (Fig. 5C). However, succinate was ineffective in reducing cytochrome b_{556} in the precipitated complex. This contrasts with the situation in isolated membranes and Triton X-100 extracts in which succinate and other respiratory substrates such as $NADH_2$ and malate (10) can be shown by spectrophotometry to effect the reduction of cytochrome b_{556} and other cytochromes present in the preparations (data not shown).

Reaction of individual subunits with anti-succinate dehydrogenase serum. The reactivity of the individual subunits of the succinate dehydrogenase complex with monospecific serum was assessed by an immunoprint technique (see above) in which SDS-polyacrylamide slab gels containing resolved ^{35}S-labeled polypeptides were overlaid with antibody-containing agarose gels. Overlay experiments conducted with membranes and membrane extracts (Fig. 6) and also

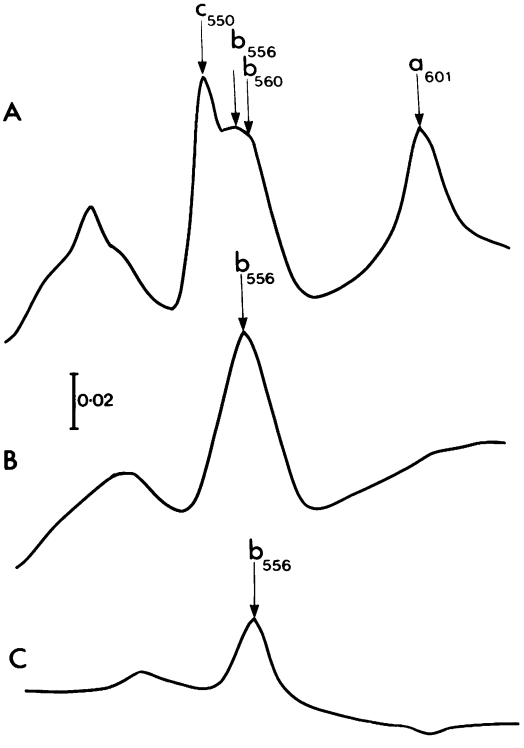

FIG. 5. Low-temperature cytochrome difference spectra (dithionite reduced minus ferricyanide oxidized). (A) *M. luteus* membranes (11.6 mg of protein per ml); (B) Triton X-100 extract of *M. luteus* membranes (6.2 mg of protein per ml); (C) succinate dehydrogenase antigen complex immunoprecipitated from a Triton X-100 membrane extract similar to that used in B. The identities of the cytochromes giving rise to the individual α-Soret absorption bands are indicated.

with immunoprecipitated succinate dehydrogenase complex (data not shown) revealed that only the subunit with a molecular weight of 72,000 was capable of interacting with the monospecific serum to form an immunoprecipitate. It seems likely, therefore, that the 72,000-molecular-weight flavopeptide is the major antigenic species of the succinate dehydrogenase complex.

Cross-reactivity of *M. luteus* succinate dehydrogenase with similar enzymes from other microorganisms. To assess the degree of antigenic similarity between *M. luteus* succinate dehydrogenase and similar enzymes from other microorganisms, membranes from a wide range of bacteria were tested by immunodiffusion against anti-*M. luteus* succinate dehydrogenase serum. Of the membranes tested (those from *M. roseus*, *M. flavus*, *S. lutea*, *Staphylococcus aureus*, *P. citreus*, *B. subtilis*, *Lactobacillus casei*, *Streptococcus pyogenes*, *Leucondstoc mesenteroides*, *C. perfringens*, *Peptococcus indolicus*, *E. coli*, *Serratia marcescens*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Rhodopseudomonas sphaeroides*), only three (i.e., those of *M. roseus*, *M. flavus*, and *S. lutea*) gave positive reactions. The reacting antigens all gave lines of partial identity with the *M. luteus* enzyme (Fig. 7). Moreover, tests of *M. roseus*, *M. flavus*, and *S. lutea* membranes by CIE revealed that in each case, succinate dehydrogenase was one of a maximum of only four antigens capable of reacting with anti-*M. luteus* membrane serum (compared with 32 antigens for *M. luteus* membranes; Fig. 1C, ref. 4).

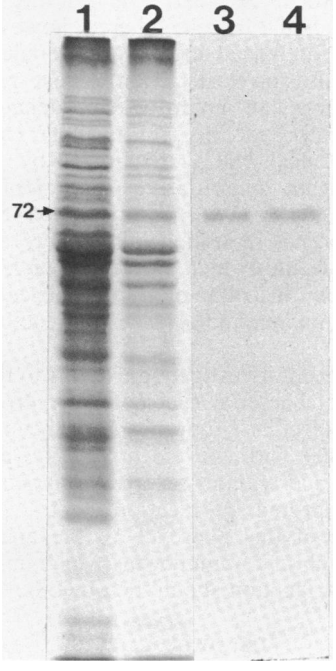

FIG. 6. Reaction of membrane polypeptides with anti-succinate dehydrogenase serum. ^{35}S-labeled membranes (58 μg of protein, track 1) and a Triton X-100 extract (52 μg of protein) of ^{35}S-labeled membranes (track 2) were analyzed by SDS-polyacrylamide gel electrophoresis. Gels were either fixed (tracks 1 and 2) or overlaid with an agarose gel containing anti-succinate dehydrogenase serum (5.4 mg of protein per ml of gel) as detailed in the text. Autoradiograms of the fixed gel (tracks 1 and 2) and of the corresponding immunoprint (tracks 3 and 4) are presented. The identity and molecular mass (in kilodaltons) of the single polypeptide reacting with the monospecific serum is indicated at the side of the figure.

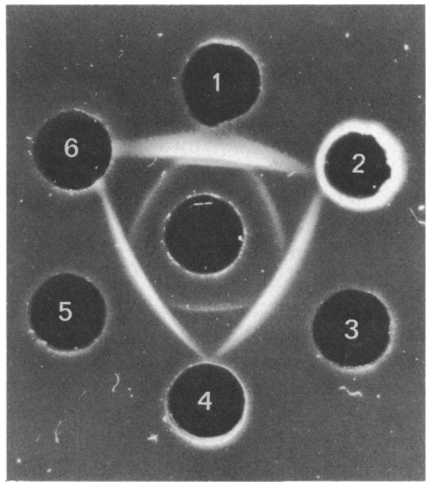

FIG. 7. The reactions of membrane antigens from *M. luteus*, *M. roseus*, *M. flavus*, and *S. lutea* with anti-*M. luteus* succinate dehydrogenase serum as determined by immunodiffusion. Triton X-100 extracts of membranes from *M. luteus* (wells 1, 3, and 5; 60 μg of protein), *M. roseus* (well 2; 54 μg of protein), *M. flavus* (well 4; 65 μg of protein), and *S. lutea* (well 6; 65 μg of protein) were tested against anti-*M. luteus* succinate dehydrogenase immunoglobulins (central well, 1.2 mg of protein).

DISCUSSION

From the results presented above and elsewhere (4), it is apparent that the Triton X-100-solubilized succinate dehydrogenase antigen of *M. luteus* is remarkably similar in its molecular properties to corresponding enzyme complexes isolated from other sources (12, 14). These similarities include the facts that (i) it is a metaloflavoantigen possessing nonheme iron and covalently bound flavin (4); (ii) its two largest subunits are present in equimolar amounts and are in the molecular weight ranges of 70,000 and 30,000; (iii) the largest (72,000-molecular-

weight) subunit contains the covalently bound flavin prosthetic group; and (iv) the complex contains additional low-molecular-weight subunits, including a *b*-type cytochrome, cytochrome b_{556}. It is notable that this cytochrome has previously been shown to copurify with two other enzymes of the *M. luteus* respiratory chain, namely NADH dehydrogenase and malate dehydrogenase (EC 1.1.1.37; ref. 19, 33).

In most, but not all, instances, *b*-type cytochromes have been shown to be composed of single polypeptide chains, and in several dehydrogenase-cytochrome *b* complexes they have been implicated not only in electron transfer but also in attachment of the dehydrogenase to the membrane (11, 14). However, at the present time it is unclear which (or indeed whether both) of the low-molecular-weight subunits consistently observed for the *M. luteus* antigen complex represents cytochrome b_{556}. Certainly, the observed 1:1:1:1 stoichiometry of the four subunits, together with results of experiments conducted with protease inhibitors, would seem to rule out the possibility that they are artifacts caused by proteolytic degradation.

An additional candidate for one of the low-molecular-weight subunits might be a quinone-binding protein similar to that observed for the succinate-ubiquinone oxidoreductase (complex II) of beef heart mitochondria (36, 37). In the mitochondrial complex, the ubiquinone-binding protein is of low molecular weight and is necessary for the expression by succinate dehydrogenase of succinate-ubiquinone oxidoreductase activity (36, 37). It is not inconceivable that analogous menaquinone-binding proteins are present in *M. luteus* membranes and in the precipitated succinate dehydrogenase enzyme complex. Certainly we have been able to demonstrate for both the membrane-bound and detergent-solubilized forms of the enzyme complex not only succinate-cytochrome b_{556} reductase activity but also succinate-ubiquinone (Q-1) reductase activity. Unfortunately, we have been unable as yet to detect either of these activities for the immunoprecipitated enzyme complex. The following observations suggest that this is probably a result of antibody-induced inhibition of catalytic activity: (i) monospecific serum almost totally inhibits phenazine methosulphate-mediated succinate-dichlorophenolindophenol reductase activity; (ii) the 72,000-dalton flavoprotein which probably houses the catalytic site of the enzyme (12) appears to be strongly antigenic; and (iii) electron spin resonance studies have indicated that iron-sulfur centers S-1 and S-3 are only partially reduced by succinate in the immunoprecipitated form of the enzyme complex but are fully reduced by substrate in the membrane-bound and Triton X-100-solubilized forms of the enzyme (B. A. Crowe et al., manuscript in preparation).

The similarities that are evident in the molecular properties of succinate dehydrogenases isolated from *M. luteus* and other sources do not appear to be reflected in the cross-relatedness of the enzymes as determined by methods of agar gel precipitation. In the current survey, antigens reacting with anti-*M. luteus* succinate dehydrogenase serum were detected in members of the genus *Micrococcus* only. Even other members of the family *Micrococcaceae* (*Staphylococcus aureus* and *P. citreus*) failed to show cross-reactive material. A similar survey conducted recently with anti-*E. coli* succinate dehydrogenase has shown slightly broader specificity, with cross-reactive antigens observed in all members of the *Enterobacteriaceae* tested (3). The *E. coli* antiserum, however, failed to react with membranes from other bacteria, including *M. luteus* (3), an observation which confirms the results of the reciprocal test performed in the present study. It should be remembered, however, that reactions of partial identity or nonidentity in immunodiffusion tests often reflect relatively small changes in amino acid sequence. Reactions of partial identity, for example, can occur for molecules which cross-react by 80% as judged from quantitative immunoprecipitin tests—a situation which can reflect less than 10% difference in sequence homology. Furthermore, molecules which differ from each other in sequence by 30 to 40% or more have been shown to exhibit no immunological cross-reactivity (30, 31).

The present investigation extends to three the number of bacterial succinate dehydrogenases whose molecular properties have been probed successfully with the aid of specific immunoglobulins and without resort to classical procedures of protein purification (3, 14, 21). The relevance of this approach to the analysis of other membrane immunogens which are refractory to purification should be obvious.

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