Solubilization, Purification, and Characterization of Succinate Dehydrogenase from Membranes of *Mycobacterium phlei*

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Succinate dehydrogenase (SDH) was solubilized from membranes of *Mycobacterium phlei* by Triton X-100 with a recovery of about 90%. The solubilized SDH was purified about 90-fold by Sephacryl S-300, DEAE-cellulose, hydroxylapatite, and isoelectric focusing in the presence of Triton X-100 with a 20% recovery. SDH was homogeneous, as determined by polyacrylamide gel electrophoresis in nondenaturing gels containing Triton X-100. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the enzyme revealed two subunits with molecular weights of 62,000 and 26,000. SDH is a flavoprotein containing 1 mol of flavin adenine dinucleotide, 7 to 8 mol of nonheme iron, and 7 to 8 mol of acid-labile sulfide per mol of protein. Using phenazine methosulfate and 2,6-dichloroindophenol as electron acceptors, the enzyme had an apparent K_m of 0.12 mM succinate. SDH exhibited a sigmoidal relationship of rate to succinate concentration, indicating cooperativity. The enzyme was competitively inhibited by fumarate with a K_i of 0.15 mM. In the absence of Triton X-100, the enzyme aggregated, retained 50% of the activity, and could be resolubilized with Triton X-100 with full restoration of activity. Cardiolipin had no effect on the enzyme activity in the absence of Triton X-100, but it stimulated the activity by about 30% in the presence of 0.1% Triton X-100 in the assay mixture. Menaquinone-9(2H), isolated from *M. phlei*, had no effect on the enzyme activity either in the presence or absence of Triton X-100.

To understand the basic bioenergetics of an organism that is dependent upon oxidative metabolism for ATP production, it is necessary to study individually the enzymes of the tricarboxylic acid cycle and their regulation and control. Succinate dehydrogenase [SDH; EC 1.3.99.1; succinate: (acceptor) oxidoreductase] is a membrane-bound enzyme and is present in all aerobic cells (12). It plays an important role in energy conservation by the succinoxidase pathway both in mammalian (29) and bacterial (23) systems. The purification and properties of reconstitutively active SDH from mammalian mitochondria have been the subject of extensive investigation (15, 24), but very little is known about its counterpart in procaryotes (12). Hatefi and co-workers (6, 7) purified SDH from both mitochondria and *Rhodospirillum rubrum* and studied the properties of the enzyme. In *Escherichia coli*, SDH was isolated as a soluble complex with cytochrome *b* and reconstituted with cytochrome oxidase to yield a functional membranous succinoxidase (23). Kalra et al. (14) partially purified SDH which was released from the membranes of *Mycobacterium phlei* by alkali treatment. This paper describes the solubilization, purification to homogeneity, and some properties of the membrane-bound SDH from *M. phlei*.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are as follows: SDH, succinate dehydrogenase; PMS, phenazine methosulfate; DCIP, 2,6-dichloroindophenol; MTT, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide; FAD, flavin adenine dinucleotide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MK-9(2H), menaquinone-9(2H).

Reagents. Triton X-100, deoxycholate, DCIP, PMS, MTT, cardiolipin, and succinic acid were purchased from Sigma Chemical Co. Sephacryl S-300 is a product of Pharmacia Fine Chemicals. DEAE-52 was obtained from Whatman, Inc. Hydroxylapatite powder and Bio-Lyte (pH 4 to 6) are products of Bio-Rad Laboratories. All other chemicals used were analytical grade.

Growth of *M. phlei* and isolation of membranes. *M. phlei* ATCC 354 was grown in the medium described by Brodie and Gray (2) for 24 h with vigorous aeration on a New Brunswick Gyrotory incubator shaker at 37°C. Cells were harvested in a Sorvall continuous flow centrifuge (model RC2-B) and washed three times with cold distilled water. The washed cells were suspended in 50 mM Tris hydrochloride (pH 8.0) and sonicated, and membranes were isolated as previously described (27). Membrane pellets were stored at -25°C until used.

Solubilization of SDH. Membrane pellets were thawed and suspended in 10 mM potassium phosphate buffer (pH 7.5), containing 1 mM succinate, to an approximate protein concentration of 20 mg/ml. The suspension was incubated at 30°C for 10 min and centrifuged at $3,000 \times g$ for 5 min to remove aggregated particles. Protein was adjusted to 15 mg/ml by dilution with the phosphate buffer and succinate. The membrane suspension was cooled in an ice slurry, and while the suspension was continuously stirred, a 10% (wt/vol) solution of Triton X-100 was added to a detergent-to-protein ratio of 1.0 (wt/wt). After 15 min in ice, the solubilized membranes were centrifuged in a Beckman Model L2-65 centrifuge with a Ti 60 rotor at $105,000 \times g$ for 1 h. The clear supernatant fluid was used for assaying SDH activity.

Assay of SDH. The enzyme was assayed at 30°C by the PMS-mediated reduction of DCIP in a final volume of 1 ml containing 0.01 ml of appropriately diluted enzyme, 0.78 ml of 50 mM Tris hydrochloride (pH 8.2), 0.1 ml of 1.5 mM DCIP containing 10 mM KCN, and 0.1 ml of PMS (1 mg/ml).

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FIG. 1. Solubilization of SDH from membranes of *M. phlei* by Triton X-100. A membrane suspension was prepared as described in Materials and Methods, and 2-ml samples (15 mg of protein per ml) were distributed into centrifuge tubes to fit in a Beckman Ti 40 rotor. A 10% (wt/vol) solution of Triton X-100 was added to give a detergent-to-protein ratio as indicated. After 15 min in ice, the detergent-solubilized membranes were centrifuged in a Beckman Model L centrifuge at $105,000 \times g$ for 1 h. The supernatant fluid was used for assaying the SDH and protein.

The reaction was initiated with 0.01 ml of 0.5 M sodium succinate (pH 8.0), and the decrease in the absorbance of DCIP was measured at 600 nm in a Cary Model 210 recording spectrophotometer. DCIP and PMS solutions were prepared in the Tris hydrochloride buffer. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 μmol of DCIP per min with an extinction coefficient of 21 $\text{mM}^{-1} \text{cm}^{-1}$ (25). Specific activity is expressed as units per milligram of protein.

Isoelectric focusing. Preparative isoelectric focusing was done in an LKB 8100-1 Ampholine Column with a capacity of 100 ml. The cathode solution was 0.25 M NaOH. The anode solution was 60% (wt/vol) sucrose containing 0.16 M H_3PO_4 . A dense sucrose solution (54 ml; 50%, wt/vol) and a light sucrose solution (54 ml; 5%, wt/vol) were prepared, each containing 0.2% Triton X-100 and 2% Bio-Lyte (pH range 4 to 6). SDH-containing fractions from the hydroxylapatite step, freed from phosphate buffer, were added to the dense sucrose solution (see Results for details). The dense and light sucrose solutions were poured into the appropriate compartments of an LKB 8121 Ampholine Gradient Mixer, and the gradient was pumped into the column. Focusing was started at a constant voltage of 400 V. After about 40 h, the voltage was raised to 800 V, and focusing was continued for 2 h. Fractions (4 ml) were collected, the pH was measured, and the SDH activity was determined.

PAGE. Gel electrophoresis was performed in tubes by the method of Davis (5) in 6% polyacrylamide gels, containing 0.1% Triton X-100, at a constant current of 3 mA per tube. At the end of the run, one set of gels was stained for protein with Coomassie blue and destained with 7% acetic acid. The other set of gels was stained for SDH activity by soaking the gels for 30 min at room temperature in 50 mM Tris hydrochloride (pH 8.2)–0.1 mM MTT–5 mM succinate. Purple

bands appeared in about 5 min and intensified up to 30 min. The liquid was removed and replaced with Tris buffer.

Slab gel electrophoresis in the presence of SDS was performed in 10% polyacrylamide gels by the Laemmli procedure (16). Protein samples in sample buffer were placed at 90°C for 3 min and then were applied to the wells of 3% polyacrylamide stacking gels and run at a 20-mA constant current. At the end of the run, proteins were fixed by soaking the gel in 50% methanol–12% acetic acid. The gels were stained for proteins by the silver stain method of Merrill et al. (20).

Determination of molecular weights of SDH subunits. The molecular weights of the subunits of SDH were determined by SDS-PAGE by the method of Laemmli (16) with 10% acrylamide gels. The molecular weight markers used were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

Preparation of cardiolipin and MK-9(2H) suspensions. Ten milligrams each of cardiolipin and MK-9(2H) was placed in thick-walled polycarbonate tubes and dried under a stream of nitrogen. A 2-ml portion of 50 mM Tris hydrochloride (pH 8.2) was added to each tube, and the tubes were sonicated in a 10-kc Raytheon magnetostriction oscillator, which was tuned to a maximum at 1.25 A, at 4°C for 10 min.

Other estimations. Flavin was dissociated with trichloroacetic acid from the pure SDH and identified as FAD by the method of Burch (3). The nonheme iron content of SDH was estimated by the method of Doeg and Ziegler (9). The acid-labile sulfide content of the enzyme was measured as described by Fogo and Popowsky (10). Protein was determined by the method of Lowry et al. (19) with crystalline bovine serum albumin as a standard. The Triton X-100 which precipitated in the Lowry procedure was removed by centrifugation at $3,000 \times g$ for 5 min before the absorbance of the sample was read. Triton X-100 had no effect on the color.

RESULTS

Purification of SDH. All the purification steps were carried out at 0 to 4°C . The extraction of SDH and protein from the membranes of *M. phlei* at various Triton X-100-to-protein ratios is shown in Fig. 1. It can be seen that 90% of the SDH and 75% of the proteins were solubilized at a ratio of 1.0. Concentrations of Triton X-100 as great as 2% (wt/vol) did not inhibit the enzyme activity.

In a typical purification of SDH, 600 to 700 mg of membrane protein (derived from 1,200 g [wet weight] of cells) was extracted with 10% (wt/vol) Triton X-100 at a detergent-to-protein ratio of 1.0. The solubilized membranes were centrifuged for 1 h at $105,000 \times g$ with a Ti 60 rotor, and the clear supernatant was used for the purification of SDH.

The Triton X-100 extract was applied to a Sephacryl S-300 column which had been equilibrated with 10 mM potassium phosphate buffer (pH 7.5) containing 1% Triton X-100 and 1 mM succinate. The enzyme was eluted with this buffer, and 11-ml fractions were collected at a flow rate of 36 ml/h. SDH was eluted as an asymmetrical peak (Fig. 2A). Active fractions were pooled and used for further purification. The enzyme recovery in these fractions was about 120%; this increase was probably a result of activation of SDH by Triton X-100.

The SDH pool from Sephacryl S-300 chromatography was loaded on a 300-ml DEAE-cellulose column which had been equilibrated with the chromatography buffer. The SDH was eluted essentially in two fractions (49 and 50, Fig. 2B) with a

FIG. 2. Purification of SDH. (A) Sephacryl S-300 chromatography. Forty-five milliliters of Triton X-100 extract (560 mg of protein) was chromatographed on a 500-ml column of Sephacryl S-300 (2.6 by 100 cm). Fractions were collected and assayed for SDH activity and protein. (B) DEAE-52 chromatography. Sixty-five milliliters of the active fractions (20 to 25) from Sephacryl S-300 chromatography were pooled (291 mg of protein) and chromatographed on a 300-ml column of DEAE-52 (5 by 15 cm). The column was washed with 600 ml of chromatography buffer, and elution was performed with a linear gradient of 0 to 0.4 M NaCl buffer. Fractions (23 ml) were collected at a flow rate of 70 ml/h. (C) Hydroxylapatite chromatography. The DEAE-52 concentrate in a volume of 5 ml (11 mg of protein) was chromatographed on a 50-ml column (1.5 by 30 cm) of hydroxylapatite as described in Results. (D) Isoelectric focusing. The hydroxylapatite concentrate (2.68 mg of protein) was subjected to electrofocusing as described in Materials and Methods.

recovery of 75%. This step resulted in a major purification of the enzyme.

Active fractions from the DEAE-52 step were concentrated to about 5 ml in an Amicon concentrator (50-ml cell) with a PM 10 membrane filter. The concentrate was diluted 10 times with the chromatography buffer to reduce the NaCl content and re-concentrated to about 5 ml. This DEAE-52 concentrate was applied to a hydroxylapatite column equilibrated with the chromatography buffer. Fractions were collected at a flow rate of 45 ml/h. Some enzyme activity

appeared in this eluate (Fig. 2C). When the enzyme activity was negligible, SDH was eluted with 50 mM potassium phosphate buffer (pH 7.5) containing 1% Triton X-100 and 1 mM succinate.

Fractions from the eluate from the hydroxylapatite column were pooled and concentrated on the Amicon membrane filter. The concentrate was diluted 10 times with 0.2% Triton X-100 to lower the PO₄ buffer concentration and re-concentrated to 2 ml. The process was repeated to achieve a negligible buffer concentration for final purification by isoelectric focusing. The final hydroxylapatite concentrate in a volume of 2 ml was added to the dense sucrose solution, and electrofocusing was done as described in Materials and Methods. Fractions 12 to 14 (Fig. 2D) were pooled and used for studying the properties of the enzyme.

The results of the purification of SDH are given in Table 1. The enzyme was purified 92-fold with about a 20% yield of activity. The enzyme was remarkably stable during the purification procedures at 0 to 4°C and during freezing at -80°C. In the absence of Triton X-100, the purified enzyme aggregated, retained about 50% of the activity, and could be resolubilized with a concentration of Triton X-100 as low as 0.1% with full restoration of activity. Succinate was routinely present during extraction of the enzyme from the membranes and in the chromatography buffer during purifi-

TABLE 1. Purification of SDH from membranes of *M. phlei*^a

Purification step	Amt of protein (mg)	Activity of SDH (U ^b)	Sp act of SDH (U/mg)	Purification (fold)	Yield (%)
Membranes	662	138	0.21	1.0	100
Triton X-100 extract	560	133	0.24	1.1	96
Sephacryl S-300	291	158	0.54	2.6	114
DEAE-52	11	110	10	47.6	80
Hydroxylapatite	2.7	46	17	81.7	33
Isoelectric focusing	1.5	29	19	92.0	21

^a Purification was performed as described in Results. Assays were performed as described in Materials and Methods.

^b One unit = one micromole of DCIP reduced per minute.

FIG. 3. Analysis by PAGE of the purity of SDH. Samples were subjected to electrophoresis in the presence of 0.1% Triton X-100, as described in Materials and Methods. (1) Triton X-100 extract of the membranes; (2) Sephacryl S-300; (3) DEAE-52; (4) hydroxylapatite; (5) isoelectric focusing; (6) analysis by SDS-PAGE of SDH from the isoelectric focusing stage. Lanes: A, protein stained with Coomassie blue; B, enzyme stained with MTT. Protein was stained by the silver stain method described by Merril et al. (20).

cation, although the enzyme was equally stable in the absence of succinate. The advantage of including succinate during the purification procedure was that the rate of reduction of DCIP was linear with no lag from the start of the reaction in the SDH assay. Unlike the mitochondrial enzyme (15), the *M. phlei* enzyme was stable in the presence of oxygen.

Analysis of the purity of SDH by PAGE. The purity of SDH was analyzed by nondenaturing PAGE in the presence of 0.1% Triton X-100 (Fig. 3). Gels were run in duplicate for each stage of purification; one set of gels was stained for protein with Coomassie blue, and the other set was stained for enzyme activity with MTT. In the Triton X-100 extract (lane 1B) and the Sephacryl S-300 pool (lane 2B), the enzyme exhibited two bands of activity. This was because most of the SDH was extracted from the membranes as a complex with cytochrome *b* and a minor portion of the enzyme was solubilized as a single entity. The association of SDH with cytochrome *b* was confirmed by the ability of succinate to reduce cytochrome *b* in the Triton X-100 extract and Sephacryl S-300 pool (data not shown). In the DEAE-52 step (lane 3B), the SDH-cytochrome *b* complex was broken down to individual components, as evidenced by a single enzyme activity band and migration of this band as a lower-molecular-weight species. Also, there was no detectable cytochrome *b* in the enzyme pool from the DEAE-52 step; the enzyme migrated as one principal band. The enzyme obtained by isoelectric focusing was free of contaminants (lane 5A).

Molecular properties. The purified SDH when subjected to SDS-PAGE exhibited two subunits with molecular weights of 62,000 and 26,000 (Fig. 3, lane 6). The isoelectric point of SDH, as determined by electrofocusing, was 4.8 (Fig. 2D). SDH is a flavoprotein containing 1 mol of FAD, 8 mol of acid-labile sulfide, and 8 mol of nonheme iron per mol of enzyme.

Catalytic properties. The pH optimum for the activity of

purified SDH was about 8.2. The enzyme had a half maximum reaction rate of 0.12 mM succinate, as determined from the Lineweaver-Burk plots (18). SDH exhibited a sigmoidal relationship of reaction rate to succinate concentration (Fig. 4A); in this experiment, the enzyme was isolated in the absence of succinate. SDH was competitively inhibited by fumarate (Fig. 4B). The K_i was 0.15 mM fumarate, as determined from a Dixon plot (8).

Effect of Triton X-100 and lipid on enzyme activity. The activity of purified SDH was unaffected by all concentrations of Triton X-100 used (Fig. 5), in contrast to the stimulation of the crude enzyme observed in Sephacryl S-300 chromatography (Table 1). Cardiolipin had no effect on the enzyme activity in the absence of Triton X-100. However, in the presence of 0.1% Triton X-100, cardiolipin stimulated the enzyme activity by about 30%. MK-9(2H), isolated from *M. phlei*, had no effect on enzyme activity either in the presence or absence of Triton X-100. Deoxycholate (0.5%) inhibited the enzyme activity by about 50%, and 0.1 mg of cardiolipin, but not MK-9(2H), reversed the inhibition by 50% (data not shown).

DISCUSSION

Most of the published work on the enzymology, composition, and reconstitutive ability of SDH concerns the mammalian mitochondrial enzyme (24). There have been several attempts to purify SDH from bacterial species (11, 13, 14, 17, 21–23), but to our knowledge the only other report of the purification of SDH to homogeneity is for *R. rubrum* (7). The great difficulty encountered in the purification of SDH is its association with cytochrome *b*. From *E. coli* (23) and *Bacillus subtilis* (11), SDH was extracted as a complex with cytochrome *b*. Extraction of the membranes of *M. phlei* with Triton X-100 also yielded SDH as a complex with cytochrome *b* (Fig. 3, lane 1B). Sephacryl S-300 chromatography of the Triton X-100 extract yielded SDH still associated with cytochrome *b* (Fig. 3, lane 2B). However, further chromatography on an anion exchange column (DEAE-52) resolved the SDH-cytochrome *b* complex to yield SDH free of the cytochrome (Fig. 3, lane 3B).

The *M. phlei* SDH is a complex enzyme containing 1 mol of FAD, 7 to 8 mol of nonheme iron, and 7 to 8 mol of acid-labile sulfide per mol of enzyme. Similar values have been reported for the enzyme from mitochondria (6) and *R. rubrum* (7). The enzyme is composed of two subunits with molecular weights of 62,000 and 26,000. The calculation of the FAD content of the *M. phlei* SDH was based on the added molecular weights of the subunits (88,000), which gave a value of 1 mol of FAD per mol of the enzyme. This is in agreement with reported values for the FAD content of SDH (6, 7).

A role for vitamin K in electron transport in *M. phlei* was proposed by Weber et al. (26), and vitamin K has been identified as a member of the NADH oxidase system (28). MK-9(2H), the natural menaquinone isolated from *M. phlei*, had no effect on the activity of the purified SDH in the presence or absence of Triton X-100 (Fig. 5). Cardiolipin, which accounts for 50% of the phospholipid content of *M. phlei* (1), activated SDH by 30% in the presence of 0.1% Triton X-100 (Fig. 5). Cardiolipin also activates and stabilizes the NADH dehydrogenase from *E. coli* (4). For membrane proteins that are in close association with lipids, it might be expected that these small molecules could modulate the functional rates of the enzymes. Kalra et al. (14) postulated that vitamin K was the intermediate electron

FIG. 4. Effects of succinate and fumarate on SDH activity. (A) Effects of various concentrations of succinate on SDH activity. SDH activity was measured as described in Materials and Methods. Ten microliters of SDH (0.67 μ g of protein) was used in each assay at the indicated concentrations of succinate. The enzyme was incubated with succinate for 5 min at 30°C in Tris hydrochloride buffer, and the reaction was started with DCIP and PMS. (B) Effect of fumarate on SDH activity. SDH was incubated with the indicated concentration of fumarate for 5 min at 30°C before being incubated with succinate.

FIG. 5. Effects of Triton X-100 and cardiolipin on the activity of purified SDH. SDH activity was measured as described in Materials and Methods. The enzyme (0.67 μ g of protein) was incubated for 5 min at 30°C in 50 mM Tris hydrochloride buffer (pH 8.2) with various concentrations of Triton X-100 (●) and either 0.1 mg of MK-9(2H) (○) or 0.1 mg of cardiolipin (×). Reactions were started with succinate. Triton X-100 at a concentration of 20 μ g/ml was carried over with the enzyme into the assay mixture.

acceptor in the transfer of electrons from succinate to cytochrome *b*. The present investigation was undertaken because of our interest in studying the interaction between the respiratory chain components of *M. phlei* and the regulation of energy transduction in this system. The unique finding that the SDH from *M. phlei* showed cooperativity with succinate and was competitively inhibited by the product fumarate will be further investigated to elucidate these effects on the mechanism of action of the enzyme.

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