The Succinate Dehydrogenase of *Escherichia coli*

**IMMUNOCHEMICAL RESOLUTION AND BIOPHYSICAL CHARACTERIZATION OF A 4-SUBUNIT ENZYME COMPLEX**

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Using EPR spectroscopy to monitor the integrity of the enzyme, conditions have been established which allow specific immunoprecipitation of the succinate dehydrogenase complex of *Escherichia coli*. The enzyme complex precipitated from Lubrol PX-solubilized membranes by monospecific antiserum in the presence of a cocktail of protease inhibitors contains four polypeptides of approximate *M*<sub>s</sub> 71,000, 26,000, 17,000, and 15,000. The 71-kDa flavopeptide is readily susceptible to proteolysis, and the enzyme complex shows unusual facile dissociation. Spectroscopic measurements indicate the presence of a [2Fe-2S] cluster (Center 1), a [3Fe-4S] cluster (Center 3), and a b-type cytochrome. In addition, a change in relaxation of Center 1 at low potentials is indicative of Center 2. Midpoint redox potentials of Centers 1–3 for both the membrane-bound and detergent-solubilized enzyme were estimated to be +10 mV, −175 mV, and +65 mV, respectively.

Succinate dehydrogenases (succinate:ubiquinone oxidoreductases) isolated from a variety of eukaryotic and prokaryotic cells appear to be similar to each other in their subunit composition and in the presence of a covalently bound flavin, iron, and labile sulfide (1–3). Of these enzymes, the succinate:ubiquinone oxidoreductase of beef heart mitochondria is arguably the best characterized. It consists of four discrete polypeptides of approximate *M*<sub>s</sub> 70,000, 27,000, 15,000, and 13,600. Analysis also indicates the presence of 1 histidyl flavin, 7–8 iron, and 7–8 labile sulfide atoms/enzyme complex. Some preparations also contain a b-type cytochrome (4–6). The 70- and 30-kDa subunits together comprise the soluble succinate dehydrogenase. Evidence from the mitochondrial system (7, 8) and from the closely related enzyme, fumarate reductase (9), implicates the two small hydrophobic polypeptides in membrane attachment and reaction with ubiquinone.

Succinate dehydrogenase is considered to have up to three redox centers designated Centers 1, 2, and 3 (3, 5). In mitochondria, Center 1 gives rise to a rhombic EPR signal at *g* = 2.03, 1.93, and 1.91 upon reduction with succinate. From the temperature dependence of the signal and from linear-electric-field effect measurements, it is proposed to be a [2Fe-2S]<sup>2+/3+</sup> cluster. Center 2 is normally detected as an increase in the intensity of the *g* = 1.93 signal at low redox potentials and has been ascribed to a second [2Fe-2S] cluster (10). However, most measurements indicate that the increased spectral intensity, when measured under nonsaturating conditions, is low (5) if it exists at all (11). Proposals to explain this phenomenon include ones based on an interaction between two [2Fe-2S] clusters, spin relaxation enhancement caused by a conformational change in the protein or by interaction between Center 1 and an EPR-silent component (2, 3, 11, 12). However, these interpretations remain controversial (2, 3). At low temperature (<20 K) and in the oxidized state, Center 3 gives rise to a narrow feature at *g* = 2.01. Recent linear-electric-field effect results have indicated that for the air-oxidized enzyme, Center 3 is a [3Fe-4S] rather than a [4Fe-4S]<sup>2+</sup> cluster (13).

The location of the redox centers in the various subunits of succinate dehydrogenase and fumarate reductase cannot yet be considered settled (see Ref. 2 for review). The only point to be established with certainty is that in both cases the histidyl flavin is located in the largest (70 kDa) subunit (1, 14, 15).

In the model Gram-negative bacterium *Escherichia coli* the nucleotide sequence of the fumarate reductase operon and more recently of the succinate dehydrogenase genes (sdhA–D) has been established (16–20). The two largest subunits (sdhA and B) of the latter enzyme show considerable homology with the corresponding gene products from fumarate reductase (frdA and B) and indicate an amino acid composition similar to that of beef heart succinate dehydrogenase. The flavopeptide subunits (sdhA and frdA) contain only one cysteine in a conserved position (16, 18), but the 27-kDa subunits contain 10, in arrangements which suggest similarities to known binding sites for iron-sulfur clusters of ferredoxins (17). However, in the absence of biophysical studies on purified enzyme preparations, interpretation of these observations with respect to their redox centers remains speculative.

*E. coli* succinate dehydrogenase has proved particularly refractory to biochemical and biophysical characterization, notwithstanding considerable effort (21–24). Biophysical studies of the metalloflavoprotein have been restricted to analyses of signals resolved by EPR spectroscopy of membranes prepared from cells grown under aerobic (25) or anaerobic (26) conditions. Cellular assignment of EPR-detectable centers to defined membrane components has not proved possible. A number of investigators have reported solubilization of succinate dehydrogenase and *M*<sub>s</sub> 100,000 and 150,000 have been quoted for the cytochrome-free enzyme.
(21) and for a succinate dehydrogenase-cytochrome bcomplex (22), respectively. However, these preparations werextremely labile, and unambiguous resolution of enzyme-specificpolypeptides has been limited to one, viz. a 71-kDa polypeptide containingthe covalently bound flavin prosthetic group (15, 23, 24).

In light of these problems we have employed alternatestrategies to elucidate the properties of E. coli succinate dehydrogenase. Recent biochemical and immunological studies have identified succinate dehydrogenase as a major membrane immunogen for E. coli cells grown aerobically on succinate (24) supplemented where appropriate with aeration on succinate (24) supplemented where appropriate with sodium succinate and lysozyme and EDTA (30) and minimal medium containing 0.2% glucose. Preparation of envelopes were cleared by centrifugation at 48,000 x g for 10 min and washed twice in solutions containing 10 mM Tris-HCl (pH 7.8), 2 mM protease inhibitors, and 1% (v/v) of the appropriate detergent. Washed immunoprecipitates were resuspended using a sonic water bath in Laemmli sample buffer (34) for SDS-Polyacrylamide gel electrophoresis or in 50 mM Tris-HCl (pH 7.8) for spectrophotometric analysis. For EPR measurements, immunoprecipitates were transferred to EPR sample tubes and rapidly frozen in liquid nitrogen. Selective precipitation in this manner of 5-mg membrane extract protein with 3.5 ml of antiserum (x10 concentrated immunoglobulin G) gave sufficient material for a pair of EPR samples. In experiments involving Triton X-100 extracts of E. coli membranes, a number of the above experimental parameters were altered in attempts to successfully immunoprecipitate the enzyme complex. These included choice of host organism (E. coli K12 was preferred over E. coli K12), type of membrane preparation (envelopes, K50b vesicles, or solubilized plasma membranes), the temperature (0-37 °C), and pH (6.8 to pH 8.6) of extraction and precipitation, or modification of procedures for washing (omission of detergent, protease inhibitors, or sonication) and processing (solubilization in SDS at 40, 60, or 100% of immunoprecipitates). Immunoprecipitation of succinate dehydrogenase from Triton X-100-solubilized membranes of Micrococcus luteus was as described elsewhere (28).

**EXPERIMENTAL PROCEDURES**

**Growth and Membrane Preparation.—** E. coli ML308-225 was grown aerobically on succinate (24) supplemented where appropriate with [U-14C]protein hydrolysate (420 μCi/liter). E. coli WGAS (JRG 653) and adh (JRG546, JRG548) or fad (JRG940) mutants were grown on minimal medium containing 0.2% glucose. Preparation of envelopes by French pressing in the presence of lysozyme and EDTA has allowed the production of the catalytic activity of enzyme antigens, including succinate dehydrogenase (29) EPR signals at 25 °C for 16 h. Immunoaffinity columns were harvested at 6,000 x g for 10 min and washed twice in solutions containing 10 mM Tris-HCl (pH 7.8), 2 mM protease inhibitors, and 1% (v/v) of the appropriate detergent. Washed immunoprecipitates were resuspended using a sonic water bath in Laemmli sample buffer (34) for SDS-Polyacrylamide gel electrophoresis or in 50 mM Tris-HCl (pH 7.8) for spectrophotometric analysis. For EPR measurements, immunoprecipitates were transferred to EPR sample tubes and rapidly frozen in liquid nitrogen. Selective precipitation in this manner of 5-mg membrane extract protein with 3.5 ml of antiserum (x10 concentrated immunoglobulin G) gave sufficient material for a pair of EPR samples. In experiments involving Triton X-100 extracts of E. coli membranes, a number of the above experimental parameters were altered in attempts to successfully immunoprecipitate the enzyme complex. These included choice of host organism (E. coli K12 was preferred over E. coli K12), type of membrane preparation (envelopes, K50b vesicles, or solubilized plasma membranes), the temperature (0-37 °C), and pH (6.8 to pH 8.6) of extraction and precipitation, or modification of procedures for washing (omission of detergent, protease inhibitors, or sonication) and processing (solubilization in SDS at 40, 60, or 100% of immunoprecipitates). Immunoprecipitation of succinate dehydrogenase from Triton X-100-solubilized membranes of Micrococcus luteus was as described elsewhere (28).

**SDS-Polyacrylamide Gel Electrophoresis.—** SDS-Polyacrylamide gel electrophoresis was performed according to Laemmli (34). Radioactive polypeptides were visualized by autoradiography and molecular weights calculated in the standard fashion using the following 16 molecular weight markers: β-galactosidase (M, 116,000), phosphorylase b (M, 94,000), bovine serum albumin (M, 66,000), catalase (M, 61,000), glutamate dehydrogenase (M, 55,400), fumarase (M, 48,500), alcohol dehydrogenase (M, 41,000), E. coli ompF protein (M, 37,200), carbonic anhydrase (M, 30,000), chymotrypsinogen A (M, 25,000), trypsin inhibitor (M, 20,100), myoglobin (M, 17,200), myoglobin I + II (M, 14,600), myoglobin (M, 14,300), myoglobin I (M, 8,200), myoglobin II (M, 6,400). Myoglobin and its cyanogen bromide fragments were used as standards in a visible spectrophotometry calibration kit. Precipitation properties were obtained from Sigma or Boehringer Mannheim.

**Spectroscopy.—** EPR spectra were recorded on a Varian E4 spectrometer fitted with a 1020E microwave bridge and an Oxford Instruments EPR liquid helium flow cryostat. Values of power of half-saturation (P1/2) were estimated by the procedures of Rupprecht et al. (35), and redox titrations were performed as previously described (29, 36). Low-temperature difference spectra were recorded by transmission between 480 and 680 nm using a dual-wavelength spectrophotometer constructed at the Johnson Foundation and fitted with a nitrogen flow temperature control. Purified cytochrome c was used as standard. Samples for both EPR and optical absorption spectroscopy were analyzed in cylindrical quartz tubes of 3.5-mm internal diameter.

**Analytical Procedures and Chemicals.—** Protein was estimated by a modification (37) of the method of Lowry et al. (38) which eliminates interference by detergent. Bovine serum albumin (A280 3.08) was used as standard. Succinate dehydrogenase activity was assayed by phenazine methosulfate-mediated reduction of dichlorophenolindophenol essentially as described (39) using a sodium phosphate (100 mM, pH 7.6) buffered system. All protease inhibitors were obtained from Sigma. [14C]Protein hydrolysate (220 μCi/mg; specific activity 100 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, United Kingdom.

**RESULTS**

**EPR Spectra of Membranes, Detergent-solubilized Membranes, and Succinate Dehydrogenase-antibody Complexes**

1 The abbreviation used is SDS, sodium dodecyl sulfate.
Membrane preparations from *E. coli* grown aerobically on the nonfermentative carbon source succinate display two prominent EPR signals, one at \( g = 2.02 \) in the oxidized state and the other at \( g = 2.03 \) and \( g = 1.94 \) in the reduced state (Fig. 1). As will become evident, these signals derive almost exclusively from succinate dehydrogenase. Since Center 3 of succinate dehydrogenase is most labile during purification (3), the intensity of the \( g = 2.02 \) signal in the oxidized state was used to monitor the integrity of the enzyme following extraction of membranes with various detergents. Of those tested (see “Experimental Procedures”), only Lubrol PX generated detergent extracts for which the amplitude of the \( g = 2.02 \) signal was consistently comparable to that of untreated vesicles (Fig. 1A, spectra 1 and 2). Triton X-100, which is frequently the detergent of choice for the solubilization of bacterial membranes and which has been employed by us in earlier studies (15, 24, 27), produced detergent extracts which showed variable amounts of signal at \( g = 2.02 \). Furthermore, immunoprecipitates obtained following treatment of Triton X-100 extracts with specific anti-succinate dehydrogenase antiserum failed to display any EPR-detectable components. X-100 extracts with specific anti-succinate dehydrogenase antibody complexes obtained by centrifugation following incubation of Lubrol PX-solubilized membranes with specific antiserum (Fig. 1A, spectrum 3). The signal had a temperature dependence expected for Center 3, having maximum intensity at 12 K and being virtually undetectable at 25 K. It is notable that the same signal was not detected for the supernatant fraction obtained following removal of immune complexes (Fig. 1A, spectrum 4).

Addition of succinate (final concentration 20 mM) caused the \( g = 2.02 \) signal detected for membrane vesicles, Lubrol PX extracts, and the precipitated enzyme complex to decrease in amplitude as the cluster became reduced. Reduction with succinate also caused the appearance in all three preparations of a signal at \( g = 2.03 \) and \( g = 1.94 \) with a line shape which is best described as axial with a slight rhombic distortion (Fig. 1B, spectra 1–3). The properties of this signal, which we assign to Center 1, are typical of a reduced [2Fe-2S] cluster. The apparent signal amplitude in Fig. 1B is somewhat low because of microwave power saturation. Even so, reduction of this center by succinate was slow and incomplete. Full reduction of the \( g = 1.94 \) signal was achieved by the addition of dithionite (Fig. 1C, spectra 1–4), the integrated intensity of this signal being about 2.5-fold that of the \( g = 2.02 \) signal from the oxidized enzyme.

The presence of Center 2, which causes a change in the electron-spin relaxation of the \( g = 1.94 \) signal, was deduced from the difference in power for half-saturation values between succinate-reduced and dithionite-reduced samples. As shown in Table I, a significant relaxation enhancement was observed in membrane vesicles, Lubrol extracts, and immunoprecipitated enzyme.

It seems clear from the complete partitioning of the prominent EPR signals into material precipitated by nonspecific anti-succinate dehydrogenase serum (Fig. 1) that (a) both signals originate from succinate dehydrogenase and (b) this enzyme represents most of the EPR-detectable centers in the membranes obtained from cells grown aerobically on succinate. Thus, while fumarate reductase is known to be present in trace amounts in these membranes (24), it cannot contribute significantly to the EPR spectra for the following reasons. First, its EPR signature is absent from supernatant fractions obtained after selective removal of succinate dehydrogenase (Fig. 1). Second, it is immunologically distinct from succinate dehydrogenase (24), does not react with our specific antiserum (27), and would not be expected to cofractionate with succinate dehydrogenase during immunoprecipitation. This sup-

**Fig. 1.** Co-precipitation of EPR-detectable centers with succinate dehydrogenase. EPR spectra 1 to 4 were obtained from *E. coli* membrane vesicles, Lubrol PX extracts, immunoprecipitated succinate dehydrogenase, and the supernatant remaining after immunoprecipitation, respectively. Samples in Panel A are oxidized as prepared and spectra are recorded at 12 K. Samples in Panels B and C are reduced with 20 mM succinate and 5 mM dithionite, respectively, and have been recorded at 26 K. Instrument settings: frequency, 9.18 GHz; microwave power attenuation, 10 dB of a nominal 200 milliwatts; modulation amplitude, 1 milliTesla; gain settings for Panels A, B, and C are in the ratio 1:5:10.
position is confirmed by the absence of the readily resolvable frdA gene product in the spectrum of sdh-specific polypeptides precipitated by the serum (see Figs. 2 and 7). Finally, the polypeptide of position is confirmed by the absence of the readily resolvable the known subunit composition of succinate dehydrogenase therein) or with antibody, a proposal consistent with the that the enzyme complex had become dissociated, possibly as

Polypeptide Composition of Succinate Dehydrogenase Immunoprecipitated from Detergent-solubilized Membranes—Initial experiments designed to establish the polypeptide profile of succinate dehydrogenase selectively precipitated from Triton X-100 extracts of 14C-labeled membrane vesicles of E. coli ML308-225 by specific serum generated the surprisingly simple profile shown in Fig. 2 (track 3). Only a single major polypeptide of M, 71,000 was resolved in marked contrast to the known subunit composition of succinate dehydrogenase from other sources (1, 4, 6). Manipulation of a number of experimental parameters (see “Experimental Procedures”) failed to resolve any other polypeptides. It seems probable that the enzyme complex had become dissociated, possibly as a result of interaction with Triton X-100 (or impurities therein) or with antibody, a proposal consistent with the absence of EPR signals from this preparation.

In contrast to the results obtained with Triton X-100-solubilized membranes, precipitation from Lubrol PX extracts of 14C-labeled membrane vesicles (in the presence of protease inhibitors) resulted in the immunopurification of an enzyme complex in which four polypeptides of M, 90%, of the precipitated radioactivity (Fig. 2, track 4); and from Lubrol PX-solubilized membranes (tracks 4 and 5) following immunoprecipitation in the absence (tracks 2 and 4) and presence (tracks 3 and 5) of protease inhibitors. Molecular masses in kilodaltons are indicated to the right of the fluorogram.

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In contrast to the results obtained with Triton X-100-solubilized membranes, precipitation from Lubrol PX extracts of 14C-labeled membrane vesicles (in the presence of protease inhibitors) resulted in the immunopurification of an enzyme complex in which four polypeptides of M, 71,000, 26,000, 17,000, and 15,000 clearly and consistently accounted for over 90% of the precipitated radioactivity (Fig. 2, track 5). The 71- and 26-kDa proteins appear analogous to the 2-subunit succinate dehydrogenase isolated from beef heart mitochondria (see Fig. 7). The two lower molecular weight subunits also appear to be integral components of the complex and not, for example, degradation products since (a) the relative intensities of the 26-, 17-, and 15-kDa polypeptides did not increase when precipitation was performed in the absence of protease inhibitors; (b) although the 71-kDa subunit was degraded in the absence of protease inhibitors, the degradation products were distinct from the three subunits of lower molecular weight (Fig. 2, tracks 2 and 4); (c) proteolytic fragments corresponding to these proteins were not resolved by digestion of the 71- and 26-kDa subunits with V8 protease in Cleveland digests (data not shown); and (d) an exchange of samples between ourselves and Dr. John Guest of the University of Sheffield indicated that the resolved polypeptides co-migrated in our SDS-polyacrylamide gel system with the putative in vitro translation products of the cloned sdh operon (Fig. 3A).

Interestingly, a single-subunit profile similar to that shown in Fig. 2 (track 3) could be generated from the 4-subunit complex (Fig. 2, track 5) by washing the immunoprecipitate with low concentrations (200 mM) of either guanidinium hydrochloride or sodium thiocyanate. This is again suggestive of unusually weak interactions between certain subunits of the enzyme and reinforces the suspicion that the major precipitating antibodies in our immunoglobulin preparation are directed against the 71-kDa protein. For these reasons, we regard with caution the significance of stoichiometry measurements performed on the 4-subunit complex which indicate a clear bias in favor of the flavoprotein subunit.

Redox Titrations of the g = 2.02 and g = 1.94 Signals—Because of limited availability of specific serum and hence of immunoprecipitated enzyme and because of the latter’s sluggish reaction with the oxidizing and reducing agents, we were unable to measure the redox potentials of the iron-sulfur centers directly. We, therefore, made these measurements in intact membranes and in Lubrol extracts. This was considered justified on the basis of the arguments presented above that the EPR-detectable centers in the membranes of E. coli ML308-225 grown aerobically on succinate arise primarily from succinate dehydrogenase.

During redox measurements made on E. coli membrane vesicles, the g = 2.02 signal titrated as a single species with a midpoint potential of +65 ± 15 mV (Fig. 4A). When measured at the relatively high temperature of 50 K and microwave power attenuation of 10 db, the g = 1.94 signal showed a principal increase with a midpoint of +10 ± 20 mV, with some
precipitated from Lubrol PX extracts of 14C-labeled membrane vesicles. Gel A: track 1, 15% polyacrylamide gel electrophoresis, and the resultant protein-stained profiles are shown in Fig. 7. The 71- and 15-kDa subunits observed for E. coli succinate dehydrogenase were very similar to those resolved for the enzyme purified from beef heart mitochondria (71 and 27 kDa). The two major subunits of succinate dehydrogenase precipitated from M.

Power Saturation Studies of the g = 1.94 Signal—Fig. 5A shows power saturation curves for g = 1.94 from redox samples (arrowed in Fig. 4) poised at −110 mV and −300 mV. Similar curves for immunoprecipitated succinate dehydrogenase partially reduced with succinate and fully reduced with dithionite are shown in Fig. 5B. In each case, the partially reduced signal showed slower relaxation than its fully reduced counterpart. However, the g = 1.94 signals had similar intensities at low power (nonsaturation conditions) suggesting that this feature arose from a single paramagnetic species, viz. Center 1. Essentially identical results (not shown) were obtained for membranes extracted with Lubrol PX.

Presence of b-type Cytochrome in Immunoprecipitated Succinate Dehydrogenase—The presence of cytochrome species in the immunoprecipitated E. coli succinate dehydrogenase was investigated by low-temperature difference spectroscopy (Fig. 6). Oxidized minus dithionite-reduced spectra of E. coli vesicles (Fig. 6A) and of Lubrol PX extracts (Fig. 6B) indicated the presence of a b-type cytochrome with an a-band at 559 nm (at 173 K). Absorption at 628 nm, presumably due to cytochrome d, was also detected. The cytochrome b species in Lubrol PX extracts was reduced by either succinate or dithionite. Succinate dehydrogenase immunoprecipitated from the extract in the presence of protease inhibitors also showed absorption at 559 nm (Fig. 6C). This suggests that one of the small subunits in E. coli succinate dehydrogenase may be a b-type cytochrome. Purified preparations of cytochrome b(559) (40) yield polypeptides which migrate on SDS gels in the region of the 17- and 15-kDa subunits of the precipitated enzyme complex (Fig. 3B).

Comparison of the Subunit Composition of Various Succinate Dehydrogenases and E. coli Fumarate Reductase—The immunoprecipitated 4-subunit E. coli succinate dehydrogenase was compared to related enzymes from other sources by SDS-polyacrylamide gel electrophoresis, and the resultant protein-stained profiles are shown in Fig. 7. The 71- and 26-kDa subunits observed for E. coli succinate dehydrogenase were very similar to those resolved for the enzyme purified from beef heart mitochondria (71 and 27 kDa). The two major subunits of succinate dehydrogenase precipitated from M.

indication of a small increase at −175 mV (Fig. 4C). In contrast, at 22 K (Fig. 4B) the increase was clearly biphasic and occurred with Em values of +10 ± 20 mV and −175 ± 20 mV. Similar results (not shown) were obtained for membranes solubilized with Lubrol PX indicating that the potentials were not significantly altered by detergent extraction. These results are in reasonable agreement with values obtained by Ingledew et al. (25) for signals in membranes from two other strains of E. coli and may be considered as confirmation of their tentative assignment of these signals to succinate dehydrogenase.

**FIG. 3.** Comparison of immunoprecipitated polypeptides with (A) sdh gene products and (B) purified cytochrome b(559) by SDS-polyacrylamide gel electrophoresis. Gel A: track 1, 15% polyacrylamide gel electrophoresis, and the resultant protein-stained profiles are shown in Fig. 7. The 71- and 15-kDa subunits observed for E. coli succinate dehydrogenase were very similar to those resolved for the enzyme purified from beef heart mitochondria (71 and 27 kDa). The two major subunits of succinate dehydrogenase precipitated from M.

**FIG. 4.** Redox titration of EPR signals in membrane vesicles. The amplitude of the g = 2.02 signal at 12 K (A) and the g = 1.94 signal at 22 K (B) and 50 K (C) were recorded as a function of redox potential. Spectra were recorded as for Fig. 1. The individual points were fitted to curves calculated from the Nernst equation, with n = 1, and assuming midpoint potentials of +60 mV for A, +10 mV for B, and −175 mV for C.

**FIG. 5.** Curves of power saturation for the g = 1.94 from membranes and immunoprecipitated succinate dehydrogenase poised at different redox potentials. Signal amplitude was recorded as a function of microwave power for the g = 1.94 signal at 25 K. Curves were fitted assuming inhomogeneous broadening (35). Panel A shows curves for membrane vesicles poised at −300 mV (○) and −110 mV (■). Panel B shows immunoprecipitated succinate dehydrogenase reduced with dithionite (●) and with succinate (■). mW, milliwatt(s).
**DISCUSSION**

The results described above clearly portray for the first time the polypeptide composition and EPR characteristics of the succinate dehydrogenase complex from the cytoplasmic membranes of *E. coli*. Several lines of evidence indicate that the four polypeptides resolved are integral components of the complex. First, enzyme activity remains high in Lubrol PX-extracted membranes. Second, and more important, the immunoprecipitated enzyme complex retains its complement of EPR-detectable iron-sulfur centers including the normally labile Center 3. Third, none of the smaller subunits correspond to products of proteolytic degradation resolved following digestion with serum or V8 proteases. Finally the four polypeptides co-migrate with the putative in vitro translation products of the cloned *sdh* operon.

Clearly there has been some conservation of the subunit composition of the succinate dehydrogenase holoenzyme. The similarity between the *E. coli* enzyme and that of beef heart mitochondria is particularly striking, a feature which extends to their biophysical properties (vide infra). The larger subunits of *E. coli* succinate dehydrogenase (*sdhA* and *sdhB*) and fumarate reductase (*frdA* and *frdB*) are also known to exhibit a high degree of homology at the protein and DNA sequence level (16, 17). Whether the associated low-molecular-weight polypeptides of the two succinate dehydrogenases are similarly conserved remains less clear but is of interest in the light of a considerable divergence between the aerobic respiratory chains of *E. coli* and mammalian mitochondria downstream from this enzyme. Certainly, in common with many succinate dehydrogenase preparations (1, 6, 22, 28), the immunoprecipitated enzyme complex from *E. coli* does contain a *b*-type cytochrome, very possibly cytochrome b$_{556}$. However, conclusive identification of either, or both, the 17- and 15-kDa succinate dehydrogenase subunits as apocytochrome of b$_{556}$ has not been achieved. Moreover, our experimental approach precludes an accurate quantitation of cytochrome content. Thus, the association of cytochrome *b* with the enzyme complex, while probably not fortuitous, may represent the presence of a substoichiometric amount of an exogenous component. We find it intriguing, however, that the gene encoding cytochrome b$_{556}$ (*cybA*) has been recently mapped to a region of the chromosome extremely close to (and possibly in) the *sdh* operon (41). Fine structure mapping is required to deter-
mine whether cybA and sdhC/D represent the same genetic locus. Certainly, in *Bacillus subtilis* an integral component (the sdaA gene product) of succinate dehydrogenase is known to be a transmembrane b-type cytochrome which can be reduced by the enzyme and which is responsible for membrane attachment (1, 42). The two hydrophobic subunits (M, 15,000 and 13,000) of *E. coli* fumarate reductase, on the other hand, have a role in membrane attachment but are not thought to be cytochromes (9, 18-20, 43). Clearly, the structural and functional interrelationships of these two *E. coli* enzymes deserve further investigation.

As judged by their EPR behavior, the iron-sulfur clusters of succinate dehydrogenase suffered minimal perturbation on membrane extraction and precipitation with specific serum and displayed a remarkable similarity to those described for the succinate dehydrogenase of beef heart mitochondria (2, 3). The g = 1.94 signal was typical of a reduced [2Fe-2S] cluster while the g = 2.02 signal showed properties consistent with a [3Fe-2S] or [4Fe-4S] cluster. Power saturation studies on membranes and precipitated succinate dehydrogenase are consistent with the view (11) that the g = 1.94 signal arose from a single [2Fe-2S] component. The biphasic increase in apparent intensity observed with decreasing redox potential measured with saturating microwave power reflects changes in spin-lattice relaxation induced by another redox center within the enzyme (Center 2), which itself does not contribute any significant intensity to the g = 1.94 signal. There is no doubt that Center 2 represents a redox species. Its reduction, as monitored by relaxation of Center 1, occurs consistently in almost all succinate dehydrogenase preparations as a n = 1 (single electron) species (2, 3, 29). It might, therefore, be expected to give rise to an EPR signal. Possibly this is too broad for detection at the signal-noise levels currently available. The function of such a center, with a midpoint potential (~175 mV) substantially less than the fumarate/succinate couple (28 mV), is not clear at present.

The redox midpoint potentials determined here for Centers 1 and 3 of *E. coli* succinate dehydrogenase (+10 mV and +65 mV, respectively) are very close to those reported for the analogous centers in the mammalian counterpart (0 mV and +60 to +120 mV; Refs. 10 and 44) but are more positive than those of Centers 1 and 3 of fumarate reductase (~20 mV and ~70 mV, respectively; Ref. 45). This is one of the few distinguishing features of otherwise extremely similar EPR spectra. Consideration of the primary structures of the two *E. coli* enzymes and comparison with homologous sequences for ferredoxins possessing well characterized iron-sulfur clusters (17-19) suggests a possible reason for this difference and shed light on the likely location and structure of the centers. In the sdbH and frdB proteins, 10 of the 11 cysteine residues (the usual ligands to the iron-sulfur clusters) are conserved. In contrast, between the flavoprotein subunits of the two enzymes there is only one cysteine in an invariant position. The likely site for at least two and possibly three of the iron-sulfur clusters would seem to be the 26-kDa polypeptide (sdbB). Within the 27-kDa subunit of fumarate reductase there are three cysteines in homologous positions to the [2Fe-2S] ferredoxins with another cysteine at position 78 which might represent the fourth (19, 46). However, in succinate dehydrogenase one of these cysteines is substituted by aspartate. Noncysteine ligands to an iron-sulfur cluster have previously been invoked for the "Rieske" iron-sulfur protein (47). If Asp 62 were a ligand it would probably result in a positive shift in redox potential for the cluster (48). Seven of the remaining cysteines in the sequence of the sdbH subunit show homologies with ferredoxins that contain either two [4Fe-4S] or a [4Fe-4S] cluster and a [3Fe-3S] cluster (17, 46). It is notable that there are no features in the sequence which indicate a second [2Fe-2S] cluster. This would suggest that Center 3 is a 4-iron cluster. Once again, if both clusters were located exclusively in sdbB, there must be additional non-cysteine ligands to iron.

The precautions taken to ensure specificity of antiserum and to minimize proteolysis and dissociation have clearly aided in the successful resolution of the succinate dehydrogenase-enzyme complex. The unusual lability of the enzyme, its tendency to dissociate, and its susceptibility to proteolysis may account, at least in part, for the complex polypeptide profile previously reported for this antigen when analyzed by precipitate excision (15) and provide a plausible reason for the difficulties encountered here and elsewhere in the biochemical purification of this important respiratory component. However, it is relevant to point out that our experimental strategy is not without its limitations. The presence of antibody, for example, precludes meaningful reconstitution experiments or even a direct assessment of catalytic activity. Irrespective of this point, it is evident that our knowledge of labile respiratory enzymes can be significantly advanced by this type of interdisciplinary approach.

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E. coli Succinate Dehydrogenase