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Succinate Dehydrogenase[1]

A PARTIAL PURIFICATION FROM MUNG BEAN HYPOCOTYLS AND SOYBEAN COTYLEDONS

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ABSTRACT

A procedure was developed for the partial purification of succinate dehydrogenase from mung bean (*Vigna radiata* L.) hypocotyls and soybean (*Glycine max* [L] Merr. v. Ransom) cotyledons. The procedure utilized a Triton X-100 extraction followed by ammonium sulfate precipitation. The final fraction was enriched in two polypeptides with approximate molecular weights of 67,000 and 30,000 daltons, exhibited a pH optima of 7.0 to 7.5, contained a *b*-type cytochrome, and exhibited the characteristic ferredoxin-type and high potential iron-sulfur protein-type electron paramagnetic resonance signals reported for the iron-sulfur centers of mammalian succinate dehydrogenase. Inhibition constants of 1.15 and 24.6 micromolar for oxaloacetate and malonate, respectively, were obtained.

SDH,[3] the only enzyme of the Kreb's cycle that is bound to the inner mitochondrial membrane, exists as part of a multiprotein complex (complex II) of the electron transport chain and functions in the transfer of electrons from succinate to ubiquinone-10 (10, 25). The purified mammalian enzyme consists of two dissimilar subunits, a polypeptide with a mol wt of 70,000 and a smaller subunit of 27,000 D (4). The 70,000 D polypeptide contains two iron-sulfur centers of the Fe_2S_2 type, named centers S-1 and S-2, and has 1 mol of covalently bound flavin adenine dinucleotide (4, 21). The 27,000 D polypeptide contains a Fe_4S_4 center referred to as iron-sulfur center S-3 (20). Complex II contains two additional polypeptides with mol wt of 15,000 (Cyt b_{560}) and 13,000 D (possibly a ubiquinone-binding protein) (1, 29).

SDH has been purified from both animal tissues and yeast, and the properties of the enzyme have been demonstrated to be essentially identical (27). Maintenance of an active preparation of SDH in a homogeneous form requires low temperatures, anaerobiosis, and the presence of protective agents (4, 10, 25). Partially purified SDH preparations from higher plants have been reported in only a few cases and in none are extensive characterizations of the properties of the resulting products presented (11, 12, 19).

Isolation procedures that have been used for the purification of SDH from mammalian mitochondria include alkaline extraction of acetone powders (26), alkaline extraction in butanol (15), extraction with cyanide (30), and perchlorate extraction of purified complex II preparations (4). Although the extraction of SDH from complex II is both rapid and simple, the purification of complex II requires a significant investment of time (several days) and material (200 g wet weight of a mitochondrial paste).

The available procedures for purificiation of SDH from mammalian mitochondria have not proven useful in the extraction of plant mitochondria mainly because of the difficulties in obtaining the required quantity of starting material necessary for the purification of complex II. In 1961, Hiatt partially purified SDH from bean root and tobacco leaf mitochondria (12). He used an acetone powder extract similar to that first described by Kearney and Singer (14) followed by elution from a calcium phosphate gel and precipitation with ammonium sulfate. This isolation procedure did not result in a purified enzyme and did not utilize the anaerobic conditions or reducing agents later shown to be required to maintain maximal enzyme activity. More recently, Nakayama and Ashi (19) and Hattori and Asahi (11) partially purified SDH from pea cotyledons and sweet potato tubers, respectively. The latter workers used deoxycholate extraction followed by ammonium sulfate fractionation and DEAE-cellulose chromatography to purify the SDH, and a limited characterization of the resulting product was reported.

In the current study, an isolation procedure is described for the isolation and partial purification of SDH from plant mitochondria. This procedure has incorporated techniques described for mammalian SDH purification to maintain maximal activity. In addition, the polypeptide composition, pH optima, EPR spectra, and inhibitor characteristics of the plant SDH are reported, and compared with those obtained with the enzyme from mammalian sources.

MATERIALS AND METHODS

Mitochondria were isolated from 3- to 4-d-old dark-grown mung bean (*Vigna radiata* L.) hypocotyls and 6- to 7-d-old soybean (*Glycine max* [L.] Merr. v. Ransom) cotyledons essentially as described by Ikuma and Bonner (13). Both the grinding and washing media, however, were supplemented with 20 mM Hepes buffer (pH 7.1) (17). Isolated mitochondria were suspended to a final concentration of 4 mg protein/ml in nitrogen-purged 20 mM Hepes buffer (pH 7.1) that contained 20 mM succinate and 5 mM β-mercaptoethanol at 4°C. Triton X-100 was added to a final

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[3] Abbreviations: SDH, succinate dehydrogenase; EPR, electron paramagnetic resonance; DPIP, 2,6-dichlorophenol-indophenol; PMS, phenazine methosulfate; DPB, 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone; TTF, 2-thenoyltrifluoroacetone; HiPIP, high potential iron-sulfur protein; OAA, oxaloacetic acid.

concentration of 0.4% and the sample was stirred for 20 min at 4°C. Following incubation with Triton X-100, the sample was centrifuged for 15 min at 40,000*g*. Solid ammonium sulfate was added to the supernatant to 27% saturation at 4°C and incubated for 5 min on ice. The supernatant was overlayed with 3 ml of 2 mM succinate and 10 mM phosphate buffer (pH 7.5) and centrifuged for 5 min at 10,000*g*. The yellow band that appeared at the interface of the two layers following centrifugation was removed and mixed at 4°C with 20 ml of 2 mM succinate, 10 mM phosphate buffer (pH 7.5) containing ammonium sulfate (27% saturated). The solution was overlayed with 3 ml of 2 mM succinate and 10 mM phosphate buffer (pH 7.5), and centrifuged for 5 min at 10,000*g*. The yellow band was removed from the interface and stored at 4°C under nitrogen.

SDH activity was determined either by continuous recording of O_2 uptake with a Clark-type electrode or by following the reduction of DPIP at 580 nm. SDH O_2 uptake assay mixtures contained 8.3 mM succinate (neutralized), 1 mM PMS, 30 mM Tricine-NaOH (pH 7.5), and enzyme. A rate-limiting concentration of succinate (0.25 mM) was utilized during analysis of the competitive inhibitors oxaloacetic acid and malonic acid with the concentrations of all other solutions the same as mentioned above. Succinate-DPIP reductase was monitored at 580 nm in assay mixtures that contained 2.0 mM succinate, 30 mM Tricine-NaOH (pH 7.5), 50 μM DPIP, and enzyme.

Separation of SDH polypeptides by SDS-polyacrylamide gel electrophoresis was carried out with the discontinuous buffer system of Laemmli (16). Electrophoresis was performed in a slab gel apparatus (28) with a continuous 12 to 16% (w/v) polyacrylamide separating gel and a 5% (w/v) stacking gel. SDH polypeptides were solubilized in a 65 mM Tris-Cl sample buffer (pH 6.8) that contained 10% (v/w) glycerol, 1% (v/v) β-mercaptoethanol, and 2% (w/v) SDS. Samples were boiled for 2 min and applied to the gel sample wells. Electrophoresis was carried out at a constant current of 35 mamp. Gels were stained for protein in a solution that contained 0.2% (w/v) Coomassie blue, 50% (v/v) methanol, and 7% (v/v) glacial acetic acid for 30 min, and were destained in 20% methanol, 7% acetic acid.

The room temperature absorption spectrum of Cyt b_{560} was recorded in a solution of 100 mM phosphate buffer (pH 7.5) that contained 2 mM succinate, plus or minus $Na_2S_2O_4$. Protein concentrations were determined by the method of Murphy and Keys (18), following the removal of Triton X-100 with BioRad SM-2 beads as described by Halloway (8).

EPR spectra were recorded with a Varian E-9 spectrometer[4] equipped with an Air Products variable temperature cryostat.

RESULTS AND DISCUSSION

Triton X-100 Solubilization of the Mitochondrial Inner Membrane. Solubilization of the mitochondrial membranes and subsequent purification of SDH in an active form requires the presence of a reductant (β-mercaptoethanol) and protection of the enzyme with succinate during fractionation. At a protein concentration of 4 mg/ml, Triton X-100 solubilization of SDH from the mitochondrial membrane was optimal at a concentration of 0.4%. Gurtubay *et al.* (7) reported similar findings for the fractionation of rat liver mitochondria with Triton X-100. They observed the highest specific activity of succinate:coenzyme Q oxidoreductase in the solubilized fraction when the membranes, at a protein concentration of 4 mg/ml, were solubilized with 0.5% Triton X-100. Because the amount of protein solubilized from rat liver mitochondrial membranes with 0.5% Triton X-100 was the same at 4, 20, and 37°C (6), the detergent fractionations in the present study were performed at 4°C.

Maintenance of SDH activity has previously been demonstrated to be temperature dependent (4, 25). Similar findings were obtained for the purified enzyme from plant tissues. The effect of storage temperatures of 4 and 25°C on the stability of succinate-PMS reductase activity in the purified preparation are shown in Figure 1. Within 1 h, 62% of the enzyme activity was lost when incubated at 25°C. If the enzyme was kept at 4°C, however, the enzyme activity remained constant for up to 5 h.

Purification of SDH from mammalian tissues has been demonstrated to require stringent anaerobic conditions during the isolation (4). To determine if similar anaerobic conditions were necessary with the plant enzyme, Triton X-100 solubilizations were conducted under both aerobic and anaerobic conditions. During the 20-min solubilization period, 22% of the succinate-PMS reductase activity was lost in the presence of O_2, whereas only 3% of the activity was lost when the solubilization was performed under argon. To minimize the loss of succinate-PMS reductase activity, solubilization of the membranes with Triton X-100 in subsequent preparations was performed under argon for 20 min.

Characterization of the Succinate-DPIP Reductase. The pH profile for the succinate-DPIP reductase activity of the SDH from mung bean mitochondria is presented in Figure 2. A pH optimum between 7.0 and 7.5 was obtained from both mung bean and soybean succinate-DPIP reductase with the activity dropping rapidly on either side of the optimum. Attempts to determine the pH optimum for succinate-PMS reductase yielded artificially high values (pH 9.0–9.5) due to PMS autoxidation, and caution must therefore be used when monitoring the autoxidation of PMS. Hiatt (12) previously reported an optimum of 7.4 for *Phaseolus vulgaris* root SDH, while the enzyme from sweet potato tuber tissue had an optimum centered around pH 8.0 (11). Values that approach pH 8.0 are common for most mammalian SDH preparations (10, 25).

To determine the purity of the SDH obtained in this study,

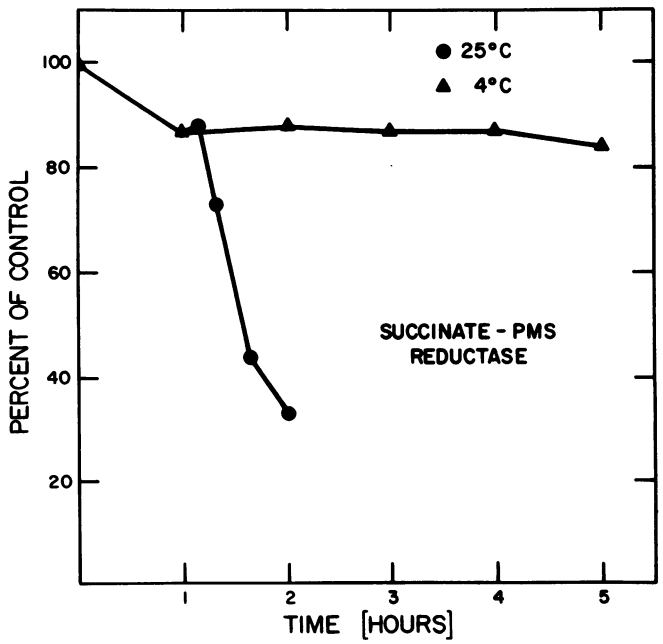

FIG. 1. The effect of storage temperatures of 4 and 25°C on the stability of purified succinate dehydrogenase. SDH assay mixtures contained 8.3 mM succinate, 30 mM Tricine-NaOH (pH 7.5), 1 mM PMS, and enzyme.

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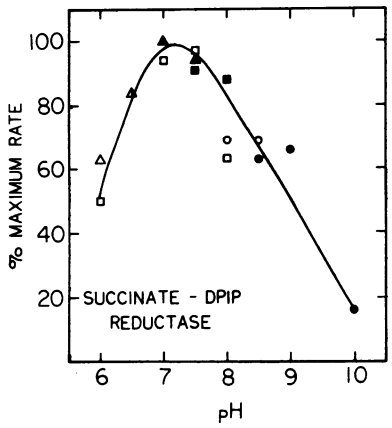

FIG. 2. The pH profile for succinate-DPIP reductase activity from mung bean mitochondria. Assay mixtures contained 2.0 mM succinate (neutralized), 50 μM DPIP, enzyme, and either 25 mM Tricine-NaOH (○), 25 mM sodium phosphate (□), 25 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid (●), 25 mM Hepes (■), 25 mM piperazine-*N*,*N*′-*bis*(2-ethanesulfonic acid) (▲), or 25 mM Mes (△) buffer at various pH adjustments.

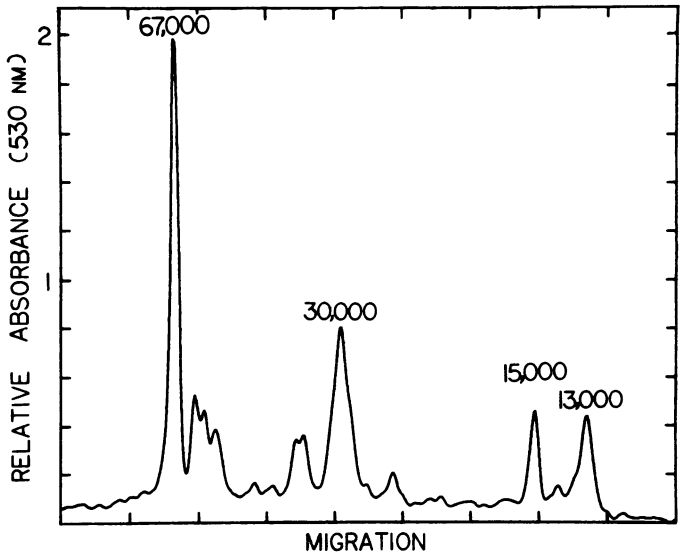

FIG. 3. A gel densitometric tracing of the polypeptide banding patterns for soybean succinate dehydrogenase following SDS, electrophoresis on a 12 to 16% polyacrylamide gel. The mol wt are given for the four polypeptides whose staining intensities suggest equimolar concentrations.

SDS-polyacrylamide gel electrophoresis was performed. A gel densitometric tracing of the peptide banding patterns for the soybean SDH fraction is shown in Figure 3. The preparation was enriched in two polypeptides with apparent mol wt of 67,000 and 30,000 D. These polypeptides migrated with mol wt similar to those reported for the two subunits from both mammalian and yeast SDH (1, 4, 10, 25). The peptides that migrated with mol wt between 67,000 and 30,000 D in the SDH preparation may represent contamination with components of complex III based upon comparison with densitometric tracings of mammalian SDH preparations (complex II) that contained contaminating polypeptides of similar mol wt (3). Two additional polypeptides appeared having staining intensities that suggested molar concentrations comparable to the two subunits of SDH. These polypeptides, of 15,000 and 13,000 D, migrated with mol wt similar to those reported as components of mammalian complex II. One of these latter polypeptides has been shown to contain a Cyt b_{560} (9) and the second has been suggested to be responsible for conferring

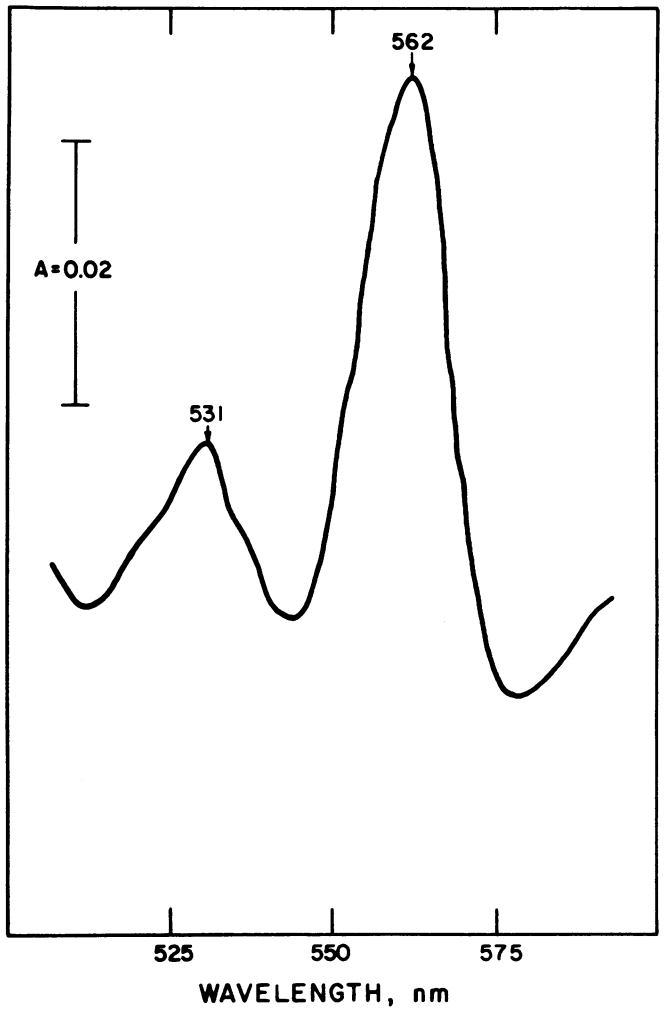

FIG. 4. The absorption difference spectrum of the dithionite-reduced minus oxidized succinate dehydrogenase preparation at ambient temperature. The SDH was dissolved in 100 mM sodium phosphate buffer (pH 7.5) that contained 2 mM succinate, plus or minus $Na_2S_2O_4$.

ubiquinone reductase activity to complex II (29). The SDH recently isolated from sweet potato tubers showed major bands at 65,000 and 26,000 D plus a variable band in the 45,000/55,000 D range (11). The sizes of the former two bands compare favorably with those found in the present preparation from soybean.

Because of the similarity in polypeptide patterns between our partially purified SDH and the complex II from mammalian mitochondria, experiments were performed to determine if Cyt b_{560} was present in our SDH fraction. The absorption difference spectrum of the dithionite-reduced minus oxidized SDH preparation at room temperature is shown in Figure 4. The resulting difference spectrum is clearly that of a *b*-type Cyt showing an α-band maximum at 562 nm. Although not shown in this figure, a Soret maximum at 430 nm was also detected. The absorption maxima for the plant Cyt are similar to those reported by Hatefi and Galante (9) for Cyt b_{560} from complex II beef heart mitochondria. They reported an α-peak maximum, room temperature, at 560 nm and a Soret peak maximum at 424 nm.

Analysis of mammalian complex II revealed that in the presence of the two low mol wt polypeptides, SDH interacted with Q analogs and complex III and, consequently, transferred electrons to Cyt *c* via an antimycin-sensitive pathway (29). The combined mixture of the two low mol wt polypeptides and the SDH closely resembled complex II in reactivity with PMS and DPB, in the characteristics of TFF inhibition of these reactions, and in relative

stabilization of the dehydrogenase (1). The SDH fraction isolated in the present study did not donate electrons to DPB and failed to show sensitivity to TTF using the PMS reductase assay. The inability of the SDH preparation to donate electrons to DPB is not necessarily surprising because in an earlier study with mammalian SDH, concentrations of Triton X-100 as low as 0.5% were reported to inhibit succinate-DPB activity by as much as 50% (1). In our isolation procedure, the concentration of Triton X-100 in the ammonium sulfate precipitate greatly exceeded the 0.5% concentration used in the earlier study.

Another study of the enzymic activity of mammalian complex II demonstrated that succinate-DPIP reductase activity has the same properties as succinate-ubiquinone reductase (31). Because soluble SDH does not react with DPIP, whereas the membrane-bound enzyme catalyzes TTF-sensitive reduction of DPIP by succinate, it has been suggested that DPIP reduction is mediated by ubiquinone (31). Our SDH fraction also reduced DPIP; however, the activity was not sensitive to TTF inhibition at concentrations as high as 2 mM. The inability of TTF to inhibit the succinate-DPIP reductase activity of our preparation again may in part be attributed to the presence of Triton X-100 in the final fraction.

EPR spectroscopy was also used to characterize the SDH preparation. In mammalian SDH, iron-sulfur centers S-1 and S-2 both give ferredoxin-type EPR signals in their reduced state (21), whereas center S-3 produces a HiPIP-type EPR signal when oxidized (20). The EPR signals from centers S-1 and S-2 both show rhombic symmetry and have very similar g-values (g_z = 2.03, g_y = 1.93, g_x = 1.91). The two centers differ in their reduction potentials (S-1 is succinate reducible, S-2 requires dithionite) and in their apparent relaxation rates (21). Center S-3 produces a relatively symmetric EPR signal centered around g = 2.01 with a peak to trough width of about 25 G (20). EPR signals associated with all three iron-sulfur centers found in mammalian SDH have been reported in plant mitochondria (22, 23).

Figure 5 shows the EPR spectra obtained with the SDH preparation from mung bean mitochondria under different redox conditions. Addition of excess (2.5 mM) ferricyanide led to the appearance of a symmetric EPR signal centered at g = 2.01 and having a peak at g = 2.02 and a trough at g = 2.00 (Fig. 5A). The signal has a peak to trough width of 27 G and rapidly decreased in intensity as the temperature was raised above 12 to 15 K. The above properties coincide with those reported previously for center S-3 from both mammalian (20) and higher plant sources (23).

The plant SDH preparation, therefore, appears to contain an intact iron-sulfur center S-3, at least as judged by the appearance of its EPR signal. The marked loss of signal amplitude above 15 K suggests that this signal is not caused by an EPR signal associated with contaminating aconitase. Aconitase gives a similar HiPIP-type EPR signal which is readily observed between 15 and 25 K (24). In isolated mammalian SDH, the EPR signal of center S-3 is extremely susceptible to breakdown in the presence of oxidants such as ferricyanide or even O_2. Further, modification of center S-3, as indicated by the lack of the EPR signal, correlated with the inability of the SDH preparation to reconstitute succinate-Cyt *c* reductase activity when combined with either soluble Cyt bc_1 complex or alkaline-treated submitochondrial particles (20). No attempts at reconstitution were made in the current study, but given the lack of quinone reductase activity, it seems unlikely that the plant preparation would be active in reconstitution experiments even though the HiPIP-type EPR signal is present. Ohnishi *et al.* (20) noted that in some mammalian SDH preparations with diminished reconstitution ability, a low field peak began to appear in the EPR spectrum at g = 2.03. Whereas the peak shown in Figure 5A is at g = 2.02, this peak was shifted closer to g = 2.03 in some preparations.

Figure 5B shows the EPR spectrum that resulted from the addition of dithionite to the mung bean SDH preparation. This leads to the appearance of an EPR signal with a low-field component at g = 2.027 and a large high-field resonance centered at g = 1.93. These two g-values correspond closely to those reported previously for iron-sulfur centers S-1 and S-2 from both plant (22) and animal (21) sources. The third g-value associated with centers S-1 and S-2 in mammalian SDH is generally reported to be at g = 1.91 to 1.92 (21) and was not seen in any of the preparations. The third g-value did not appear even when the slight power saturation and modulation broadening effects associated with Figure 5B were eliminated. High values of microwave power and modulation amplitude were required to generate reasonable EPR spectra given the low concentrations of iron-sulfur centers present in the samples. A weak trough at g = 1.89 appears as a third feature in the EPR spectrum of the reduced preparation, but this g-value is much too low to be associated with the high-field feature of SDH. In spite of the shortcomings, these results clearly indicate that iron-sulfur centers with EPR spectra similar to those reported previously are present in the plant SDH preparation.

Finally, it should be pointed out that there was no appearance of a ferredoxin-type EPR signal in the resting state enzyme even though the preparation was maintained in the presence of 2 mM succinate. Further, in some, though by no means all, preparations, the HiPIP signal was present in the resting enzyme. Succinate should have reduced both centers S-1 and S-3 which would lead

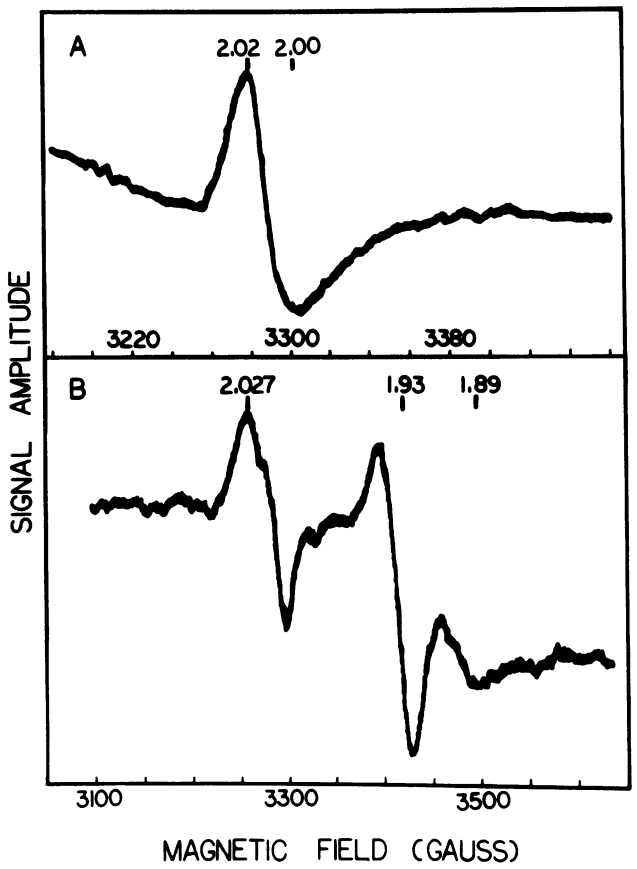

FIG. 5. EPR spectra of mung bean succinate dehydrogenase under different redox conditions. Enzyme was prepared as described in "Materials and Methods." A, Ferricyanide was added to the sample to a final concentration of 2.5 mM, immediately frozen, and maintained in liquid N_2 until EPR measurements were made. B, As in A, except the sample was reduced with excess sodium dithionite. EPR operating conditions were: modulation frequency, 100 kHz; microwave frequency, 9.24 GHz; and as follows: A, 40 mw power at 10 K with 20 G modulation amplitude; B, 10 mw power at 15 K with 20 G modulation amplitude.

to the appearance of roughly half the total ferredoxin-type EPR signal and no HiPIP signal. Such was not the case, however, for reasons that currently are not clear. Interestingly, the addition of 1 mM KCN to the resting SDH preparation resulted in the loss of most of the HiPIP signal and the appearance of a ferredoxin-type signal equivalent to about 60% of the amplitude present in the dithionite-treated enzyme (data not shown).

Inhibition of SDH by malonate and other dicarboxylate anions is the classic example of competitive enzyme inhibition (5). The competitive inhibitor malonate resembles succinate in having two ionized carboxyl groups at pH 7.0; however, SDH is unable to dehydrogenate malonate. Competitive inhibitors of SDH include D-chlorosuccinate, D-methyl succinate, malonate, methylene succinate, maleate, acetoacetate, OAA, bicarbonate, formate, glycolate, and glyoxylate (5). Inhibition constants for both OAA and malonate were determined with mung bean and soybean SDH. Mung bean SDH exhibited inhibition constants of 0.34 μM for OAA and 21.8 μM for malonate, whereas soybean SDH exhibited constants of 1.15 μM for OAA and 24.6 μM for malonate. These inhibition constants are comparable to the 1.5 μM for OAA and 18 μM for malonate reported for mammalian SDH (5). OAA inhibition of succinate oxidation in the present study supports the data from mammalian SDH analyses which demonstrate that OAA is the more potent of the two competitive inhibitors. OAA regulation of SDH activity may function *in vivo* because tightly bound OAA has been demonstrated to be in most SDH preparations in amounts equimolar to the deactivated fraction (2).

In summary, we have reported a procedure for the isolation of an SDH-containing fraction from higher plants that uses limiting amounts of mitochondrial starting material. Judging from the polypeptide pattern and by comparison with various preparations previously isolated from mammalian sources, this purified fraction should more properly be considered a complex II preparation rather than purified SDH *per se*. Not unexpectedly, the spectral and kinetic properties of the plant SDH do not differ significantly from those of SDH from mammalian and yeast sources. Additional fractionation studies using plant mitochondria are required to determine how similar, or different, the remaining plant mitochondrial electron transfer complexes are to those from nonplant sources.

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