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Selection of Succinic Dehydrogenase Mutants of *Neurospora crassa*

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A method is described which permits the selection of mutants of *Neurospora crassa* that are deficient in succinic dehydrogenase activity. The method relies on the observation that succinic dehydrogenase-deficient strains fail to reduce the dye nitrotetrazolium blue when overlaid with the dye in the presence of succinate and phenazine methosulfate. Wild-type colonies reduced the dye and turned blue, whereas mutant colonies remained colorless. In this communication we present studies of a mutant, SDH-1, isolated by this method. The mutant had 18% of the succinic dehydrogenase activity of the parent strain used in the mutation experiments as determined from the ratio of V_{max} activities obtained from Lineweaver-Burk plots. The SDH-1 mutant segregated in a Mendelian manner when back-crossed to its parent strain. Succinate oxidase activity in SDH-1 was low and was markedly inhibited by adenosine 5'-diphosphate. The succinate oxidase activity of the parent strain was high and was not affected by the presence of adenosine 5'-diphosphate.

The enzyme succinic dehydrogenase (EC 1.3.99.1) is the only component of the citric acid cycle that is firmly bound to the mitochondrial inner membrane. It has been purified from beef heart mitochondria (2) and from chromatophores from *Rhodospirillum rubrum* (3) and in both instances consists of two polypeptide chains. The enzyme from beef heart has subunits of 26,000 and 70,000 molecular weight, whereas the enzyme from *R. rubrum* has subunits of 25,000 and 60,000. Both enzymes contain covalently bound flavin as well as iron-sulfur centers. A recent review of biochemical and biophysical properties of succinic dehydrogenase is given in reference 11.

Soderberg et al. (13) have reported the selection of mutants defective in succinic dehydrogenase activity from Chinese hamster fibroblasts. Mitochondria isolated from these cells had less than 5% of the succinic dehydrogenase activity found in wild-type cells. The absence of succinic dehydrogenase activity in whole cells of these mutants could be demonstrated by a histochemical staining technique, using nitrotetrazolium blue as an indicator. Cells with wild-type activity reduce the due and turn blue, whereas mutant cells remain colorless.

Our laboratory has been interested for some time in the assembly of the mitochondrial inner membrane in the fungus *Neurospora crassa*. Our approach has been to select for mutants that are defective in mitochondrial respiratory chain components and then to attempt to analyze these mutants biochemically. In this communication we report the selection of mutants deficient in succinic dehydrogenase activity using the inability to reduce nitrotetrazolium blue as a selective screen. Mutants isolated in this manner are deficient in succinic dehydrogenase activity when the enzyme is measured either in crude extracts or in activated submitochondrial particles. A preliminary genetic analysis of one of these indicates that the defect is due to mutation in a nuclear gene.

MATERIALS AND METHODS

Strains. The ANT-1 strain of *N. crassa* used in these studies has been described previously (5, 8). Crosses were carried out on corn meal agar as described by Davis and DeSerres (4).

Enzyme assays. Succinic dehydrogenase was assayed using the reduction of phenazine methosulfate (PMS) and 2,6-dichloroindophenol as described by Baginsky and Hatefi (1). Submitochondrial particles prepared according to Edwards and Unger (8) were activated by incubation at 38°C in the presence of 20 mM succinate for 15 min before assay. Succinate oxidase and NADH oxidase activities were measured on isolated mitochondria as described by Edwards et al. (7).

Selection of succinic dehydrogenase mutants. Conidia from ANT-1 were mutagenized with UV light and submitted to inositol-less death on acetate medium for 2 to 4 days at 30°C as described by Edwards et al. (6). Survivors of the inositol-less death were plated on petri dishes containing Vogel medium N (15), sucrose (2%), and inositol (50 μg/ml). The colonies were grown for 2 days at 30°C and overlaid with

a solution containing 137 mM NaCl, 5 mM KCl, 0.7 mM NaH_2PO_4, 25 mM Tris-hydrochloride (pH 7.3), 0.5% agar, 0.55 mM PMS, 0.40 mM nitrotetrazolium blue, and 15 mM succinate (13). The overlaid plates were incubated at 37°C for 20 min and scored for nitrotetrazolium blue reduction. Colonies which failed to reduce the dye under these conditions were retained and tested further for succinic dehydrogenase activity. Control experiments carried out with ANT-1 cells overlaid as above with succinate and oxaloacetate (19 mM), a competitive inhibitor of succinic dehydrogenase, failed to reduce the dye, indicating that the procedure does, in large part, measure succinic dehydrogenase activity. The nitrotetrazolium blue-negative colonies isolated as above were tested for succinic dehydrogenase activity in crude cell extracts. Cultures of the presumptive mutants were grown for 15 h at 30°C and ground with alumina as described by Edwards and Unger (9). The extracts were clarified by centrifugation at 1,000 × *g* for 5 min, and the supernatant was activated by incubation at 38°C in the presence of 20 mM succinate for 15 min. Extracts which displayed a succinic dehydrogenase activity of 40% of the ANT-1 level or less were retained, and the activity of the enzyme was measured in submitochondrial particles. Submitochondrial particles were prepared as described by Edwards and Unger (9) and activated in the presence of 20 mM succinate for 15 min at 38°C before assay.

Miscellaneous procedures. Protein was measured by the method of Lowry et al. (12), using bovine serum albumin as a standard. Mating type was determined as described by Davis and DeSerres (4).

RESULTS

Selection of presumptive mutants. Colonies which failed to reduce nitrotetrazolium blue as described in Materials and Methods were retained and transferred to slants of appropriately supplemented media. Liquid shaking cultures were prepared by inoculating conidia from individual isolates into Vogel medium N, supplemented with inositol and sucrose and grown for 15 h at 30°C. Crude extracts were prepared by grinding mycelia with alumina as described by Edwards and Unger (9). The extracts were assayed for succinic dehydrogenase activity, and the results were compared with those obtained with ANT-1. Isolates which had a specific activity of 40% or less of the ANT-1 value (micromoles of dichloroindophenol reduced per minute per milligram) were considered presumptive mutants and were retained for further study. Approximately 10% of the nitrotetrazolium blue-negative colonies that we isolated fell into this category.

Characterization of succinic dehydrogenase activity of submitochondrial particles. Submitochondrial particles were prepared from ANT-1 and from the presumptive succinic dehydrogenase mutants according to the method of Edwards and Unger (9). Cultures were grown for 15 h at 30°C and assayed as described in Materials and Methods. Figure 1 shows the succinic dehydrogenase activity for submitochondrial particles from ANT-1 and a presumptive succinic dehydrogenase mutant, SDH-1, as a function of the concentration of PMS. The activities in both sets of particles increases as the concentration of PMS increases, although the activity in the SDH-1 particles is greatly reduced compared with ANT-1. Both sets of particles showed an optimal succinic dehydrogenase activity at 1.63 mM PMS, and this concentration of PMS was used in all subsequent experiments. This value compares well with the previously reported PMS optimum for beef heart succinic dehydrogenase reported by Davis and Hatefi (2).

Figure 2A shows succinic dehydrogenase activity in submitochondrial particles from ANT-1 and SDH-1 as a function of succinate concentration using the optimal concentration of PMS. The activity of the ANT-1 particles is again much greater than that of the SDH-1 particles at all concentrations of succinate used. The optimal concentration of succinate for each set of particles is approximately 10 mM. Figure 2B shows a Lineweaver-Burk plot of the same data. The data extrapolate to the same K_m value, which is 2.04 mM. The V_{max} for SDH-1 is 0.13 μmol/min per mg as compared with a value of 0.71 for ANT-1.

Stability of the enzyme. We noted in our preliminary studies of succinic dehydrogenase in submitochondrial particles that the activity of

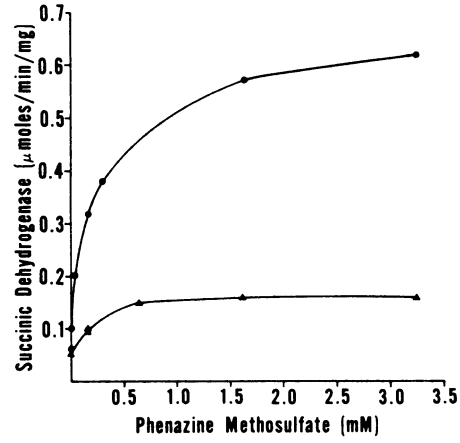

FIG. 1. *Succinic dehydrogenase activity in submitochondrial particles as a function of PMS concentration. Submitochondrial particles were prepared from 15-h cultures as described. The particles were activated in the presence of 20 mM succinate for 15 min before assay and assayed according to Baginsky and Hatefi (1). Symbols:* (●) *particles from ANT-1;* (▲) *particles from SDH-1.*

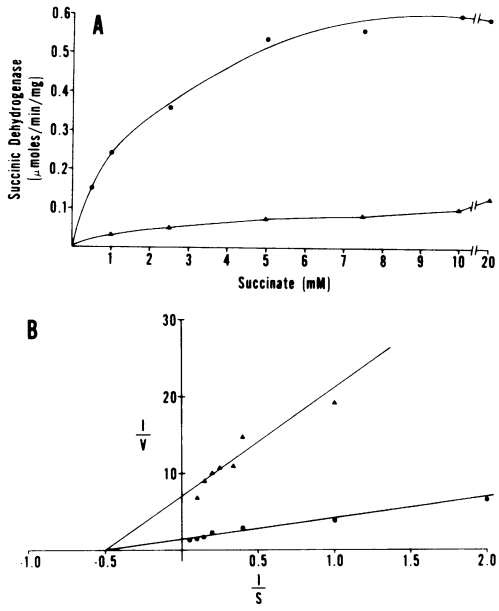

FIG. 2. *(A) Succinate dehydrogenase activity in submitochondrial particles as a function of succinate concentration. Particles were prepared and assayed as in Fig. 1. Symbols are the same as in Fig. 1. The concentration of PMS used was 1.63 mM. (B) Lineweaver-Burk plot of the data in (A). V is micromoles of dichloroindophenol reduced per minute per milligram. S is micromolarity.*

the enzyme was labile and decayed with a half-life of 3 to 4 h when incubated at 38°C. Figure 3 shows the succinic dehydrogenase activity of particles from ANT-1 in the presence and absence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The figure shows that PMSF greatly protects the activity in particles from ANT-1 as compared with particles prepared in the absence of PMSF. The figure also shows that succinic dehydrogenase activity in particles from SDH-1 decline in activity with a half-life of 3 to 4 h even in the presence of PMSF. Succinic dehydrogenase activity in these particles is much more labile than in comparable particles from ANT-1.

Mitochondrial respiration. Table 1 shows the respiration rates of mitochondria isolated from ANT-1 and SDH-1. The rate of oxidation of NADH is elevated in SDH-1 as compared with ANT-1. Increased mitochondrial respiration is a phenomenon that was also recently observed in an oligomycin-resistant mutant of *Neurospora* (D. L. Edwards and B. W. Unger, submitted for publication). The cause of the increased respiration is not known, but it is speculated to be a regulatory response due to defective mitochondrial function. Succinate oxidase activity is markedly decreased in SDH-1, as would be expected for a mutant deficient in succinic dehydrogenase activity. We were surprised to observe that the low succinic oxidase activity of SDH-1 was inhibited markedly by the addition of ADP to the medium (state 3 respiration). This inhibition does not occur with ANT-1 mitochondria.

Genetic analysis. Data from a random spore analysis of succinic dehydrogenase activity in

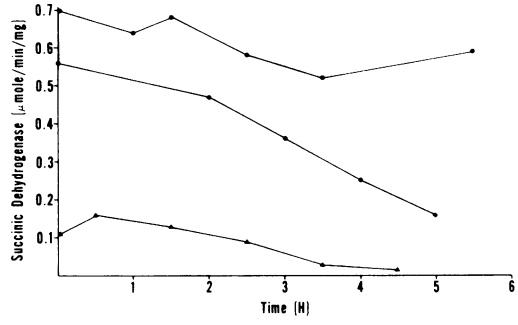

FIG. 3. *Succinic dehydrogenase activity as a function of time. Submitochondrial particles were prepared as in Fig. 1 and incubated at 38°C. The particles were assayed at various times as described (1). Symbols: (●) particles from ANT-1 prepared in the presence of 2 mM PMSF; (○) particles from ANT-1 prepared without PMSF; (▲) particles from SDH-1 prepared in the presence of 2 mM PMSF.*

TABLE 1. *Mitochondrial respiration rates*[a]

Strain	Substrate	Respiration rate (μmol/min per mg)	
		State 4	State 3
ANT-1	NADH	0.074 ± 0.001 (6)	0.130 ± 0.004 (6)
SDH-1	NADH	0.120 ± 0.005 (8)	0.217 ± 0.0001 (8)
ANT-1	Succinate	0.128 ± 0.009 (9)	0.134 ± 0.003 (8)
SDH-1	Succinate	0.047 ± 0.002 (7)	0.017 ± 0.001 (7)

[a] Mitochondria were isolated from 15-h cultures by the method of Edwards and Unger (8). The concentrations of substrates used were: NADH, 0.5 mM; succinate, 10 mM. The concentration of ADP added to induce state 3 respiration was 5 mM. Rates are presented as the mean ± standard error. The numbers in parentheses are the number of determinations made.

the progeny of a cross between ANT-1 and SDH-1 are shown in Table 2. Progeny with succinic dehydrogenase activity similar to that of either ANT-1 or SDH-1 are present in approximately equal numbers. This result indicates that the SDH-1 defect is due to a mutation in a nuclear gene. This is consistent with previous observations in other organisms that succinic dehydrogenase activity is not decreased when cells are incubated in the presence of ethidium bromide to inhibit mitochondrial transcription (14) and that synthesis of the enzyme is carried out on cycloheximide-sensitive ribosomes (13), a general property of nuclear gene products.

DISCUSSION

The method described here appears to us to be a reasonable one for detecting mutants of *Neurospora* deficient in succinic dehydrogenase activity. In this communication, we present data for a single mutant, SDH-1, that was isolated using this procedure. We have examined in detail the succinic dehydrogenase activity of three additional mutants isolated in our initial search, and all of these vary from ANT-1 both in the value of the Michaelis constant, K_m, and in V_{max}. The kinetic parameters of ANT-1 and of the mutants are shown in Table 3. In the absence of a genetic analysis of these mutants, however, we are unable to determine which mutants, if any, are allelic. A complete genetic analysis of these mutants is currently underway in our laboratory and will be published subsequently.

TABLE 2. *Succinic dehydrogenase activity in the progeny of a cross of SDH-1 to ANT-1*[a]

Strain	Sp act (μmol/min per mg of protein)	W/M
ANT-1	0.602	W
SDH-1	0.145	M
P-1	0.476	W
P-2	0.102	M
P-3	0.069	M
P-4	0.051	M
P-5	0.047	M
P-6	0.068	M
P-7	0.470	W
P-8	0.665	W
P-9	0.491	W
P-10	0.389	W
P-11	0.531	W
P-12	0.295	W
P-13	0.101	M
P-14	0.068	M
P-15	0.071	M
P-16	0.426	W
P-17	0.498	W
P-18	0.119	M
P-19	0.393	W
P-20	0.428	W

[a] Succinic dehydrogenase activity was measured on activated submitochondrial particles prepared from 15-h cultures, using 20 mM succinate as the substrate. The designations P-1 to P-20 refer to individual progeny from the cross. The column W/M represents our assignment of the activity of the isolate as either wild type (W) or mutant (M).

The data in Table 2 indicate that the gene responsible for the SDH-1 defect segregates in a Mendelian manner in a cross to ANT-1 and is therefore of nuclear origin. The cross was carried out in such a way that if the SDH-1 mutation had been extrachromosomal in nature, all of the progeny would have displayed the phenotype of SDH-1. When the progeny from the cross in Table 2 were scored for mating type, all of the mutant progeny were determined to be of mating type A. We then selected three additional random progeny of mating type A and found that all of these had mutant succinic dehydrogenase activity. This is a preliminary indication that SDH-1 is linked to the mating type locus which is on linkage group I. Mapping studies of SDH-1 are continuing in our laboratory.

The inhibition of succinate oxidase by ADP in SDH-1 mitochondria (Table 1) is a surprising result because mitochondrial respiration is generally stimulated by the state 4 to state 3 transition. Gutman et al. (10) have reported that succinic dehydrogenase activity in rat liver mitochondria is inhibited by the addition of ADP, and these authors have concluded that the level of reduction of coenzyme Q has a regulatory effect on the activity of succinic dehydrogenase, with greater reduction of coenzyme Q resulting in greater succinic dehydrogenase activity. A possible explanation of our results, in view of the work of Gutman et al., would be that the lowered rate of succinic dehydrogenase activity in SDH-1 becomes even further reduced by the addition of ADP so as to become the rate-limiting step in the reaction. There is sufficient succinic dehydrogenase activity in ANT-1 so that this phenomenon does not occur.

TABLE 3. *K_m and V_{max} parameters of succinic dehydrogenase mutants*[a]

Strain	K_m (mM)	V_{max} (μmol/min per mg)
ANT-1	2.04	0.71
SDH-1	2.04	0.13
SDH-160	1.17	0.22
SDH-163	1.37	0.16
SDH-127	13.30	0.08

[a] K_m and V_{max} parameters were determined graphically as in Fig. 2B. Measurements were made on activated submitochondrial particles prepared from 15-h cultures in the presence of PMSF.

In addition to these observations, we have found that succinic dehydrogenase activity, as measured by the PMS assay in cyanide-poisoned submitochondrial particles, is inhibited 40 to 60% by the following compounds: ATP, GTP, UTP, CTP, IDP, and AMP. The concentration of each of these compounds was 15 mM. We have noted that ATP and IDP are reported to be activators of mammalian succinic dehydrogenase in submitochondrial particles (11). Because these compounds markedly inhibit succinic dehydrogenase activity in submitochondrial particles from *Neurospora*, it seems very likely to us that the regulation of this activity occurs by a different mechanism than that reported for the mammalian enzyme. Studies along these lines are continuing in our laboratory.

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