A SUCCINIC DEHYDROGENASE ACTIVITY IN "MESOSOMES" OF NEUROSPORA CRASSA

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A succinic dehydrogenase (SDH) activity can be demonstrated histochemically in association with the plasma membranes and "mesosomes" of Neurospora crassa. This finding is consistent with the presence of a SDH activity in the microsomal fraction from N. crassa which is unlikely to be due to mitochondrial contamination. The role of this extra mitochondrial SDH activity is not certain.

Membranous structures resembling bacterial mesosomes have been reported to occur in electron micrographs of Neurospora crassa (1-3). The functional significance of these membranous structures, which are termed "mesosomes" for convenience, is not certain. Beck and Greenawalt (1) found that the number of these mesosomes varies during the growth of the culture and with changes in the composition of the culture medium. They suggested that these mesosomes play a role in biogenesis of mitochondria or in general cellular synthesis. Demonstration of mitochondrial components associated with these mesosomal membranes could serve as evidence for a mesosomal role in mitochondrial biogenesis. The present communication reports evidence for SDH associated with these mesosomal structures by histochemical methods. Consistent with this finding is the presence of a SDH activity in the microsomal fraction.

Materials and Methods

Conidia of wild type N. crassa were grown on Horowitz solid agar medium and mycelia in Vogel's liquid medium according to the procedure given in (4). Mycelia were harvested approximately 12 hours after inoculation, i.e., beginning of the exponential phase of growth. Two histochemical methods were employed to localize SDH activity in mycelia; 1) the potassium ferricyanide-copper sulfate technique of Ogawa et al. (5), and 2) the TNBT (tetra nitro blue tetrazolium

chloride) procedure given by Leene and van Iterson (6). Mycelia subjected to these histochemical reactions were either freshly harvested or prefixed for 10 minutes in cold 3% formaldehyde or glutaraldehyde (in 0.1 M phosphate buffer, pH 7.3). They were incubated in the reaction mixture for 30 minutes at 27°C with constant agitation. After incubation the mycelia were collected by filtration, washed, and fixed in formaldehyde or glutaraldehyde at room temperature for up to 12 hours. In some cases, the mycelia were postfixed for 3 hours in 2% OsO_4 (0.1 M phosphate buffer, pH 7.3). The fixed mycelia were routinely embedded in Araldite or Epon and thin sections examined without staining in an electron microscope.

Possible extra-mitochondrial SDH activity was assayed for in sub-cellular fractions of mycelia obtained by the technique given in Fig. 1. SDH activity in the fractions was determined by spectrophotometrically following the reduction of 2,6-dichlorophenolindolphenol (8). Protein concentration was

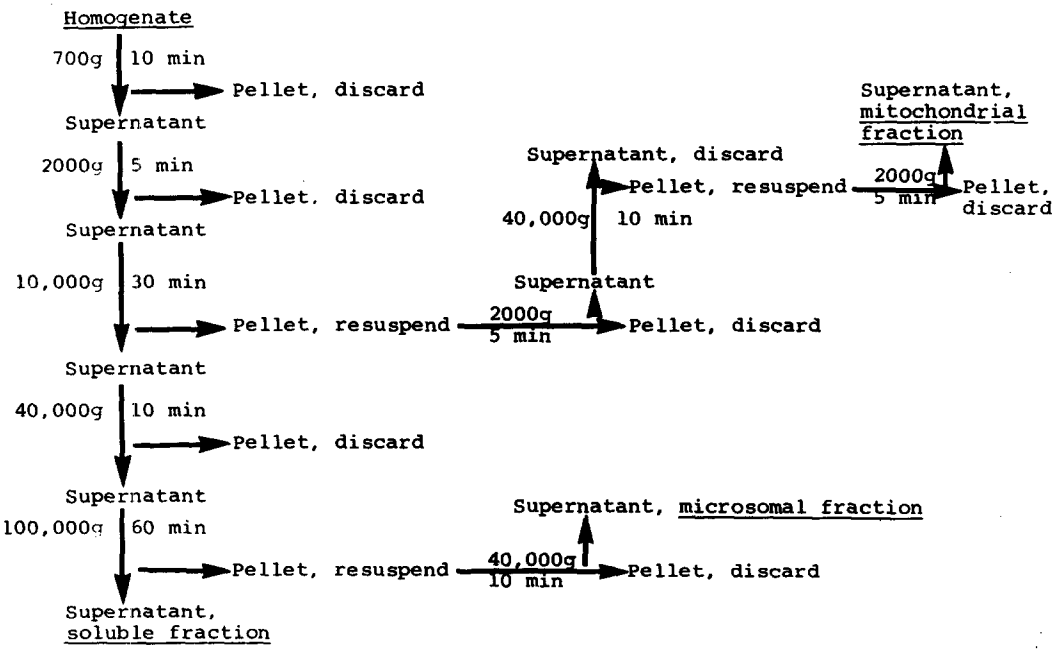

Fig. 1. Isolation method for obtaining mitochondrial, microsomal, and soluble fractions from N. crassa. The mycelia are homogenated by grinding with sand in a medium consisting of 0.25 M sucrose, 0.1 % BSA, 0.003 M EDTA, 0.05 M tris-HCl at pH 7.5, and then differentially centrifuged as indicated.

Exp.	Mitochondrial SDH Activity	Microsomal SDH Activity	% SDH Activity As Microsomal	Mitochondrial F_1 ATPase Activity	Microsomal F_1 ATPase Activity	% F_1 ATPase as Microsomal
1	0.21	0.010	9.40	-	-	-
2	0.20	0.030	9.60	-	-	-
3	0.17	0.020	8.40	-	-	-
4	0.24	0.016	6.25	-	-	-
5	0.25	0.018	6.70	5.43	0.21	2.3
6	-	-	-	6.81	0.09	0.9
7	0.24	0.030	7.50	5.80	0.10	1.1
Averages	0.22	0.021	7.97	6.01	0.13	1.4

Fig. 2 Summary of SDH and oligomycin sensitive (F_1) ATPase activities in fractions from N. crassa. SDH activity was determined at 37°C in a solution consisting of in one ml: 10.0 µmoles PO_4, pH7.4; 500 µg BSA; 2.0 µmoles KCN; 20 µg 2,6-dichlorophenolindolphenol; 10 µmoles Na succinate; and 100 µg mitochondrial protein or 500 µg microsomal protein. Succinate was added separately to initiate the reaction, and the reaction was followed by observing the reduction in O.D. at 600 mµ of the assay mixture every 30 seconds against a blank identical to the sample except succinate was deleted. F_1 ATPase was assayed at 37°C in a medium containing in one ml: 250 µmoles sucrose; 10 µmoles $MgCl_2$; 50 µmoles tris-HCl, pH7.5; 1.0 µmole ATP; 10 µg oligomycin; and 0.5 mg mitochondrial protein or 1.0 mg microsomal protein. The reaction was followed by sampling at intervals and determining the amount of Pi released (7). SDH activity units equal reduction in O.D. at 600 mµ/min/mg protein, and F_1 ATPase activity units equal µg Pi released/min/mg protein.

determined by the method of Lowry et al. (9) using crystalline BSA as a standard. Purity of the fractions was checked by electron microscopical examination of negatively stained (2% PTA pH 7) samples and by assaying for oligomycin sensitive (F_1) ATPase (Fig. 2).

Results

Deposits of electron opaque copper ferrocyanide are observed on the mitochondrial membranes, mesosomes and the plasma membrane in micrographs of sections of fresh mycelia incubated in complete ferricyanide medium (Fig. 3). Not all mitochondria show the same degree of electron dense deposit, some reveal a uniform deposit, while others show a patchy deposit, and still others show no deposit at all. The plasma membrane and mesosomal membranes also show a

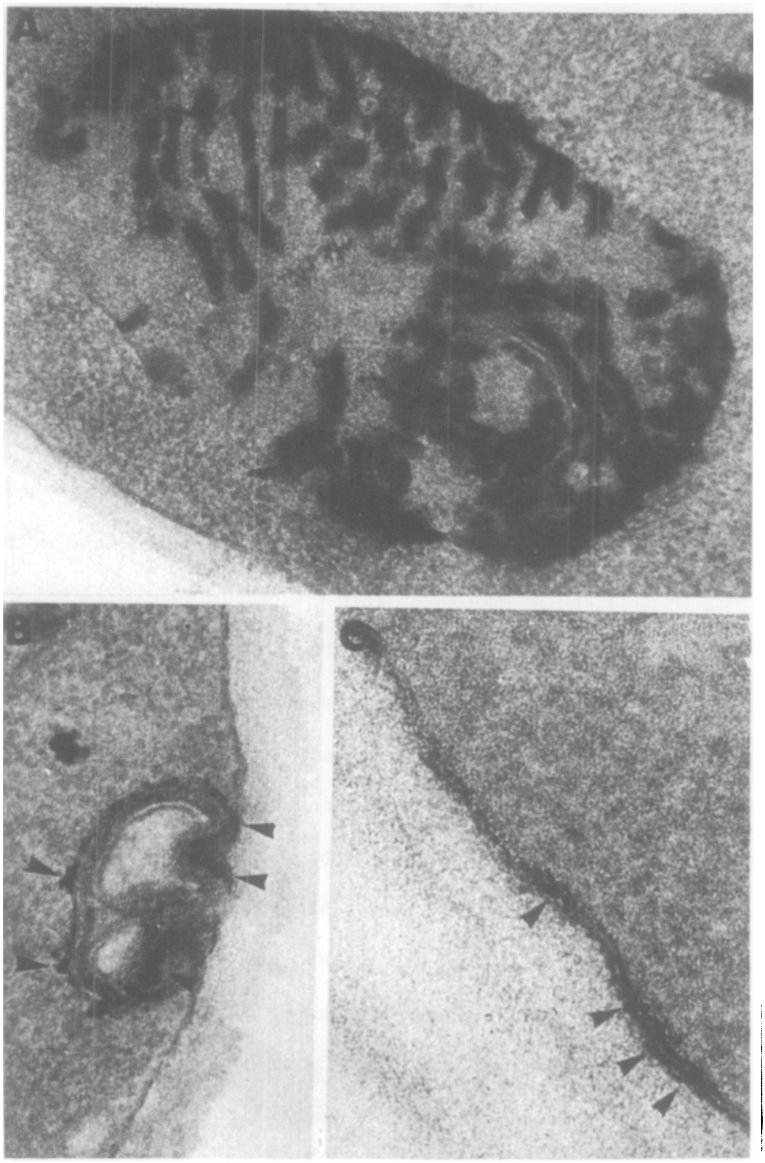

Fig. 3. Electron micrographs of N. crassa showing dense reaction product resulting from ferricyanide reaction for succinic dehydrogenase in a mitochondrion (A), mesosome (B), and plasma membrane (C). Arrows in B and C indicate dense reaction product. A, x 90,000; B, x 110,000; C, x 223,000.

similar heterogeneous response. Sections of fresh mycelia incubated in ferricyanide medium without succinate show a sparse deposit on mitochondrial membranes and no deposit on the plasma and mesosomal membranes. No electron dense deposits are seen in sections of fresh mycelia incubated in medium to which malonate has

been added. In sections of mycelia prefixed before incubation in complete ferricyanide medium, a slight deposit is observed on the mitochondrial membranes, and no deposit on the mesosomes or plasma membrane. No deposit at all is seen in sections of prefixed mycelia incubated in ferricyanide medium from which either succinate has been omitted or to which malonate added.

Incubation of fresh mycelia in TNBT reaction mixtures gives results that are essentially similar to those obtained by incubation in ferricyanide medium. However, difficulty is experienced in recognizing the reaction product of the TNBT (formazan) in thin sections in the electron microscope, because of the low electron density of the formazan. Therefore, the ferricyanide technique is preferred, as the reaction product is easily recognized in electron micrographs.

Biochemical assays for SDH activity in fractions isolated from Neurospora mycelia show SDH activity in the microsomal fraction (Fig. 2). This activity is linear with time and protein concentration, and makes up about 8% of the total SDH activity (mitochondrial + microsomal). No SDH activity could be demonstrated in the soluble fraction. Electron microscopic examination of negatively stained samples of the microsomal fractions show a small amount of mitochondrial contamination. However, approximately 98% of the (F_1) ATPase activity is found in the mitochondrial fraction (Fig. 2) and less than 2% of the oligomycin sensitive ATPase activity can be detected in the microsomal fraction. These observations suggest that only about 25% of the SDH activity of the microsomal fraction could be due to mitochondrial contamination.

Discussion

The results of the histochemical investigations indicate the presence of an activity which oxidizes succinate and concomitantly reduces an electron acceptor (ferricyanide or TNBT). This activity is visualized in electron micrographs in association with mesosomes and the plasms membrane, as well as the mitochondrial membranes. The dependence of this activity on the

presence of succinate, and its inhibition by malonate, indicate that the activity is dependent upon the presence of a succinic dehydrogenase.

Assays of SDH activity in fractions from N. crassa mycelia show approximately 8% of the total SDH activity of a given volume of cell free extract to be associated with the microsomal fraction. Electron microscopical examination of the microsomal fraction, and the distribution of (F_1) ATPase activity in the fractions indicate that cross contamination of the microsomal fraction by mitochondrial material is low. The amount of mitochondrial contamination (about 2%) is considerably less than the amount of SDH activity (about 8%) found in the microsomal fraction. Therefore, it seems that mitochondrial contamination of the microsomal material could not account for the entire SDH activity observed in the microsomal fraction.

The extra-mitochondrial finding of SDH activity is not unique to Neurospora. Two different cytoplasmic succinic dehydrogenases (or fumarate reductases) have been demonstrated in yeast (10). McEwen et al. (11) have presented evidence for a unique nuclear SDH activity in calf thymus nuclei.

Riess (12) reported that the ferricyanide technique of Ogawa et al. (5) is not applicable to N. crassa because of inhibitory action of $CuSO_4$ and $K_3Fe(CN)_6$ on its SDH activity. He found 95% inhibition of tetrazolium reduction by succinate in intact mycelia in the presence of 3 μmoles/ml $CuSO_4$, the amount present in the ferricyanide medium. Riess also stated that, even after prolonged incubation in the ferricyanide medium, distinct granular reaction products are not observed. However, reaction product produced by incubation in ferricyanide medium is not discerned with the light microscope, but considerable amounts of electron opaque reaction product can be easily visualized in sections of mycelia processed for electron microscopy. Shepherd (13) reported that Cu^{++} is inhibitory to SDH of N. crassa in cell free extracts when methylene blue is used as the electron acceptor. This apparent contradiction from the results of present experiments in which reduction of ferricyanide in the presence of Cu^{++} has been observed in situ could result from

acceptance of electrons at different levels in the respiratory chain by the different acceptors. Singer and Kearney (14) indicate this possibility in their extensive discussion of SDH from various sources.

The significance of the extra-mitochondrial SDH activity observed in N. crassa is not certain but this activity could be related to biogenesis of mitochondria. Further investigations into the nature and characteristics of this activity are in progress.

Acknowledgement

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