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An antimycin-insensitive succinate–cytochrome *c* reductase activity in pure reconstitutively active succinate dehydrogenase

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Antimycin-insensitive succinate–cytochrome *c* reductase activity has been detected in pure, reconstitutively active succinate dehydrogenase. The enzyme catalyzes electron transfer from succinate to cytochrome *c* at a rate of 0.7 μmole succinate oxidized per min per mg protein, in the presence of 100 μM cytochrome *c*. This activity, which is about 2% of that of reconstitutive (the ability of succinate dehydrogenase to reconstitute with coenzyme ubiquinone-binding proteins (QPs) to form succinate-ubiquinone reductase) or succinate-phenazine methosulfate activity in the preparation, differs from antimycin-insensitive succinate–cytochrome *c* reductase activity detected in submitochondrial particles or isolated succinate–cytochrome *c* reductase. The K_m for cytochrome *c* for the former is too high to be measured. The K_m for the latter is about 4.4 μM, similar to that of antimycin-sensitive succinate-cytochrome *c* activity in isolated succinate–cytochrome *c* reductase, suggesting that antimycin-insensitive succinate-cytochrome *c* activity of succinate–cytochrome *c* reductase probably results from incomplete inhibition by antimycin. Like reconstitutive activity of succinate dehydrogenase, the antimycin-insensitive succinate-cytochrome *c* activity of succinate dehydrogenase is sensitive to oxygen; the half-life is about 20 min at 0 °C at a protein concentration of 23 mg/ml. In the presence of QPs, the antimycin-insensitive succinate-cytochrome *c* activity of succinate dehydrogenase disappears and at the same time a thenoyltrifluoroacetone-sensitive succinate–ubiquinone reductase activity appears. This suggests that antimycin-insensitive succinate–cytochrome *c* reductase activity of succinate dehydrogenase appears when succinate dehydrogenase is detached from the membrane or from QPs. Reconstitutively active succinate dehydrogenase oxidizes succinate using succinylated cytochrome *c* as electron acceptor, suggesting that a low potential intermediate (radical) may be involved. This suggestion is confirmed by the detection of an unknown radical by spin trapping techniques. When a spin trap, α-phenyl-*N*-*tert*-butylnitrone (PBN), is added to a succinate oxidizing system containing reconstitutively active succinate dehydrogenase, a PBN spin adduct is generated. Although this PBN spin adduct is identical to that generated by xanthine oxidase, indicating that a perhydroxy radical might be involved, the insensitivity of this antimycin-insensitive succinate–cytochrome *c* reductase activity to superoxide dismutase and oxygen questions the nature of this observed radical.

Abbreviations: DCIP, dichlorophenolindophenol; Q, ubiquinone; QP, coenzyme Q binding protein; PBN, α-phenyl-*N*-*tert*-butylnitrone; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

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Introduction

Succinate dehydrogenase, which is the first enzyme in the succinate oxidase region of the mitochondrial electron transfer chain, has been isolated and purified to homogeneity in several

laboratories [1–3]. Pure enzyme contains two protein subunits with molecular weights of 70000 and 27000, one mole FAD, 8 moles non-heme iron, and 8 moles of acid labile sulfide per mole of enzyme. The M_r 70000 subunit is an FAD containing protein; the M_r 27000 subunit is an iron sulfur protein. The identity and function of the iron sulfur clusters in succinate dehydrogenase have been the subject of extensive investigation [4–9], and significant refinements have been made recently [10–12]. The complete amino acid sequence of the M_r 27000 subunit [13], and the partial sequence of the FAD binding site in the M_r 70000 subunit [14] are available. The recent isolation of QPs [15], and other similar preparations [16,17], which converts succinate dehydrogenase into succinate–ubiquinone reductase, permits mechanistic studies concerning its interaction with a neighboring component. Much of the structural information on succinate dehydrogenase and succinate–ubiquinone reductase has been obtained through genetic manipulation of bacterial mutants [12].

Depending on the reagents and conditions employed in solubilization of succinate dehydrogenase from the membrane, two kinds of preparations result: reconstitively active and inactive forms. Both preparations are capable of catalyzing the oxidation of succinate to fumarate but they use different electron acceptors [18]. When prepared in the absence of succinate under aerobic conditions, the resulting enzyme can utilize only redox dyes, such as phenazine methosulfate, or high concentrations of ferricyanide as electron acceptors; this type of succinate dehydrogenase does not reconstitute with QPs to form succinate–ubiquinone reductase, i.e., use ubiquinone as an electron acceptor. When prepared in the presence of succinate under anaerobic conditions, the resulting enzyme can use not only phenazine methosulfate and high concentrations of ferricyanide but also low concentrations of ferricyanide as the electron acceptor [19]. In addition, it can reconstitute with QPs to form succinate–ubiquinone reductase, using ubiquinone as electron acceptor, or with soluble cytochrome b-c_1 complex (a QPs containing ubiquinol–cytochrome c reductase) to form succinate–cytochrome c reductase, using cytochrome c as electron acceptor. The reconstitutive activity of succinate dehydrogenase is sensitive to oxygen and is directly proportional to succinate-low K_m ferricyanide reductase activity [19]. When reconstitutively active succinate dehydrogenase is exposed to air at 0 °C for one hour, the reconstitutive and succinate-low K_m ferricyanide reductase activities disappear, whereas the succinate-high K_m ferricyanide and succinate–phenazine methosulfate reductase activities remain unchanged. The loss of reconstitutive activity is concurrent with the disappearance of a high potential iron-sulfur cluster (S-3) from the preparation [20]. The discovery of succinate-low K_m ferricyanide reductase activity and establishment of its correlation with the reconstitutive activity have simplified the reconstitutive activity measurement which otherwise requires QPs or cytochrome b-c_1 complex. However, the succinate-low K_m ferricyanide reductase assay has experimental difficulties because the enzyme is denatured rapidly under assay conditions.

Recently we found that reconstitutively active succinate dehydrogenase can catalyze electron transfer from succinate to cytochrome c without added QPs or soluble cytochrome b-c_1 complex. This succinate–cytochrome c reductase activity is antimycin insensitive, thus different from the succinate–cytochrome c reductase activity observed in submitochondrial particles, isolated succinate–cytochrome c reductase, or reconstituted succinate–cytochrome c reductase formed from reconstitutively active succinate dehydrogenase and soluble cytochrome b-c_1 complex. This antimycin-insensitive succinate–cytochrome c reductase activity is sensitive to oxygen and correlates with its reconstitutive activity, thus providing an easier way to assay the reconstitutive activity of isolated succinate dehydrogenase. It was later observed that succinylated cytochrome c instead of cytochrome c can also be used as electron acceptor, suggesting that a low potential radical is involved in the succinate dehydrogenase catalyzed electron transfer from succinate to cytochrome c. Herein we report the properties of this antimycin-insensitive succinate–cytochrome c reductase activity in pure succinate dehydrogenase, and the EPR detection of the unidentified radical.

Experimental procedures

Materials. Horse cytochrome *c*, type III, dichlorophenolindophenol (DCIP), phenazine methosulfate, xanthine oxidase, superoxide dismutase, and antimycin were from Sigma. Myxothiazol was from Boehringer Mannheim, F.R.G. 5-*n*-Undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) was synthesized according to Friedman et al. [21]. Succinylated cytochrome *c* was prepared by reacting cytochrome *c* with succinic anhydride according to Klotz [22]. α-Phenyl-*N*-*tert*-butylnitrone (PBN) was from Aldrich. Other chemicals were of the highest purity commercially available.

Methods. Succinate–cytochrome *c* reductase [23], pure and reconstitutively active succinate dehydrogenase [3], succinate–ubiquinone reductase [23], and two-subunit QPs [24] were prepared according to methods previously described. Reconstitutively inactive succinate dehydrogenase was prepared by aging the active preparation in air at 0 °C for 2 h, or by isolating it from succinate–cytochrome *c* reductase using a procedure similar to that for reconstitutively active enzyme but omitting succinate and using aerobic conditions. The antimycin-insensitive succinate–cytochrome *c* reductase activity was measured using the same mixture as for succinate–cytochrome *c* reductase but in the presence of 5 μM of antimycin. For the assay of antimycin-insensitive succinate–cytochrome *c* reductase activity in succinate–cytochrome *c* reductase, the reductase was treated with a 2-fold excess of antimycin (based on cytochrome c_1) before assay. EPR measurements were done in a Bruker ER 200D spectrometer. The detection of the free radical by the spin trapping technique using PBN is essential according to reported method [25].

Results and Discussion

The presence of an antimycin-insensitive succinate–cytochrome c reductase activity in pure succinate dehydrogenase

Reconstitutively active succinate dehydrogenase not only possesses the ability to reconstitute with QPs [15] to form a thenoyltrifluoroacetone-sensitive succinate–ubiquinone reductase or with soluble cytochrome *b*-c_1 complex [26] to form an antimycin-sensitive succinate–cytochrome *c* reductase but also to catalyze the electron transfer from succinate to an artificial electron acceptor, such as ferricyanide or phenazine methosulfate. In addition, reconstitutively active succinate dehydrogenase (without soluble cytochrome *b*-c_1 complex) catalyzes the electron transfer from succinate to cytochrome *c*. Unlike succinate–cytochrome *c* reductase activity in isolated or in submitochondrial particle preparations, this activity is insensitive to antimycin treatment. Table I compares antimycin-insensitive succinate–cytochrome *c* reductase activity with other activities of pure succinate dehydrogenase; such as reconstitutive, succinate–phenazine methosulfate, and succinate–ferricyanide (high K_m and low K_m) reductase activities. Pure succinate dehydrogenase catalyzes electron transfer from succinate to cytochrome *c* with a specific activity of 0.7 μmole succinate per min per mg protein at 23 °C, about 2% of that of its reconstitutive or succinate-phenazine methosulfate activity. It should be stressed that the antimycin-insensitive succinate–cytochrome *c* reductase activity observed in pure suc-

TABLE I

ENZYMATIC ACTIVITIES OF PURE, RECONSTITUTIVELY ACTIVE SUCCINATE DEHYDROGENASE

Activities measured	Specific activity at 23 °C (μmol succinate · $min^{-1} \cdot mg^{-1}$)
Reconstitutive [a] (succinate-Q)	32 [b]
Succinate-phenazine methosulfate	34
Succinate-ferricyanide (low K_m)	30
Succinate-ferricyanide (high K_m)	34
Succinate-cytochrome *c*	0.7
Succinate-succinylated cytochrome *c*	0.2

[a] Refers to the reconstituted activity of succinate–ubiquinone reductase after reconstitution with excess QPs, or antimycin-sensitive succinate–cytochrome *c* reductase after reconstitution with excess soluble cytochrome *b*-c_1 complex. The calculation of specific reconstitutive activity is based on the protein content of succinate dehydrogenase.

[b] This high reconstitutive activity is obtained in the freshly prepared succinate dehydrogenase. In general, when succinate dehydrogenase is stored at −70 °C after the preparation, the specific activity decreases to around 20. However, prolonged storage at −70 °C does not show any further decrease in activity.

cinate dehydrogenase is not due to contamination with succinate–cytochrome *c* reductase. Several lines of evidence support this claim: (1) pure succinate dehydrogenase shows only two polypeptides in SDS-polyacrylamide gel electrophoresis; (2) there is no detectable cytochrome *b* or c_1 in the preparation; (3) no ubiquinol–cytochrome oxidase activity is associated with the preparation; (4) antimycin-insensitive succinate–cytochrome *c* reductase of succinate dehydrogenase is insensitive to myxothiazol, UHDBT, or thenoyltrifluoroacetone treatment. Therefore, the observed antimycin-insensitive succinate–cytochrome *c* reductase activity is an intrinsic activity of succinate dehydrogenase.

Correlation between antimycin-insensitive succinate–cytochrome c reductase activity and reconstitutive activity of succinate dehydrogenase

It is well established that reconstitutive activity of succinate dehydrogenase is sensitive to oxygen with a half life of about 20 min at 0 °C. Succinate-high K_m ferricyanide and succinate-phenazine methosulfate activities are stable in the presence of oxygen. The antimycin-insensitive succinate–cytochrome *c* reductase activity of succinate dehydrogenase is also sensitive to oxygen. As shown in Fig. 1, when succinate dehydrogenase is incubated at 0 °C in the presence of air, the antimycin-insensitive succinate–cytochrome *c* reductase activity decreases as incubation time increases. The rate of inactivation of antimycin-insensitive succinate–cytochrome *c* reductase activity parallels inactivation of reconstitutive activity.

Surprisingly, at lower concentrations of succinate dehydrogenase, the rate of inactivation is slower than at higher concentrations; the half-lifes are 17 min and 60 min for protein concentrations of 23 mg/ml and 0.5 mg/ml, respectively. It is important to know whether or not the observed stability of succinate dehydrogenase at different protein concentrations results from the intrinsic properties of succinate dehydrogenase or from factors introduced during isolation procedures. In the last step of the succinate dehydrogenase purification procedure ammonium sulfate precipitation is used. Pure succinate dehydrogenase is recovered in the 35%–55% ammonium sulfate precipitate and dissolved in a minimum volume of oxygen-free

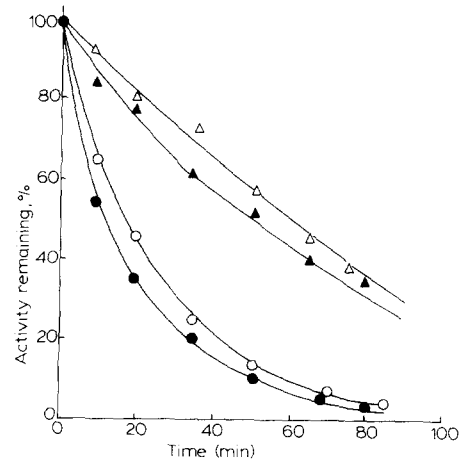

Fig. 1. Stability of antimycin-insensitive succinate–cytochrome *c* and reconstitutive activities of succinate dehydrogenase at different protein concentrations. Pure succinate dehydrogenase which has been frozen at −80 °C under an argon atmosphere was thawed and adjusted anaerobically to protein concentrations of 23 mg/ml and 0.46 mg/ml, respectively, with an oxygen-free buffer containing 50 mM sodium potassium phosphate (pH 7.8) and 20 mM succinate. After assaying succinate–cytochrome *c* reductase and reconstitution with QPs, an air stream was passed through samples for 1 min. Samples were then kept at 0 °C, and at indicated time intervals, two 2-μl and 100-μl aliquots were withdrawn from the sample of 23 mg/ml (○, ●) and of 0.46 mg/ml (△, ▲), respectively. One was used for assaying succinate–cytochrome *c* reductase (○, △) and the other was used to reconstitute with QPs (●, ▲), The activity of reconstituted succinate–ubiquinone reductase was assayed after incubation at 0 °C for 20 min.

buffer before freezing at −80 °C. Thus, isolated succinate dehydrogenase, especially at high protein concentrations, contains a high concentration of ammonium sulfate.

It is possible that the ammonium sulfate present in the preparation accelerates inactivation of both antimycin-insensitive succinate–cytochrome *c* reductase and reconstitutive activities of succinate dehydrogenase in the presence of air. To test this, we diluted succinate dehydrogenase (25 mg/ml) to 0.5 mg/ml with oxygen-free buffer containing 50 mM sodium potassium phosphate (pH 7.8), 20 mM succinate, and various concentrations of ammonium sulfate, and followed the decay of antimycin-insensitive succinate–cytochrome *c* reductase activity with time after exposure to air. When the ammonium sulfate concentration is less than 0.2 M, no effect is observed;

at higher concentrations the rate of inactivation of antimycin-insensitive succinate–cytochrome *c* reductase is proportional to the increased concentration of ammonium sulfate. These results indicate that the higher inactivation rate observed for more concentrated succinate dehydrogenase is due to the presence of higher concentrations of ammonium sulfate and not to the intrinsic properties of the enzyme. This salt effect on the stability of succinate dehydrogenase is apparent only under aerobic conditions. At a constant salt concentration, pure succinate dehydrogenase behaves like most enzymes; it is more stable in a concentrated protein solution than in a diluted solution.

Effect of cytochrome c concentration on the antimycin-insensitive succinate–cytochrome c reductase activity of succinate dehydrogenase

Since a small antimycin-insensitive activity is always observed in isolated, fully active succinate–cytochrome *c* reductase, it is important to know whether or not this antimycin-insensitive activity is the same as that observed in pure succinate dehydrogenase. The antimycin-insensitive activity accounts for less than 2% of the total succinate–cytochrome *c* activity in isolated succinate–cytochrome *c* reductase. One way to differentiate antimycin-insensitive activity of succinate dehydrogenase from that in succinate–cytochrome *c* reductase is to compare their kinetic parameters.

Table II shows the K_m for cytochrome *c* of antimycin-sensitive and -insensitive succinate–cytochrome *c* reductase in isolated and reconstituted succinate–cytochrome *c* reductase and of antimycin-insensitive activity in pure succinate dehydrogenase. The K_m for cytochrome *c* for antimycin-insensitive activity in isolated and reconstituted succinate–cytochrome *c* reductases is around 4.4 μM, whereas the K_m for cytochrome *c* for antimycin-insensitive activity of succinate dehydrogenase is too high to be measured, indicating that these antimycin-insensitive activities are different. The fact that similar K_m values were obtained for antimycin-sensitive and -insensitive activities in isolated or reconstituted succinate–cytochrome *c* reductase suggests that the insensitive activity may result from incomplete inhibition by antimycin. In contrast, the antimycin-insensitive

TABLE II

K_m FOR CYTOCHROME *c* FOR ANTIMYCIN-INSENSITIVE SUCCINATE–CYTOCHROME *c* ACTIVITY OF SUCCINATE DEHYDROGENASE, ANTIMYCIN-INSENSITIVE AND -SENSITIVE SUCCINATE–CYTOCHROME *c* ACTIVITIES OF ISOLATED AND RECONSTITUTED SUCCINATE–CYTOCHROME *c* REDUCTASES

Antimycin-insensitive succinate–cytochrome *c* reductase activity of intact and reconstituted succinate–cytochrome *c* reductases was measured by pretreatment of reductase preparations with a 2-molar excess antimycin (base on cytochrome c_1). The assay mixture contains no antimycin.

Activities	K_m for cytochrome *c*
Succinate dehydrogenase	
Antimycin-insensitive succinate-cytochrome *c*	∞
Succinate–cytochrome *c* reductase	
Antimycin-sensitive succinate-cytochrome *c*	4.4
Antimycin-insensitive succinate-cytochrome *c*	4.2
Reconstituted succinate–Cytochrome *c* reductase	
Antimycin-sensitive succinate-cytochrome *c*	4.4
Antimycin-insensitive succinate-cytochrome *c*	4.7

activity of pure succinate dehydrogenase is proportional to the concentration of cytochrome *c*, at concentrations of cytochrome *c* up to 3 mM, no K_m can be obtained, suggesting that the reduction of cytochrome *c* by succinate dehydrogenase is indirect. Cytochrome *c* may not be accepting electrons directly from succinate dehydrogenase; it rather may obtain electrons from an electron acceptor for succinate dehydrogenase. Perhaps the dehydrogenase transfers electrons from succinate to an unknown acceptor, and cytochrome *c* is then reduced by this acceptor. In such a reaction the rate of reduction of cytochrome *c* will be governed by mass action and will depend on the concentration of cytochrome *c*; hence no K_m for cytochrome *c* will be observed.

Effect of QPs on the antimycin-insensitive succinate–cytochrome c activity of succinate dehydrogenase

As described above, although the antimycin-insensitive succinate–cytochrome *c* reductase activity in pure succinate dehydrogenase, measured at 100 μM cytochrome *c*, is only about 2% of the reconstitutive activity, this activity correlates very well with its reconstitutive activity. This suggests

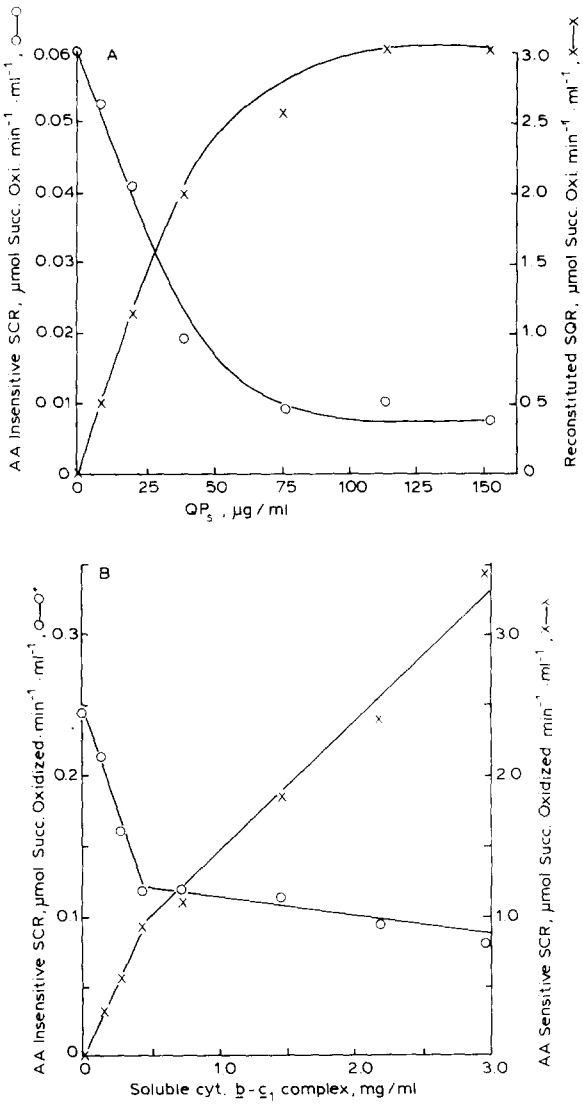

Fig. 2. Effect of QPs on antimycin-insensitive succinate–cytochrome *c* activity of succinate dehydrogenase. (A). Reconstitution with two-subunit QPs. 10-μl aliquots of pure succinate dehydrogenase, 19 mg/ml, in 0.1 M sodium potassium phosphate buffer (pH 7.8) containing 20 mM succinate were added anaerobically to 1 ml of oxygen-free buffer containing 20 mM Tris-succinate (pH 7.8) and indicated amounts of QPs. Samples were kept at 0 °C for 5 min under anaerobic conditions before succinate–cytochrome *c* reductase (O——O) and succinate–ubiquinone reductase (×——×) activities were measured. Succinate–ubiquinone reductase was assayed in the presence and absence of 100 μM thenoyltrifluoroacetone. (B) Reconstitution with soluble cytochrome b-c_1 complex. 50-μl aliquots of succinate dehydrogenase, 16 mg/ml, in 0.1 M sodium potassium phosphate buffer (pH 7.8) containing 20 mM succinate were added anaerobically to 0.95 ml aliquots of oxygen-free solution containing 20 mM Tris-succinate and indicated amounts of soluble cytochrome b-c_1 complex. After incubation at 0 °C for 20 min under anaerobic conditions, succinate–cytochrome *c* activity was assayed in the presence and absence of 5 μM antimycin A. The curve with circles (O——O) and with crosses (×——×) represent the antimycin-insensitive and -sensitive succinate cytochrome *c* activities, respectively.

that this activity apears when succinate dehydrogenase is detached from the membrane or from QPs. Fig. 2A shows the effect of QPs on the antimycin-insensitive activity of succinate dehydrogenase. When succinate dehydrogenase is titrated with QPs, the antimycin-insensitive succinate–cytochrome *c* reductase activity of succinate dehydrogenase decreases as the reconstituted succinate–ubiquinone reductase activity increases. Since the rate of disappearance of antimycin-insensitive succinate–cytochrome *c* reductase correlates well, it is suggested that the former activity is changed to the latter. Apparently the presence of QPs has prevented the electrons from succinate dehydrogenase from leaking out to a non-physiological acceptor, and directed the electrons to the native acceptor, Q bound to QPs. In a similar manner, when succinate dehydrogenase is titrated with soluble cytochrome b-c_1 complex, the appearance of reconstituted antimycin-sensitive succinate–cytochrome *c* reductase parallels the disappearance of antimycin-insensitive succinate–cytochrome *c* reductase activity (see Fig. 2B). Again, these results suggest that the antimycin-insensitive activity results from the detachment of succinate dehydrogenase from its electron transfer partner, QPs, or corresponding proteins under different terms, such as CII-3,4 or cytochrome b_{560}. Evidence for CII-3,4 serving as an anchor for succinate dehydrogenase to the mitochondrial inner membrane has been reported [27].

Involvement of free radicals in the antimycin-insensitive succinate–cytochrome c reductase activity of succinate dehydrogenase

To test the involvement of the free radical in antimycin-insensitive activity of succinate dehydrogenase, two approaches are employed: the use of succinylated cytochrome *c* as an electron acceptor in the assay system and the detection of

the free radical by the spin trapping techniques using PBN.

Unlike cytochrome *c*, succinylated cytochrome *c* is not reduced by ascorbate or by succinate in the presence of succinate–cytochrome *c* reductase. It can, however, be reduced by low potential reductants such as $O_2^- \cdot$. Reconstitutively active succinate dehydrogenase is able to catalyze the oxidation of succinate with succinylated cytochrome *c* as electron acceptor (see Table I). The rate is much slower than it is when cytochrome *c* is the electron acceptor. The rate of reduction of succinylated cytochrome *c* is increased slightly when measured under anaerobic conditions. This is because reduced succinylated cytochrome *c* is autoxidizable.

Although the use of succinylated cytochrome *c* as electron acceptor by reconstitutively active succinate dehydrogenase for succinate oxidation in the absence of QPs may suggest involvement of the $O_2^- \cdot$ radical, the failure to observe a decrease in the rate of reduction of cytochrome *c* under anaerobic conditions or a significant difference in the rate of O_2 consumption between reconstitutively active and inactive succinate dehydrogenases raises questions concerning the participation of O_2 in cytochrome *c* reduction. The insensitivity of this activity toward superoxide dismutase has further questioned the role of $O_2^- \cdot$ in this reaction. Unless we assume that the K_m for O_2 is extremely low and the residual O_2 in the anaerobic system can accept electrons from succinate dehydrogenase, it is difficult to explain why the rate of reduction is not slower under anaerobic conditions.

A better way to prove the participation of the free radical in the reduction of cytochrome *c* is to detect its formation by spin trapping techniques. Fig. 3 shows the generation of the PBN spin adduct in the presence of reconstitutively active and inactive succinate dehydrogenases, and reconstituted succinate–ubiquinone reductase. When reconstitutively active succinate dehydrogenase is added to an assay mixture containing 0.1 M phosphate buffer (pH 7.8), 0.02 M succinate and 0.1 M PBN, a PBN spin adduct is generated (see Fig. 3, spectrum A). When a reconstitutively inactive succinate dehydrogenase, at the same protein concentration is used, little PBN spin adduct is generated (about 20% of that generated by the active preparation) see (Fig 3, spectrum B). When succinate dehydrogenase is reconstituted with QPs to form succinate–ubiquinone reductase, the PBN spin adduct is generated at the level observed with inactive succinate dehydrogenase (see Fig. 3, spectrum C). QPs alone shows no PBN spin generation. These results clearly indicate that the PBN spin adduct results from the oxidation of succinate catalyzed by reconstitutively active succinate dehydrogenase in the absence of its electron partner, QPs. The free radical observed in reconstitutively inactive succinate dehydrogenase may be different from those observed in active succinate dehydrogenase, as the former is not affected by the addition of QPs.

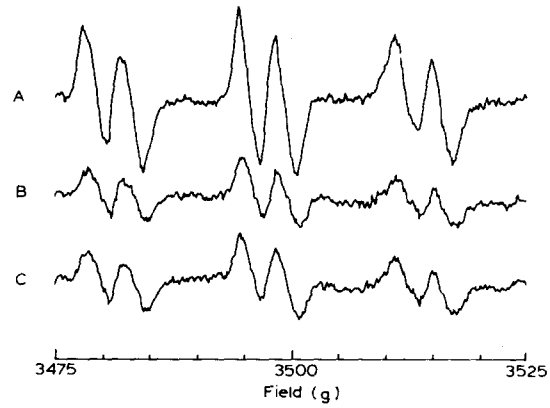

Fig. 3. PBN spin adduct generation in the succinate oxidation catalyzed by reconstitutively active and inactive succinate dehydrogenases, and reconstituted succinate–ubiquinone reductase. To a buffer solution containing 100 mM phosphate-borate (pH 7.8), 20 mM succinate, and 100 mM PBN, at room temperature, were added (A) reconstitutively active succinate dehydrogenase; (B) reconstitutively inactive succinate dehydrogenase; and (C) a mixture of reconstitutively active succinate dehydrogenase and QPs. The final concentration of succinate dehydrogenase in each sample was 0.7 mg/ml. The EPR spectra were taken at room temperature and the data were subtracted from their corresponding blank. The instrument settings were: modulation frequency, 100 kHz; modulation amplitude, 2 G; time constant, 0.1 s; microwave frequency, 9.76 GHz; microwave power, 2 mW; and scan rate, 200 s.

The generation of PBN spin adduct is reaction time dependent, and is inhibited by oxalacetate, a substrate analog inhibitor of succinate dehydrogenase. Fig. 4 shows the time dependent PBN spin generation of reconstitutively active and inactive succinate dehydrogenases. In this experiment reac-

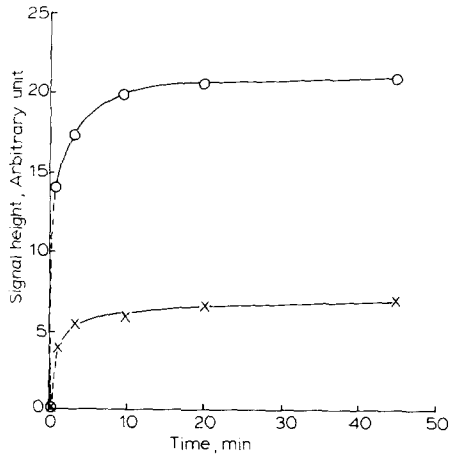

Fig. 4. Time dependence of PBN radical formation by succinate oxidation catalyzed by reconstitutively active and inactive succinate dehydrogenases. Samples were prepared as in Fig. 3. At indicated times samples were withdrawn from incubation and frozen in an isopentane bath at approx. 115 K. The EPR instrument settings were: microwave frequency, 9.34 GHz; microwave power, 5 mW; modulation amplitude, 5 G; time constant, 0.1 s; scan rate, 0.5 G/s; temperature, 150 K. The circles (O——O) and the cross (×——×) represent reconstitutively active and inactive succinate dehydrogenases, respectively.

tions were stopped by placing samples in liquid nitrogen. Although the amount of PBN spin adduct generated by reconstitutively inactive succinate dehydrogenase is small compared to that generated by reconstitutively active succinate dehydrogenase, the kinetic behavior on the adduct formation is very similar. The radicals are generated rapidly during the first one min; very slowly thereafter. It is not known whether the radical generated in the slower phase is the same as that generated in the fast phase. Even though the PBN adduct is similar to that of perhydroxy radical generated by xanthine oxidase, the identity of this PBN spin adduct remains obscure, as the role of O_2 in the reaction of antimycin-insensitive succinate–cytochrome *c* reductase has been questioned. At this moment we can only consider the identity of this free radical unknown, further investigation is needed before the role of this radical in antimycin-insensitive succinate–cytochrome *c* reductase of succinate dehydrogenase can be established.

Acknowledgements

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