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# STUDIES ON THE SUCCINATE DEHYDROGENATING SYSTEM

# II. RECONSTITUTION OF SUCCINATE-UBIQUINONE REDUCTASE FROM THE SOLUBLE COMPONENTS

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## Summary

1. A protein fraction containing three polypeptides (the major one with $M_r < 13\,000$) was isolated by means of Triton X-100 extraction of submitochondrial particles specifically treated to remove succinate dehydrogenase.

2. The mixing of the protein fraction with the soluble reconstitutively active succinate dehydrogenase results in formation of highly active succinate-DCIP reductase which is sensitive to thenoyltrifluoroacetone or carboxin.

3. The maximal turnover number of succinate dehydrogenase in the succinate-DCIP reductase reaction revealed in the presence of a saturating amount of the protein fraction is slightly higher than that measured with phenazine methosulfate as artificial electron acceptor.

4. The protein fraction greatly increases the stability of soluble succinate dehydrogenase under aerobic conditions.

5. The titration of soluble succinate dehydrogenase by the protein fraction shows that smaller amounts of the protein fraction are required to block the reduction of ferrycyanide by Hipip center than that required to reveal the maximal catalytic capacity of the enzyme.

6. The apparent $K_m$ of the reconstituted system for DCIP depends on the amount of protein fraction; the more protein fraction added to the enzyme, the lower the $K_m$ value obtained.

7. A comparison of different reconstituted succinate-ubiquinone reductases described in the literature is presented and the possible arrangement of the

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; Hipip, high-potential Fe-S cluster.

native and reconstituted succinate-ubiquinone region of the respiratory chain is discussed.

## Introduction

Succinate dehydrogenase (EC 1.3.99.1) is a membrane-bound enzyme which donates electrons directly to the respiratory chain. In fact, the enzyme itself is a part of the respiratory chain and no significant change in its properties has been found in some phospholipids containing resolved preparations such as succinate-cytochrome *c* reductase [1,2] or succinate-ubiquinone reductase [3] compared to the 'native' submitochondrial particles; As is evident from its high succinate-artificial acceptor reductase activities, the soluble lipid free enzyme still has succinate dehydrogenating machinery consisting of active site sulfhydryl group [6—9], covalently bound flavin [10], and iron-sulfur complexes [11—14]. Some types of the soluble enzyme are capable of binding to the membrane of the enzyme-depleted particles [5,15—17] with restoration of most of the properties of the native system, while others are not [18]. The studies on reconstitution [12,13], reactions with artificial electron acceptors [19—21], and ESR experiments [12,13] have revealed that the Hipip center of the enzyme is responsible for the functional link between succinate dehydrogenase and the rest of respiratory chain. The most important properties of the Hipip center have been reviewed [14].

The nature of factor(s) belonging to the membrane which are responsible for binding the enzyme and for restoration of succinate-natural acceptor reductase activity is far less known. It is generally accepted that ubiquinone serves as a natural acceptor for both succinate and NAD · H dehydrogenase. Several models for dehydrogenase-ubiquinone interaction in the mitochondrial membrane have been suggested [22—25]. Recently, a 15 000 dalton protein has been isolated in nearly pure form, which is capable of reconstitution of succinate-ubiquinone reductase by admixing with soluble succinate dehydrogenase [4,26]. The starting material for isolation of this protein was the soluble $b$-$c_1$ complex [2]. The experimental procedure for preparation of this ubiquinone-binding protein (as it is called by the authors) has not been published.

In this paper we will report a simple preparation procedure for a soluble protein fraction active in reconstitution of succinate-ubiquinone reductase by admixing with soluble succinate dehydrogenase. Some observations on the reconstitution process and the properties of the reconstituted system relevant to the arrangement of the succinate-ubiquinone region of the respiratory chain are also presented.

## Materials and Methods

*Preparation of succinate dehydrogenase*

The soluble reconstituvely active succinate dehydrogenase was prepared by our adaptation of the original method of King [27]. Keilin-Hartree bovine heart muscle preparation was isolated exactly as described in Ref. 28. 100 ml of the suspension containing 33 mg protein per ml as determined by the biuret

method [31] was added to a three-neck flask placed on a magnetic stirrer; 200 ml water was added and the suspension was slowly bubbled with Ar for 2 h at room temperature. 13 ml 1 M potassium succinate, pH 7.5, was then added and the flask was equipped with a combined glass pH electrode and a separatory funnel. The flask was placed in an ice/salt bath and cooled to 0°C. The addition of succinate causes an immediate change in color of the suspension from reddish brown to greenish brown. Once the temperature had reached 0°C, the pH of the mixture was adjusted to 9.5 with NaOH and oxygen free *n*-butanol cooled to −20°C was slowly added (10 ml per 100 ml of the suspension) from the attached separatory funnel. After the addition of butanol, the mixture was constantly stirred for 30 min and then the pH was adjusted to 6.0 with 1 M $O_2$-free acetic acid. The contents of the flask were rapidly transferred into closed centrifuge tubes and the suspension was centrifuged at 1600 × *g* for 20 min. Two layers are formed after centrifugation: one is tightly packed sediment, and the other is clear yellow supernatant containing succinate dehydrogenase. The supernatant was carefully decanted into the flask with an inlet for Ar, and calcium phosphate gel (4 mg dry weight per ml extract) was added as the supernatant was bubbled with Ar. The gel was precipitated by centrifugation at 1000 × *g* for 5 min. The precipitate was washed with 200 ml of $O_2$-free water and precipitated as before. The sedimented gel was placed into a flask and 150 ml of an $O_2$-free mixture comprising 0.1 M potassium phosphate, 10 mM potassium succinate and 100 μM EDTA, pH 7.8, was added. The suspension was stirred for 15 min and centrifuged at 10 000 × *g* for 10 min. The pH of the clear yellow supernatant was adjusted to 7.0 with acetic acid, and it was placed in closed polyethylene vials and stored in liquid $N_2$. The samples were taken out of liquid $N_2$ and thawed immediately before use. If necessary the enzyme may be precipitated by the addition of an equal volume of oxygen free $(NH_4)_2SO_4$ saturated at room temperature, and stored as pellets in liquid nitrogen.

The activity of the enzyme prepared as described and measured at 23°C in a mixture containing 10 mM succinate/10 mM Tris-sulfate buffer (pH 7.8)/ 0.1 mM EDTA with 0.1 mM Wurster's Blue as acceptor is about 20 μmol succinate oxidized per min per mg protein.

*Preparation of submitochondrial particles*

Submitochondrial particles were derived from heavy beef-heart mitochondria prepared according to Löw and Vallin [29]. The procedure for sonication and isolation was essentially as described by Lee and Ernster for EDTA particles [30], except that sucrose was substituted for 0.1 M phosphate buffer.

*Analytical methods*

Succinate-DCIP reductase was measured in the standard assay mixture comprising 20 mM potassium succinate, 10 mM Tris-sulfate buffer, pH 7.8, 0.1 mM EDTA, 1 mM KCN and 0.1 mM DCIP at 23°C. Succinate-phenazine methosulfate reductase was measured in the same way as was succinate-DCIP reductase except that freshly prepared phenazine methosulfate was added just before the enzyme. Succinate-ferricyanide reductase was measured spectrophotometrically as described previously [19]. Electrophoresis in the presence

of sodium dodecyl sulfate (SDS) was performed exactly as described by Weber and Osborn [49] using 10% gel. The chemicals for electrophoresis were from Reanal (Hungary). The gels were stained by Coomassie Brilliant Blue R 250 for 4 h. The absorbance of the gels was measured at 560 nm.

Protein was determined by the biuret method [31]. The protein content in the samples containing Triton X-100 was determined as described by Yu and Steek [32].

All in the chemicals used were of highest quality commercially available, Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) was a kind gift from Professor H. Lyr (Institute of Plant Protection Research, G.D.R.) obtained through Dr. T. Schewe.

## Results

*The preparation of the soluble fraction capable of reconstitution of succinate-ubiquinone reductase*

The sedimented submitochondrial particles derived from 1 g mitrochondrial protein were suspended in 50 ml of a solution comprising 0.1 M potassium phosphate, pH 7.2, 0.5 M $NaNO_3$ and 0.1 M semicarbazide (to remove tightly bound oxaloacetate). This suspension was incubated at 25°C for 1 h. The suspension was then diluted 10 times with 0.1 M potassium phosphate, pH 7.2, centrifuged for 40 min at 105 000 × *g* and suspended in 0.2 M sucrose. The suspension (28 mg protein per ml) was diluted to a protein content 16 mg/ml with 10 mM Tris-sulfate (pH 7.0)/0.1 mM EDTA. The pH was adjusted to 9.5 with 0.1 M NaOH and the mixture was placed in a water bath at 37°C with constant stirring for 1.5 h. The pH was monitored and adjusted to 9.5 (if necessary) every 15 min. After incubation the pH was adjusted to 8.0, the mixture was cooled in ice and diluted with an equal volume of a solution comprising 4 M urea, 4 mM EDTA and 0.1 M Tris-sulfate, pH 8.0. The mixture was incubated at 0°C for 30 min and then centrifuged for 40 min at 105 000 × *g*. The yellow supernatant was discarded and sedimented particles were washed with 320 ml 10 mM Tris-sulfate (pH 7.0)/0.1 mM EDTA and centrifuged again. The final sediment was suspended in 50 ml of 10 mM Tris-sulfate (pH 7.0)/0.1 mM EDTA (the protein content was 11 mg/ml).

To 50 ml of the suspension, 12.5 ml of a 6% solution of Triton X-100 in 10 mM Tris-sulfate, pH 7.0, were added and the mixture was allowed to incubate with constant stirring for 15 min and then was centrifuged for 40 min at 105 000 × *g*. The supernatant was collected, placed into small vials and stored in liquid nitrogen.

The protein fraction obtained as described contains no succinate dehydrogenase as evidenced by the absence of any measurable succinate-acceptor reductase activity and by the polypeptide composition (Fig. 1). As seen from the polypeptide pattern, the Triton X-100-solubilized fraction is surprisingly pure. About 80% of the protein (as justified by integrated color intensity of SDS-gel) is represented by the peptide(s) with $M_r$ less than 13 000.

*General properties of reconstitution of succinate-ubiquinone reductase*

When soluble succinate dehydrogenase is mixed with protein fraction, the

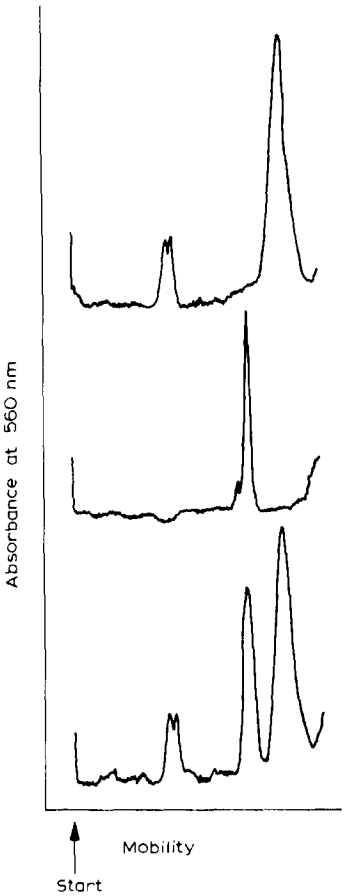


Fig. 1. Sodium dodecyl sulfate gel electrophoretic pattern of a protein fraction solubilized by Triton X-100. Top, a protein fraction (approx. 15 μg); middle, cytochrome *c* (Sigma, type IV, approx. 5 μg); bottom, a protein fraction plus cytochrome *c* (approx. 15 and 5 μg, respectively).

system is able to catalyse the succinate-DCIP reductase reaction * (Fig. 2). Neither succinate dehydrogenase nor the protein fraction reacts with DCIP. The activity appearing after the mixing is completely sensitive to carboxin (or to thenoyltrifluoroacetone, not shown). As seen from the actual tracing of DCIP reduction, a notable lag is observed when succinate dehydrogenase is added to the protein fraction or vice versa. This phenomenon was studied in more detail in experiments in which different amounts of the protein fraction were added

* Since no water-soluble ubiquinone analog was available in this laboratory, succinate-DCIP reductase without added cofactors was measured. It has been shown [3] that succinate-DCIP reductase activity of complex II has the same properties as succinate-ubiquinone reductase. Since soluble succinate dehydrogenase does not react with DCIP whereas the membrane-bound enzyme catalyses thenoyltrifluoroacetone-sensitive reduction of DCIP by succinate, it is safe to conclude that DCIP reduction is mediated by ubiquinone. The terms succinate-DCIP reductase and succinate-ubiquinone reductase are, therefore, used interchangeably throughout the text.

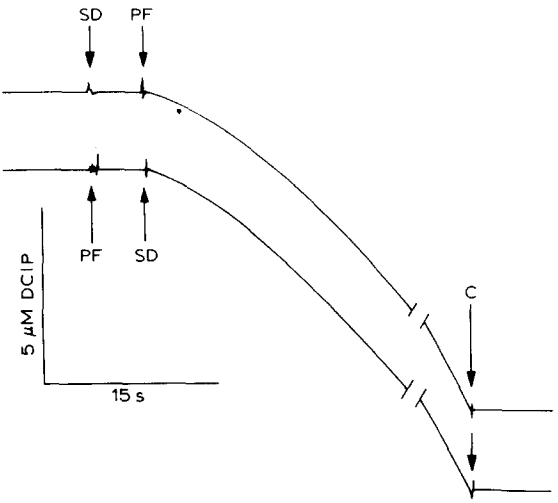


Fig. 2. The reduction of DCIP by succinate catalyzed by soluble succinate dehydrogenase (SD) in the presence of a protein fraction (PF). 12 μg succinate dehydrogenase and 100 μg protein fraction were added as indicated to a 1.5 ml spectrophotometric cuvette containing the standard assay mixture (see Materials and Methods); 20 μM carboxin (ethanolic solution) was then added (c).

to a fixed amount of succinate dehydrogenase and the initial rate of DCIP reduction was measured as a function of time (Fig. 3). The half-times of formation of active DCIP reductase are 15, 30 and 50 s for 60, 120 and 240 μg, respectively, of protein fraction added into 1 ml of the reconstitution mixture. The final level of the activity is also a function of the amount of protein fraction added (see below for the further details).

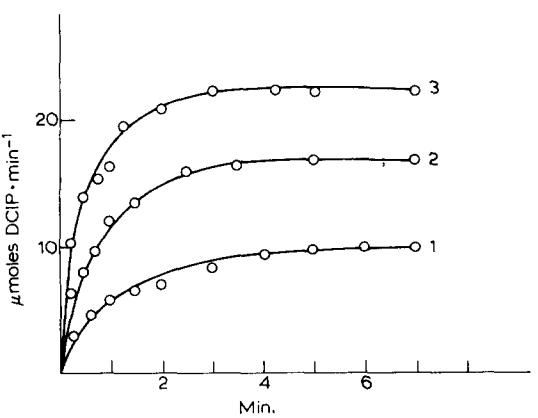


Fig. 3. Reconstitution of succinate-DCIP reductase as a function of time. 5 μg of succinate dehydrogenase was added at zero time to 0.5 ml of the mixture comprising 10 mM Tris-sulfate (pH 7.0) 10 mM potassium succinate, 0.1 mM EDTA, 1% Triton X-100 and 30, 60 and 120 μg of protein fraction (curves 1, 2 and 3, respectively). The mixture was incubated at 23° C and the reaction was started by the addition of 0.2 ml of the mixture to the assay cuvette. The initial rate of DCIP reduction was measured.

TABLE I

THE RELATIVE ACTIVITIES OF SUCCINATE DEHYDROGENASE AND PROTEIN FRACTION SOLUBILIZED BY TRITON X-100 IN THE RECONSTITUTED SYSTEM

The preparations indicated were admixed in a medium comprising 10 mM Tris-sulphate, pH 7.0, 20 mM potassium succinate, 0.1 mM EDTA, 1% Triton X-100 and the proper amount of the proteins. The mixture was incubated for 10 min and cooled in ice, and the activities were assayed as indicated in Materials and Methods. All the activities were sensitive to carboxin.

| Preparations | Activity (μmol DCIP reduced per min per mg limiting protein) |
|---|---|
| 1. Succinate dehydrogenase | 0.01 |
| 2. Protein fraction | 0.02 |
| 3. Succinate dehydrogenase (excess) + protein fraction | 3.0 |
| 4. Protein fraction (excess) + succinate dehydrogenase | 16.0 |
| 5. Alkali-treated, urea washed submitochondrial particles (AUP) | 0.01 |
| 6. Succinate dehydrogenase (excess) + AUP | 0.75 |
| 7. AUP (excess) + succinate dehydrogenase | 14.2 |

It was of interest to compare the relative activities of succinate-DCIP reductase reconstituted from succinate dehydrogenase and alkali-trated submitochondrial particles or solubilized protein fraction. The results presented in Table I show that when succinate dehydrogenase is present in limiting amounts its electron transferring activities are practically the same whether the complete respiratory chain or purified protein fraction used to mediate DCIP reduction. It may thus be concluded that, at least qualitatively, the solubilized protein fraction contains all the components required for reduction of ubiquinone by succinate dehydrogenase. On the other hand, the relative turnover numbers of the protein fraction (based on protein concentration) are quite different. The comparison of the latter would suggest that an approximately 4-times purification is achieved by the solubilization procedure. This is apparently the lower limit, since the rate of DCIP reduction should depend on ubiquinone content in the reconstituted system. Indeed, in two experiments we were able to observe the strong stimulation of succinate-DCIP reductase by added ubiquinone-6 (see footnote to p. 17).

*General properties of the reconstituted system*

The results presented above shown that the protein fraction is able to confer the reactivity of succinate dehydrogenase towards DCIP. The question arises of whether this is due to the random protein-protein collisions in solution or to the formation of a complex operating as a single unit. Since the reconstituted system is not sedimented by high-speed centrifugation, it is not easy to answer this question without development of a special technique for isolation of the complex (if it exists). In an attempt to differentiate these two possibilities the activity of the reconstituted system was measured as a function of dilution. As seen from Fig. 4, the activity after reconstitution is a linear function of the

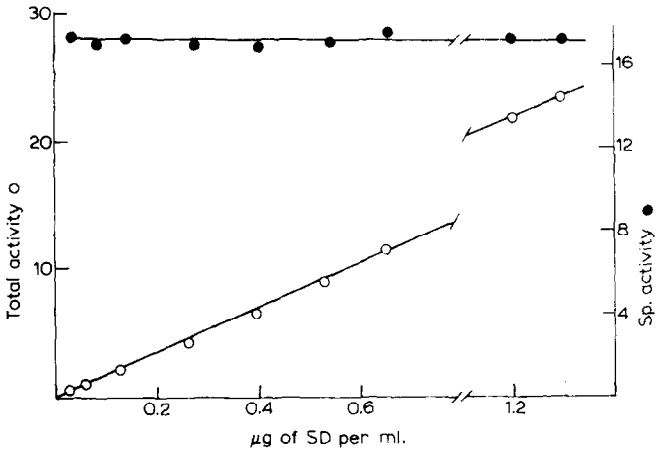


Fig. 4. Effect of dilution on the activity of reconstituted system. 340 μg protein fraction and 20 μg of succinate dehydrogenase were mixed and incubated at 23° C for 10 min as described for Fig. 3. The reaction was started by the addition of the mixture (3—120 μg of the total protein) to the assay medium. No deviation from linearity was observed during the recording of DCIP reduction. Upper line, the specific activity (sp. activity) (per mg succinate dehydrogenase); lower line, the total activity.

amount of reconstituted system and the specific activity is constant over a wide range of dilutions. This suggests that the reconstituted system forms a single unit which does not dissociate in solution under the conditions of the assay.

Another approach to the problem was to compare the stabilities of soluble succinate dehydrogenase and reconstituted system under aerobic conditions.

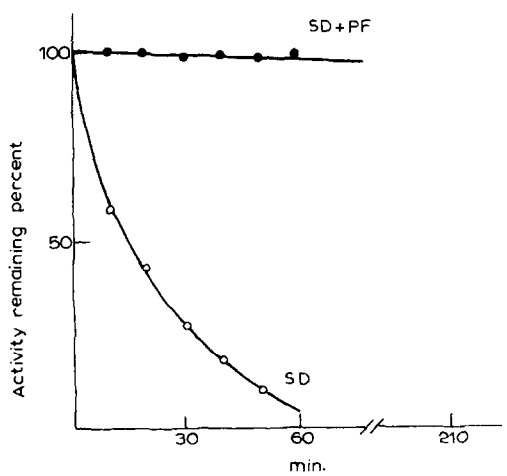


Fig. 5. Stability of soluble succinate dehydrogenase (SD) and reconstituted system. Two portions of soluble succinate dehydrogenase were treated as follows: an excess of protein fraction (PF) was added to the first sample and incubation was continued for the time indicated; the small aliquots from the second sample were mixed with the same amount of the protein fraction at the time indicated in abscissa, incubation was continued for 5 min and the activities were then measured. The conditions for reconstitution are indicated in Fig. 3; temperature was 23° C.

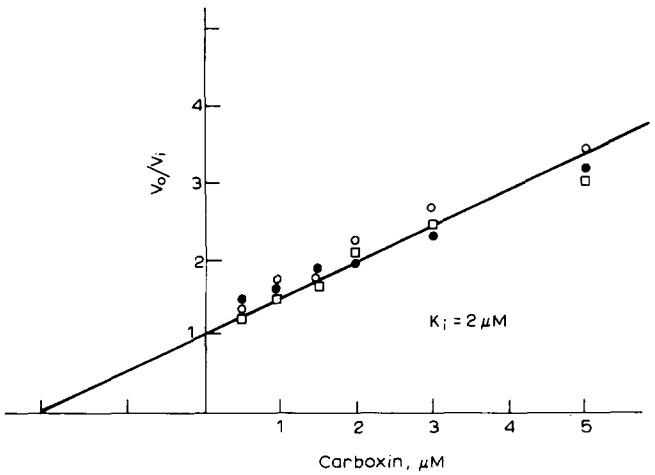


Fig. 6. The determination of the $K_i$ value for carboxin in the native and reconstituted systems. The reconstitution was performed as described for Fig. 3. ○, 0.27 and 0.01 mg of protein fraction and succinate dehydrogenase per ml of the reconstitution medium, respectively; ●, 0.06 and 0.07 mg protein fraction and succinate dehydrogenase, respectively. □, submitochondrial particles. The equivalent amount of Triton X-100 was added to submitochondrial particles before assay to eliminate possible interference of detergent with inhibition by carboxin. Carboxin (methanolic solution) was added in the assay mixture in the final concentrations indicated in abscissa.

Fig. 5 demonstrates that the reconstituted system is far more stable than soluble succinate dehydrogenase alone.

It is known that thenoyltrifluoroacetone and carboxin inhibit succinate oxidation in particulate preparations [3,33,34—37], whereas these inhibitors do not affect succinate-artificial acceptor reductase reactions catalysed by the soluble enzyme [36,37]. Fig. 6 shows that the sensitivity of reconstituted succinate-DCIP reductase is the same as that of submitochondrial particles. What is more interesting is that the $K_i$ for carboxin does not depend on the relative amount of the protein fraction added to succinate dehydrogenase.

The results of titration of succinate dehydrogenase by the protein fraction compared to phenazine methosulfate are presented in Fig. 7. As seen from the titration curve, the maximal level of activity reached in the presence of saturating amounts of protein fraction is slightly higher than that obtained with artificial acceptor. This is in agreement with the results from this laboratory on the reactivity of soluble and membrane-bound succinate dehydrogenase towards different electron acceptors [19,21,38]. The titration of protein fraction by succinate dehydrogenase (Fig. 7b) also shows the saturation behavior with the maximal level of activity proportional to the amount of protein fraction taken.

It has been shown that the activity of soluble reconstitutively active succinate dehydrogenase as revealed by the reaction with low concentrations of ferricyanide is a measure of functional intactness of the Hipip component of the enzyme [12,19,20,39]. This activity is not seen in membrane-bound preparations of the enzyme [19,41]. It is possible therefore to correlate the binding of the enzyme with the appearance of succinate-ubiquinone reductase.

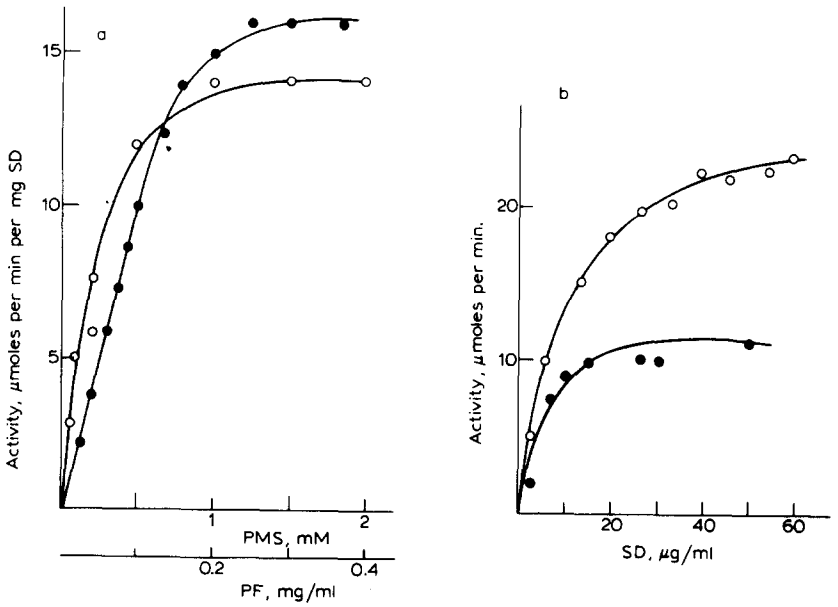


Fig. 7. The titration curves of DICP reduction by succinate catalyzed by succinate dehydrogenase in the presence of protein fraction (PF) or phenazine methosulfate (PMS). a, •, soluble succinate dehydrogenase (SD) was reconstituted with different amounts of protein fraction as indicated in Fig. 3 and the activity was measured as a function of protein fraction added per ml of the reconstitution medium. ○, DCIP reduction by succinate in the presence of soluble succinate dehydrogenase and different concentrations of phenazine methosulfate in the assay mixture. b. Activity of reconstituted system as a function of the amount of soluble succinate dehydrogenase added to protein fraction at two levels of the protein fraction in the reconstitution mixture (○, 56 and •, 28 μg per ml).

As depicted in Fig. 8, the amount of the protein fraction needed to block ferricyanide reductase is less than that required to reach the full catalytic capacity of succinate dehydrogenase. The same correlation was found when two different concentrations of succinate dehydrogenase in the reconstitution medium

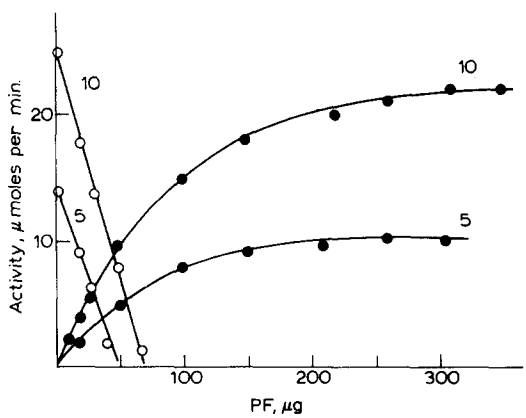


Fig. 8. Comparative titration of succinate-ferricyanide and succinate-DCIP reductases by protein fraction (PF). The reconstitution was performed as in Fig. 3 with different amounts of succinate dehydrogenase (SD) and protein fraction per ml of the reconstitution medium (indicated by the figures on the curves and abscissa). ○, succinate-ferricyanide reductase (100 μM $K_3Fe(CN)_6$); •, succinate-DCIP reductase.

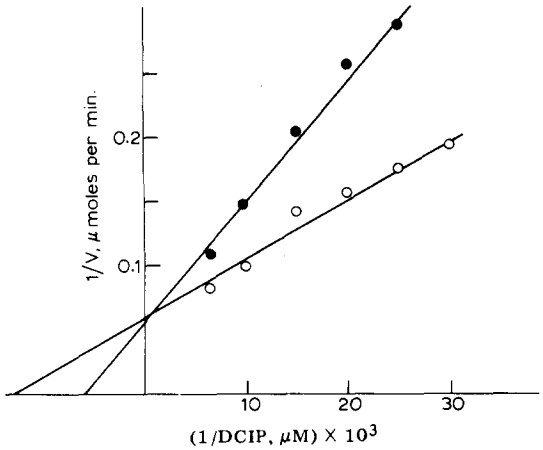


Fig. 9. Determination of $K_m$ values for DCIP in the reconstituted systems with different proportions of succinate dehydrogenase and protein fraction. The reconstitution was performed as in Fig. 3. •, 40 μg of succinate dehydrogenase and 56 μg of protein fraction were mixed in 1 ml before assay; ○, 10 μg of succinate dehydrogenase and 332 μg of protein fraction were mixed in 1 ml before assay.

were used. The amount of succinate dehydrogenase which is accessible for ferricyanide may be assumed to be a measure of unbound enzyme. Therefore, these results establish clearly that the requirements for the binding itself are different from those for the formation of fully active succinate-ubiquinone reductase.

As follows from the model proposed for two component catalytic redox system reacting according to the Law of Mass Action [21], the apparent $K_m$ value for the final electron acceptor must be in inverse proportion to the amount of the species reacting with the acceptor. This is the case for the systems reconstituted from soluble succinate dehydrogenase and protein fraction (Fig. 9). Formally, the protein fraction can be considered as the activator of succinate-ubiquinone reductase interacting with the system at the site of DCIP reduction.

## Discussion

Several preparations capable of reconstitution of succinate-ubiquinone reductase when added to the soluble succinate dehydrogenase have been described in the literature. Some of those are summarized in Table II. Five out of the seven preparations cited in Table II have high cytochrome *b* content. It was proposed that, in addition to its function as an electron carrier, cytochrome *b* has a structural role in the formation of succinate-ubiquinone reductase [43]. In light of recent findings on reconstitution of succinate-ubiquinone reductase, it seems that this proposal must be discarded. None of cytochrome *b* preparations which have been used for reconstitution is pure enough to exclude the possibility that some contaminating protein is responsible for ubiquinone reactivity-conferring property. Moreover, the preparation of QP-S protein described by Yu et al. [4,26] has no peptide other than the 15 000 dalton.

TABLE II

THE PROPERTIES OF SUCCINATE—UBIQUINONE REDUCTASES RECONSTITUTED FROM SOLUBLE SUCCINATE DEHYDROGENASES (SD) AND PROTEIN-CONTAINING FRACTIONS (PF) DERIVED FROM MITOCHONDRIAL PREPARATIONS DIFFERENT DEGREE OF RESOLUTION

| Authors | SD used | Composition of PF | Activity of reconstituted system * (μmol/min) | | Activity in phenazine methosulfate assay (μmol/min per mg SD) | References |
|---|---|---|---|---|---|---|
| | | | per mg SD | per mg PF | | |
| King and Takemori | Butanol extracted ** | cytochromes *b* and $c_1$; other proteins; phospholipids, ubiquinone | 1.4 (23°C) | 0.43 (23°) | 16.5 *** (23°C) | [47] |
| Baginsky and Hatefi | Tris-HCl extracted; chemically reactivated | cytochrome *b*; other proteins, phospholipids | 11.5 (38°C) | 6.0 (38°C) | 37 (38°C) before chemical treatment | [42] |
| Bruni and Racker | Butanol extracted ** | cytochrome *b*; factor $F_4$; other proteins; phospholipids | 1.8 (23°C) | 0.9 (23°C) | 4 (23°C) | [43] |
| Hanstein et al. | Perchlorate extracted | cytochrome *b*; other proteins; phospholipids | 13 (38°C) in succinate oxidase assay | 42.5 (38°C) | 110 (38°C) | [5] |
| McPhail and Cunningham | Butanol extracted ** | cytochrome *b*; other proteins; phospholipids | 0.25 | 0.5 | not determined | [48] |
| Yu et al. | Butanol extracted ** | *M* 15 000 protein; phospholipids (less than 20%) | 21 (23°C) | 30 (23°C) | 18 (23°C) | [4,26] |
| Vinogradov et al. | Butanol extracted ** | three polypeptides; major fraction with *M* 13 000; Triton X-100 | 16 (23°C) | 3.0 (23°C) | 14 (23°C) | this paper |

* All the activites were measured in the presence of added ubiquinone-2 [3] except for those by King and Takemori and Vinogradov et al. The reduction of DCIP was sensitive to thenoyltrifluoroacetone. The figures in the table are given as presented by the aurhors or recalculated for comparison.

** The principal procedure of Keilin and King [15] as modified in different laboratories.

*** This value taken from Ref. 16.

The comparison of protein fraction SDS-electrophoresis pattern with that of QP-S described by Yu et al. [4] shows a remarkable similarity. The difference in molecular weight reported by Yu et al. (15 000) compared to our finding (less than 13 000 for the major fraction) may well be explained by some differences in electrophoresis procedure. QP-S and our protein fraction reveal almost the same catalytic activity of succinate dehydrogenases prepared by similar procedures. 10-times lower turnover of our protein fraction, based on the protein content, compared to that of QP-S [4] may be explained by the difference in the assay procedure used by each group (the addition of ubiquinone-2 in the method of Ziegler and Doeg [3] used by Yu et al. and no addition of exogenous ubiquinone in our work).

The reconstituted system shows most of the properties of the native succinate-ubiquinone region of the respiratory chain. Its activity does not depend on dilution, is perfectly stable under aerobic conditions and sensitive to thenoyltrifluoroacetone and carboxin. Some of the points concerning the arrangement of the succinate-ubiquinone region of the respiratory chain deserve special discussion.

(i) Judging from the kinetics of ubiqquinone reductase formation (Fig. 3) and independence of the activity on dilution, it might be concluded that a simple complex with fixed stoichiometry between succinate dehydrogenase and protein fraction is formed during reconstitution. The existence of such a complex as a natural constituent of the respiratory chain has been suggested in earlier papers by Green [44] and seemed to be supported experimentally [5]. (ii) No sharp end-point in titration curves is, however, observed (Fig. 7a and b) when the formation of the complex is measured through the appearance of the active succinate-ubiquinone reductase. Qualitatively the same behavior has been observed when succinate-ubiquinone reductase [2,5] or succinate oxidase [15,16] have been reconstituted in experiments in which one component was titrated by the other. (iii) The comparison of the titration curves for binding (measured as disappearance of ferricyanide reactive site [19,20] and parallel increase of activity (Fig. 8)) shows that a clear quantitative difference exists between these two. (iv) The dependence of the $K_m$ for DCIP on the relative proportions of succinate dehydrogenase and the protein fraction is consistent with the model in which two redox components react according to the Law of Mass Action [21], e.g., in homogeneous solution.

The apparent contradictions between the points mentioned above can be solved by utilizing the following model for arrangement of the succinate-ubiquinone region of the respiratory chain. Succinate dehydrogenase (like, perhaps, NAD · H dehydrogenase) reacts with a specific component in the protein fraction with the formation of a tight complex which is identical to that existing in the native respiratory chain. The active component in the protein fraction may be considered, therefore, as a binding site on the mitochondrial membrane for succinate dehydrogenase. The other function of this component is to make the Hipip center of succinate dehydrogenase reactive with the bulk of ubiquinone freely solubilized in the hydrophobic region of the membrane. The latter function may be fulfilled either by the specific binding of ubiquinone at the active site of the protein fraction in a form which is highly reactive towards Hipip or by the changing of the Hipip center in such

a way that it becomes reactive with lipid-soluble ubiquinone. This model is consistent with the experimental results reported in this paper. It also explains the absence of simple stoichiometry between succinate dehydrogenase and cytochromes in the respiratory chain [45,46].

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