Biochimica et Biophysica Acta 809 (1985) 145–159
Elsevier

BBA 41816

Studies on the succinate dehydrogenating system. Isolation and properties of the mitochondrial succinate-ubiquinone reductase

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(Received March 5th, 1985)
(Revised manuscript received May 30th, 1985)

Key words: Succinate-ubiquinone reductase; Succinate dehydrogenase; Protein-detergent interaction; Respiratory chain; (Bovine heart mitochondria)

A simple procedure for preparation of highly purified soluble succinate-ubiquinone reductase from bovine heart mitochondrial particles is described. The enzyme exhibits four major bands on sodium dodecyl sulfate gel electrophoresis and contains (nmol per mg protein): covalently bound flavin, 6; non-heme iron, 53; acid-labile sulfur, 50; cytochrome *b*-560 heme, 1.2. The enzyme catalyzes thenoyltrifluoroacetone, or carboxin-sensitive (pure non-competitive with Q_2) reduction of Q_2 by succinate with a turnover number close to that in parent submitochondrial particles. The succinate reduced enzyme exhibits ferredoxin-type iron-sulfur center EPR-signal ($g = 1.94$ species) and a semiquinone signal ($g = 2.00$). An oxidized preparation shows a symmetric signal centered around $g = 2.01$. An unusual dissociation of the enzyme in the absence of a detergent is described. When added to the assay mixture from a concentrated protein-detergent solution, the enzyme does not reduce Q_2 being highly reactive towards ferricyanide ('low K_m ferricyanide reactive site'; Vinogradov, A.D., Gavrikova, E.V. and Goloveshkina, V.G. (1975) Biochem. Biophys. Res. Commun. 65, 1264–1269). The ubiquinone reductase, not the ferricyanide reductase was observed when the enzyme was added to the assay mixture from the diluted protein-detergent solutions. Thus the dissociation of succinate dehydrogenase from the complex occurs in the absence of a detergent dependent on the concentration of the protein-detergent complex in the stock preparation where the samples for the assay are taken from. An active antimycin-sensitive succinate-cytochrome *c* reductase was reconstituted by admixing of the soluble succinate-ubiquinone reductase and the cytochrome b-c_1 complex, i.e., from the complexes which both contain the ubiquinone reactivity conferring protein (QP_s). Cytochrome *c* reductase was also reconstituted from the succinate-ubiquinone reductase and succinate-cytochrome *c* reductase containing inactivated succinate dehydrogenase. The reconstitution experiments suggest that there exists a specific protein-protein (or lipid) interaction between QP_s and a certain component(s) of the b-c_1 complex.

Abbreviations: Q_2, Q_6, homologs of ubiquinone having two or six isoprenoid units in position 6 of the quinone ring; WB, (Wurster's Blue), a semiquinonediimine radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol; carboxin, 5,6-dihydro-2-methyl-1,4-oxathiin-3-carbaxanilide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *p*-CMB, *p*-chloromercuribenzoate; Mes, 4-morpholineethanesulfonic acid.

Introduction

Despite the significant progress has been achieved in several laboratories on the structure and catalytic properties of the succinate-ubiquinone region of the respiratory chain, much

still remains to be learned about the minimal structure and catalytic mechanism of the enzyme which catalyze the reduction of ubiquinone by succinate. Since the early isolation of complex II from the inner mitochondrial membrane [1,2], this preparation has been widely used for the kinetic studies of the succinate-ubiquinone oxido-reductase reaction [3–5] and as a starting material for the resolution and reconstitution studies [2,6,7]. It may be deduced from the results of the recent reconstitution experiments [6–9] that the mammalian succinate-ubiquinone reductase consists of the two-subunit iron-sulfur flavoprotein (reconstitutively active succinate dehydrogenase, EC 1.3.99.1 [10]), and one or two smaller peptides which are responsible for the specific catalytically competent interaction between ubiquinone and the dehydrogenase iron-sulfur center S-3 [11,12]. Until recently, such a deduction has not been substantiated by the isolation of three or four peptide-containing succinate-ubiquinone reductase from the inner mitochondrial membrane. Complex II as prepared in different laboratories [13,14] contains up to ten peptides; four of them were shown to be an intrinsic part of the enzyme, whereas the rest are the contaminations originated mostly from complex III. Recently, Yu and Yu [15] have succeeded in the resolution of the succinate-cytochrome *c* reductase on succinate-ubiquinone reductase and *b*-c_1 complex.

During our continuous efforts to elucidate the mechanism of succinate oxidation by the mammalian respiratory chain, a simple high yield procedure for the preparation of the soluble succinate-ubiquinone reductase was developed. The aim of this communication is to describe this procedure and to report some molecular and catalytic properties of the enzyme. Some results obtained with the enzyme prepared by our method have been published elsewhere [16–19].

Materials and Methods

Preparative procedures

The Keilin-Hartree bovine heart muscle preparation [20] and submitochondrial particles [21] were prepared by the conventional procedures. The soluble reconstitutively active succinate dehydrogenase was prepared as described [9], using succinate-ubiquinone reductase (see Results section) instead of the Keilin-Hartree preparation as a starting material. The succinate-cytochrome *c* reductase and soluble *b*-c_1 [22] complex were prepared according to the published procedures. The *N*-ethylmaleimide-inhibited succinate-cytochrome *c* reductase was prepared as follows. The dialyzed (24 h) succinate-cytochrome *c* reductase was resuspended (2 mg/ml) in a mixture containing: 50 mM phosphate/10 mM succinate/1.5 mM cyanide (sodium salts, pH 7.8) and incubated for 30 min at 30°C. 2 mM *N*-ethylmaleimide was then added and incubation was continued until the succinate-PMS reductase activity dropped to zero. The mixture was diluted 10-times with the same cold buffer and centrifuged at $20\,000 \times g$ for 30 min. The sediment was washed with the same buffer by the repeating centrifugation and suspended (7.5 mg/ml) in 20 mM sodium phosphate/10 mM sodium succinate (pH 7.4). The final preparation showed no the succinate-PMS reductase activity. The 'activated' *b*-c_1 complex [24] was prepared as follows. 8 mg of the extensively dialyzed *b*-c_1 complex [22] were incubated with 30 μg Q_6 at room temperature for 15 min in a mixture containing 0.25 M sucrose/25 mM sodium phosphate (pH 7.4)/0.2 mM EDTA; 4 mg sonicated phospholipids were then added and incubation was continued for 20 min. The preparations prepared as described were used in the reconstitution experiments. Partially purified soya bean phospholipids [23] were used for 'activation' of the *b*-c_1 complex.

Analytical methods

The succinate-acceptor oxidoreductase activities were measured in the standard mixture comprising 20 mM Hepes, 20 mM potassium succinate, 0.004% Triton X-100, the electron acceptors and 0.2 mM EDTA (pH 7.8) at 25°C and expressed as micromoles of succinate oxidized per min per mg protein. The optical spectra were recorded on a Hitachi-200-2 spectrophotometer at 20°C. EPR-measurements were made, using Varian E-109E spectrometer with temperature control device. The samples (0.2 ml) were frozen in quartz tubes and stored in liquid nitrogen. The quantitation of spins were made, using Cu-EDTA standards.

SDS-12.5% acrylamide gel electrophoresis was

performed according to Laemmli [25]. The protein bands were stained with Coomassie brilliant blue R-250 for 4 h.

The protein content was determined by the biuret method [26] after precipitation by trichloroacetic acid or as described for Triton X-100 containing samples [27]. Acid labile sulfide [28] and acid non-extractable flavin [29] were estimated by the published procedures. Iron content was determined essentially as described [30] except for extraction procedure which was performed in 1 M HCl for 1 h at 100°C. The samples taken for analysis of flavin, iron and acid-labile sulfide were extensively dialysed against triple-distilled water. Heme content was determined spectrophotometrically and by the hemochromogen method [31]. Triton X-100 content was determined colorimetrically [32]. Ubiquinone was extracted and determined essentially as described by Redfearn [33].

WB was prepared from *N,N,N',N'*-tetramethyl-*p*-phenylene diamine [34]. Calcium phosphate gel was prepared as described and stored for 2 weeks before use [35]. The special chemicals were: Triton X-100 from Loba chemie (Austria), Cholic acid from Aldrich (U.S.A.), PMS from Lawson (U.K.), DCIP from General Biochemicals (U.S.A.), Q_2 from Ferak, Berlin. Carboxin was a kind gift from Prof. H. Lyr (Institute of Plant Protection Research, G.D.R.). All other chemicals used were of highest quality commercially available. For other experimental details see Legends to the figures and tables.

Results

Preparation of the succinate-ubiquinone reductase

Step 1. The Keilin-Hartree bovine heart muscle preparation was suspended (final protein content, 20–30 mg per ml) in a solution comprising 0.1 M phosphate/40 mM succinate/1 mM EDTA (sodium salts, pH 7.5) and equal volume of a mixture containing 0.1 M phosphate/4 mM EDTA (sodium salts, pH 7.5) and 4 M urea was added. The suspension was incubated with a constant stirring for 30 min and centrifuged for 90 min at 105 000 × *g*. The supernatant containing about 30% of the original protein was discarded, and the sediment was suspended in the buffer containing 20 mM Tris-sulfate/20 mM succinate/0.1 mM EDTA (pH 7.5) and washed by repeated centrifugation. The particles obtained were suspended in 0.25 M sucrose and frozen at −20°C overnight.

Step 2. The suspension was thawed, thoroughly homogenized (glass/teflon) and diluted to the final protein content of 10 mg per ml in the washing buffer (see above). 10% Triton X-100 solution was added upon constant stirring up to the final concentration of the detergent 0.8 mg per mg of protein, the mixture was incubated for 30 min, pH was then adjusted to 5.8 by the addition of 1 M acetic acid, and the mixture was centrifuged for 30 min at 40 000 × *g*. The yellow supernatant was collected and pH was adjusted to 7.4. The sediment was resuspended in 0.8 vol. of the same buffer, pH of the suspension was adjusted to 7.4, and the extraction procedure was repeated with the amount of Triton X-100 of 0.5 mg per mg of the original protein. Two extracts containing up to 90% of the succinate-ubiquinone reductase activity were combined and subjected to further purification; the residue was discarded or saved for preparation of other constituents of the respiratory chain. To the combined extracts equal volume of cold (−20°C) acetone was slowly added, a copious precipitate was immediately collected by centrifugation for 5 min at 3000 × *g* and suspended in a small volume of a buffer containing 0.1 M phosphate/20 mM succinate/0.2 mM EDTA (sodium salts, pH 7.8).

Step 3. The volume of the suspension was adjusted by the same buffer up to one half of the combined Triton X-100 extract volume and sodium cholate (the final concentration 1%) and solid $(NH_4)_2SO_4$ (30% of saturation) were added, while pH was maintained at 7.8 by the addition of concentrated ammonium hydroxide. The mixture was stirred for 30 min and centrifuged for 15 min at 20 000 × *g*. The residue was discarded, and ammonium sulfate concentration in the supernatant was adjusted to 50% of saturation. After 30 min incubation the precipitated material was collected by centrifugation for 15 min at 20 000 × *g* and dissolved in the buffer containing 20 mM phosphate/20 mM succinate/0.1 mM EDTA (sodium salts, pH 7.8)/0.1% Triton X-100. The concentration of protein was adjusted to about 10 mg per ml.

Step 4. 7 vol. of water and calcium phosphate

gel (10 mg dry wt per mg protein) were added, and after stirring for 10 min the gel containing adsorbed protein was collected by centrifugation for 10 min at $15000 \times g$, and washed by 20 mM neutralized succinate solution containing 0.1% Triton X-100 and 0.1 mM EDTA. The washed gel was collected and thoroughly suspended in a solution containing 20 mM phosphate/20 mM succinate/0.1 mM EDTA (sodium salts, pH 7.8) and 0.1% Triton X-100. The amount of the eluting buffer was 2-times higher than that used for adsorbtion of the enzyme on calcium phoshate gel.

Step 5. To the clear yellow eluate solid ammonium sulfate was slowly added up to 50% of saturation and after 15 min the turbid mixture was centrifuged for 20 min at $15000 \times g$. The oily brown floating material was collected, dissolved in a small volume of a desired buffer containing succinate and EDTA and frozen. The enzyme can be stored for indefinitely long time in liquid nitrogen; it slowly loses the activity during the storage at $-20°C$ (about 30% during 3 weeks). If desired the protein can be made insoluble by the repeating precipitation by cold acetone (see step 2) without significant loss of the activity. The catalytic activity of the preparation measured with either PMS or Q_2 as electron acceptors at 25°C (pH 7.8) varies from 17 to 30 μmol succinate oxidized per min per mg protein. No detectable cytochrome *c* oxidase or NADH-ferricyanide reductase activities were found in the purified preparations. More than 15 preparations were made in this laboratory without any significant variations. The Keilin-Hartree heart muscle preparation or ultrasonic submitochondrial particles used as a starting material produce the same final preparations in terms of their polypeptide composition and the molecular activity. A protocol of a typical preparation procedure is shown in Table I.

Polypeptide composition and chemical constituents

The succinate-ubiquinone reductase prepared by our methods exhibits four major bands when subjected to SDS-electrophoresis (Fig. 1). Two of them with M_r 74 and 26 kDa belong to succinate dehydrogenase as proved by isolation of the soluble reconstitutively active succinate dehydrogenase from the complex. The resolution of low molecular weight region of the gel slab is not high and two or three small peptides is seen in the complex. When gel slabs were subjected to microfluorometric scanning after trichloroacetic acid treatment one fluorescence band coincident with the largest peptide was detected suggesting that this subunit contains covalently bound flavin (panel B, lowest trace). The chemical composition of the complex was

TABLE I

PURIFICATION OF SUCCINATE-UBIQUINONE REDUCTASE

Fraction	Volume (ml)	Protein (mg)	Total activity (succinate → Q_2) [a] (μmol per min)	Specific activities (μmol per mg per min)		Purification (-fold)	Yield (%)
				Succinate → PMS	Succinate → Q_2 [a]		
Keilin-Hartree preparation	240	4500	3240 [b]	0.7	0.7	1	100
Urea washed particles	340	3125	3000 [b]	0.9	0.9	1.3	93
Combined Triton X-100 extracts	600	750	3000	4.0	4.0	5.7	93
Cholate-$(NH_4)_2SO_4$ extract (step 3)	345		2500				77
Calcium phosphate gel eluate	150	50	1190	24	24	34	37
$(NH_4)_2SO_4$ precipitate	5	30	713	24	24	34	22

[a] Carboxin-sensitive Q_2-mediated reduction of WB.

[b] Activated by incubation for 30 min at 25°C in the presence of 20 mM succinate and measured in the presence of 2 mM cyanide.

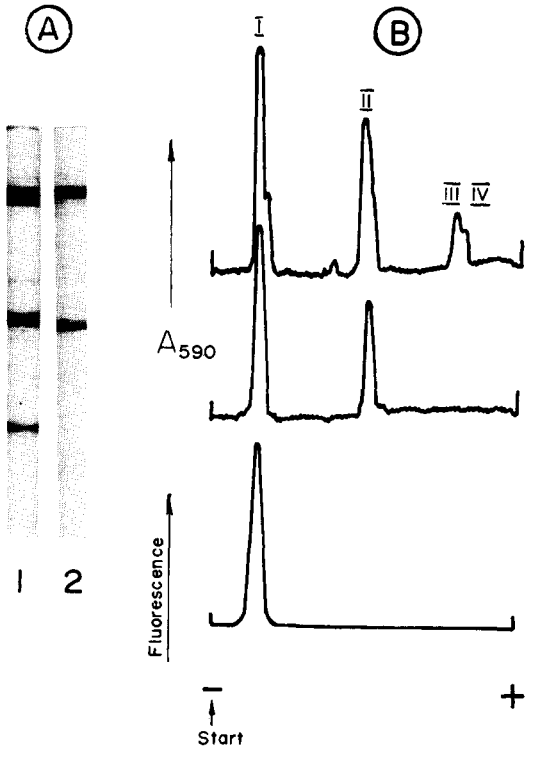

Fig. 1. Polypeptide composition of succinate-ubiquinone reductase. (A) SDS-electrophoretic patterns of succinate-ubiquinone reductase (40 μg, 1) and succinate dehydrogenase isolated from succinate-ubiquinone reductase (20 μg, 2) as appear after protein staining with Coomassie brilliant blue R-250. (B) Two upper curves are the densitometric traces of the gels 1 and 2 (panel A), respectively; lower curve is the microfluorometric scan of trichloroacetic acid fixed succinate-ubiquinone reductase after SDS-electrophoresis (excitation at 410 nm, emission at 540 nm). The molecular masses for the peptides I–IV determined from the calibration plot are 74, 26, 16 and 15 kDa, respectively.

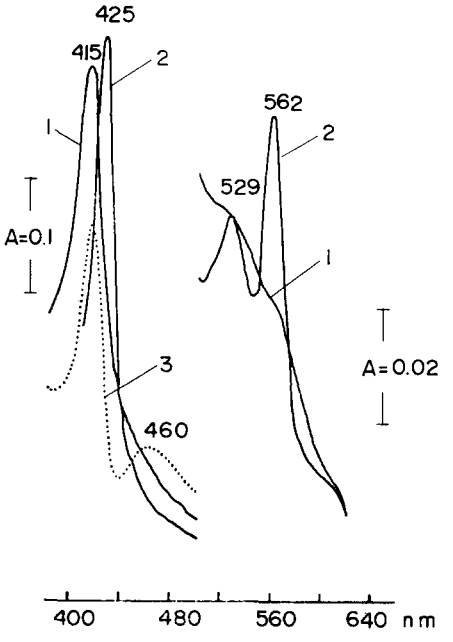

Fig. 2. Absorption spectra of succinate-ubiquinone reductase (0.7 mg per ml in 20 mM Hepes/2 mM succinate/0.2 mM EDTA/0.1% Triton X-100, pH 7.8, 23°C). (1), the enzyme as prepared; (2), reduced by $Na_2S_2O_4$; (3), oxidized enzyme (20 mM malonate and 0.5 mM ferricyanide were added to the sample and to the control cuvette).

found as follows (nmol or ngatom per mg of protein): acid non-extractable flavin, 5.9–6.3; non-heme iron, 52–54; acid-labile sulfur, 49–50; ubiquinone, 2–3; heme *b*, 1.2 (by pyridine hemochromogen method). No cytochrome c_1 heme was detected.

Spectral properties

The enzyme prepared by the described procedure is soluble and gives perfectly transparent coloured solution in the absence of a detergent. Approx. 2 mg of Triton X-100 per mg of purified preparation was determined in the final preparation by the colorimetric procedure [32]. The absorption spectra of the enzyme in the visible region are shown in Fig. 2. The spectrum of the enzyme as prepared (i.e., reduced by succinate) exhibits one sharp band at 415 nm and the shoulders at 529 and 562 nm. The reduction by $Na_2S_2O_4$ results in a shift of the peak to 425 nm, and in an appearance of two other bands with maxima at 529 nm and 562 nm, thus indicating the presence of dithionite reducible cytochrome *b* [36]. An oxidized enzyme (ferricyanide as oxidant) shows the same 415 nm peak as that detected in the succinate-reduced preparation and a distinct broad band with a maximum at 460 nm. Since the strong interference between the absorption due to heme *b*, iron-sulfur centers and flavin should be expected, the clear interpretation of the spectra is hardly possible. The most pronounced difference between the oxidized and succinate-reduced enzyme is the band at 460 nm which, most probably, belongs to an oxidized flavin.

The EPR-spectra of the reduced and oxidized

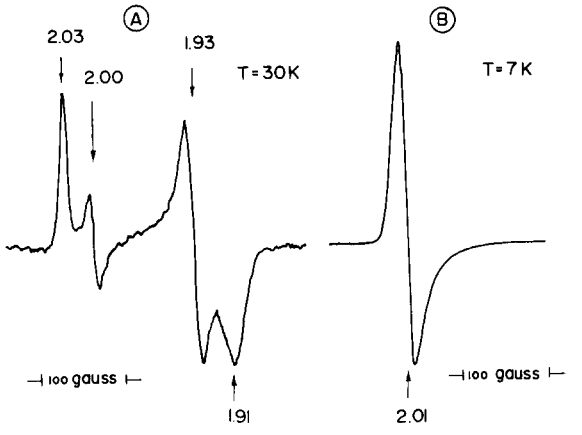

Fig. 3. EPR spectra of succinate-ubiquinone reductase. (A) Succinate-reduced enzyme (5.6 mg per ml in 20 mM Tris-sulfate/0.2 mM EDTA/20 mM succinate, pH 7.5). (B) Oxidized enzyme. The protein was diluted in the mixture containing 20 mM Tris-sulfate/5 mM malonate/0.2 mM EDTA (pH 7.5) and dialyzed for 1 h at room temperature against the same buffer; 70 mM malonate/50 μM PMS/200 μM potassium ferricyanide were then added and the mixture was incubated for 30 s before freezing; the final protein concentration was 5.6 mg per ml. EPR operating conditions: modulation frequency, 100 kHz; modulation amplitude, 6.3 gauss; microwave power, 1 and 3 mW for (A) and (B), respectively.

preparations are shown in Fig. 3. The succinate-reduced enzyme exhibits ferredoxin-type iron-sulfur center signal ($g = 1.94$ species) shown in panel A and a semiquinone signal with $g = 2.00$. A relatively symmetric signal centered around $g = 2.01$ is seen in oxidized preparations of the enzyme which is identical to that described for the membrane-bound [3,37,38] and for the soluble reconstitutively active succinate dehydrogenase [39,40] as center S-3. When spin concentrations in the succinate reduced and PMS/ferricyanide oxidized preparations were quantitated, the ratio of 1 : 0.83 : 0.1 : 0.97 for acid non-extractable flavin, S-1 center, semiquinone and S-3 center, respectively, was determined.

The substrate binding site and reactivity of the enzyme with the electron acceptors

The enzyme catalyzes the oxidation of succinate by a number of artificial electron acceptors demonstrating a simple hyperbolic dependence of the reaction rate on succinate concentration and a simple hyperbolic competitive inhibition by malonate and fumarate. The values of K_m and K_i determined at 25°C in 20 mM Hepes (pH 7.8) by the conventional procedures are: $K_m^{succinate}$, 20 μM (3 μM Q_2 and 40 μM WB as the electron acceptors) $K_i^{fumarate}$, 40 μM; $K_i^{malonate}$, 0.25 μM. No significant difference in the kinetic constants determined for soluble succinate-ubiquinone reductase and those recently reported for submitochondrial particles [41] was found, which suggests that no allotropic changes occur in the substrate binding site containing subunit of succinate dehydrogenase after solubilization.

The relative reactivities of the enzyme with some commonly used artificial electron acceptors are given in Table II. The same activities were revealed when Q_2 was the only acceptor or Q_2-mediated WB reduction was measured suggesting that the reduction of WB by the reduced quinone is not rate limiting. Although the small fraction of Q_2-mediated WB reduction is carboxin insensitive (apparently due to the direct electron transfer from flavoprotein to WB [42]) this assay system seems to be the most satisfactory one because of its convenience and high sensitivity. About only 10% of the total carboxin-sensitive activity was revealed with WB in the absence of exogenous quinone, thus indicating that the enzyme is deficient in functionally active ubiquinone. As expected the enzyme rapidly reduces PMS and a fraction of the electron flow in this assay is blocked by carboxin thus suggesting the existence of at least two sites of PMS reduction. In agreement with the earlier observations [8,9] and in contrast with the soluble reconstitutively active succinate dehydrogenase [12,43], the enzyme does not interact with hydrophylic ferricyanide. However, as will be shown below, the reactivity of the enzyme to ferricyanide can be induced under certain specific assay conditions.

Stability of the enzyme

As shown in Fig. 4, the enzyme is remarkably stable: it can be stored at pH 6.0 in the presence of 0.1% Triton X-100 for about 20 h without detectable loss of activity even at room temperature. The enzyme is almost instantly inactivated at pH below 5 and the apparent optimum of the stability at pH 6.0 was found.

TABLE II

DYE REDUCTASE ACTIVITIES OF THE SUCCINATE-UBIQUNONE REDUCTASE

25°C, 20 mM potassium succinate/20 mM Hepes (pH 7.8)/0.004% Triton X-100.

Electron acceptors	Relative activity		Inhibition (%)
	no inhibitor	+20 μM carboxin	
Q_2 (3 μM) → WB (40 μM)	1.00 [a]	0.10	90
Q_2 (3 μM)	0.90	– [b]	
Q_0 (3 μM) → WB (40 μM)	0.24	0.06	75
Q_1 (3 μM) → WB (40 μM)	0.36	0.08	88
Q_6 (3 μM) → WB (40 μM)	0.16	0.08	50
WB (150 μM)	0.43	0.20	53
PMS (2 mM) → DCIP (50 μM)	0.87	0.56	35
DCIP (50 μM)	0.11	0.00	100
Ferricyanide (0.2 mM)	0.06	0.02	67
Ferricyanide (2 mM)	0.05	0.02	60
WB (40 μM)	0.09	0.06	33

[a] The arbitrary unit corresponds to the activity of 32 μmol succinate oxidized per min per mg of protein.

[b] Due to high absorption of carboxin at 275 nm the sensitivity to the inhibitor in the direct succinate-Q_2 assay [17] was not measured.

Effect of the detergent on the catalytic properties of the enzyme

Although the enzyme as prepared contains considerable amounts of Triton X-100, the rate of Q_2 reduction strongly depends on the concentration of the detergent in the assay mixture (Fig. 5). The inhibitory effect of high Triton X-100 concentrations can be partially relieved by the higher level of Q_2 in the assay.

At the beginning of our work it has been noted that the activities determined without Triton X-100 added to the assay system were poorly reproduci-

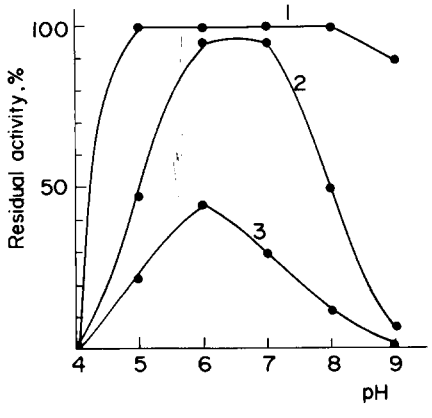

Fig. 4. Stability of succinate-ubiquinone reductase. The enzyme (0.2 mg/ml) was incubated at 20°C in a mixture containing 20 mM Mes, 20 mM Tris, 20 mM succinate and 0.2 mM EDTA (potassium salts) at pH indicated on abscissa for 1, 20 and 44 h (curves 1, 2 and 3, respectively). The succinate-ubiquinone reductase was then assayed in the standard reaction mixture with 3 μM Q_2 as the acceptor. 100% correspond to the original activity of 27 μmol succinate oxidized per min per mg of protein.

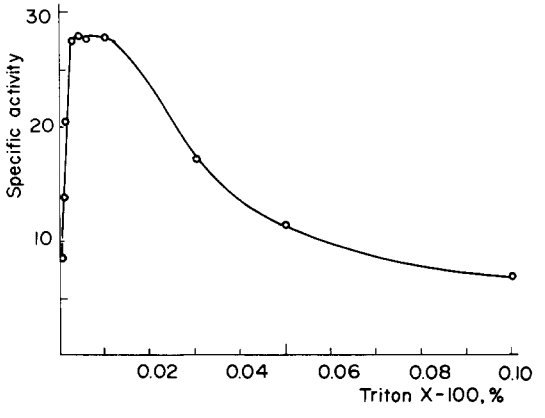

Fig. 5. Effect of Triton X-100 in the assay mixture on the activity of succinate-ubiquinone reductase. The Q_2-mediated WB reduction was measured in the standard assay mixture containing indicated amount of Triton X-100. Zero concentration of Triton X-100 on abscissa corresponds to the actual value of $1.7 \cdot 10^{-4}$% which was added with the enzyme preparation.

ble even when the same batches of the enzyme were assayed. Much time was spent in attempts to find out possible reasons for this apparently uncontrolled variations in the activity, and finally an unusual property of the enzyme to exist in two alternative states was discovered. This property is demonstrated in Fig. 6, where the specific succinate-ubiquinone reductase activity is plotted as a function of protein concentration in the stock solution where the samples for the assay were taken from. As shown in Fig. 6 the specific activity revealed in the carboxin-sensitive Q_2 reduction assay without added Triton X-100 is strongly dependent on protein concentration in the stock solution (note that the final concentrations of all components in the assay mixture are identical for any point on the curves). No such difference in the specific activity was found when optimal concentration of Triton X-100 (0.004%, see Fig. 5) was present in the assay mixture, or when the succinate-PMS reductase was measured. It was found

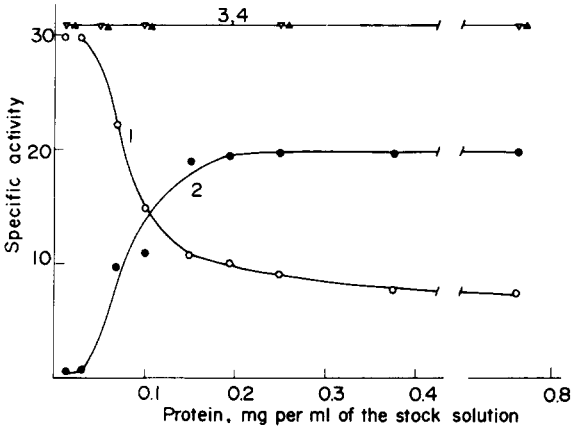

Fig. 6. Dependence of the succinate-acceptor reductase activities of succinate-ubiquinone reductase assayed without added Triton X-100 on the protein concentration in the stock solution of the enzyme. The protein was diluted to the concentrations indicated on abscissa and the activities were assayed in the standard mixture containing no Triton X-100 with 3 μM Q_2 (curve 1), ferricyanide (curve 2, V_{max} values obtained by extrapolation using 50, 100, 150 and 200 μM of the acceptor), and 2 mM PMS (curve 3). Curve 4, the assay mixture contained 3 μM Q_2 and 0.004% Triton X-100. All the points on each curve correspond to the same final concentrations of the protein (and detergent) in the assay mixtures which were: 0.2 mg/ml for the curves 1, 3 and 4), and 2 mg/l for curve 2.

in the special experiments (data are not shown) that: (i) the enzyme is perfectly stable during storage at room temperature at any concentrations shown in abscissa of Fig. 6 for the time of the experiments; (ii) the same rates of Q_2 reduction were reached when the reaction was started either by the enzyme or by Q_2; (iii) the linear dependence of Q_2 reduction rate on the amount of protein added to the assay mixture was observed at all protein concentrations in the stock solutions providing that the amount of Triton X-100 added with the protein does not exceed 0.0004%.

The results presented in Fig. 6 suggest that succinate-ubiquinone reductase appears in the assay mixture either in the active or inactive states depending on the concentration of the protein-detergent complex in the stock solution.

It is well established that the membrane-bound succinate dehydrogenase or native and reconstituted succinate-ubiquinone reductase do not reduce low concentrations of ferricyanide, whereas the soluble reconstitutively active succinate dehydrogenase catalyzes succinate low 'K_m' ferricyanide reductase reaction [12,42]. Thus it seemed of interest to examine the reactivity of 'inactive' succinate-ubiquinone reductase towards low concentrations of ferricyanide under the conditions where the quinone reactivity was inhibited. As shown in Fig. 7 the 'inactive' succinate-ubiquinone reductase (taken from the concentrated solution) rapidly reduces 150 μM ferricyanide, whereas the 'active' enzyme (taken from diluted solution) does not interact with the hydrophylic electron acceptor. The reactivities of the enzyme towards Q_2 and ferricyanide as a function of protein concentration in the stock solution were found to be reciprocal and the sum of both activities is constant and equal to that determined in the presence of Triton X-100 with Q_2 or with PMS (Fig. 6).

These results strongly suggest that the inactivation of succinate-ubiquinone reductase after rapid dilution (100-fold and more) in the assay without Triton X-100 is due to the dissociation of the succinate dehydrogenase from the complex. Additional evidence for such an interpretation is given in Table III, where some parameters of the 'low K_m ferricyanide reductase' activities of soluble succinate dehydrogenase and 'inactive' succinate-ubiquinone reductase are compared. We have

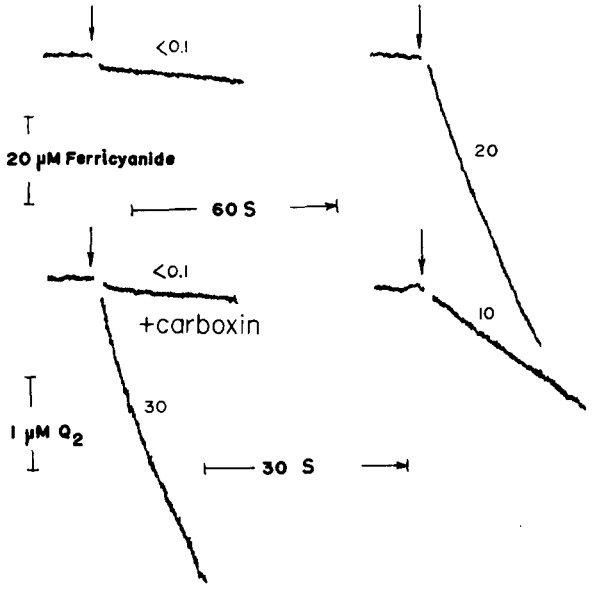

Fig. 7. The succinate-acceptor reductase activities of succinate-ubiquinone reductase assayed without Triton X-100. Upper curves, 150 μM ferricyanide was used as the acceptor; on the left, the reaction was started by addition of the enzyme taken from diluted solution (0.03 mg/ml); on the right, the reaction was started (indicated by the arrows) by the addition of the enzyme taken from concentrated solution (0.6 mg/ml); the final concentration of the protein in the assay mixture was 2 mg/l. Lower curves, 3 μM Q_2 was used as the acceptor; left and right curves, the reaction was started by the enzyme taken from diluted and concentrated enzyme solution respectively as indicated above. The final protein concentration in the assay mixture was 0.2 mg/l. 20 μM carboxin was added to the assay mixture where indicated. The figures on the curves indicate the specific activities of the enzyme.

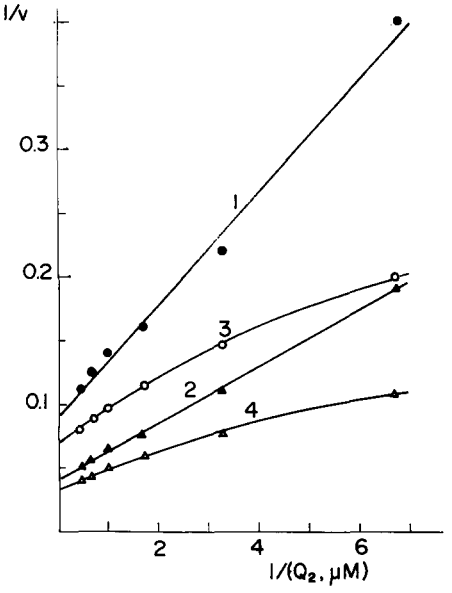

Fig. 8. The kinetics of Q_2 reduction by succinate-ubiquinone reductase. Q_2-mediated WB reduction rates were determined in the standard mixture as a function of Q_2 concentration without added Triton X-100 (lines 1 and 2) or in the presence of 0.004% Triton X-100 (curves 3 and 4). Curves 1 and 3, 2 μM carboxin was present in the assay mixture.

shown recently that carboxin protects an alkali-induced dissociation of succinate dehydrogenase from submitochondrial particles [44]. In agreement with those results it was found that the appearance of ferricyanide reductase activity induced by rapid dilution of succinate-ubiquinone reductase is also sensitive to carboxin (the results are not shown).

Kinetics of Q_2 reduction

In contrast with other purified succinate-ubiquinone reductases [1,2,15], our preparation does not significantly reduce WB or DCIP without exogenous quinone (Table III). Thus kinetic studies of quinone interaction with the specific site of the enzyme seemed to be a promising approach. Fig. 8 demonstrates the simple saturation kinetics

TABLE III

SOME PROPERTIES OF RAPIDLY DILUTED SUCCINATE-UBIQUINONE REDUCTASE AND, SOLUBLE RECONSTITUTIVELY ACTIVE SUCCINATE DEHYDROGENASE

Succinate-ubiquinone reductase was taken from concentrated stock solution (0.3 mg/ml) and rapidly added to the standard assay mixture containing no Triton X-100 (the final protein concentration of the enzyme in the assay mixture was 0.8 mg/l). Reactivity with ferricyanide was measured immediately and during further incubation in the assay medium at 25°C. The same experimental procedure was done using soluble succinate dehydrogenase prepared as described in Ref. 9 (the final protein concentration was 0.8 mg/l).

Parameters	Diluted succinate-ubiquinone reductase	Soluble succinate dehydrogenase
Reactivity towards ferricyanide	+	+
$K_m^{\text{ferricyanide}}$ (μM)	80	100
Stability of ferricyanide reductase ($t_{1/2}$, min)	22	16
Sensitivity to CMB [a]	+	+

[a] 10 μM *p*-CMB was added to the assay mixture before the enzyme preparation.

of Q_2 reduction in the absence of added Triton X-100. Since OH_2 instantly reduces WB in aqueous solutions [45] (this is not the case for DCIP), the hyperbolic dependence of the reaction rate on Q_2 apparently corresponds to saturation of the specific binding site by the added quinone. The linearization procedure gives an apparent K_m value of 0.5 μM. The reduction of Q_2 was almost completely sensitive to carboxin and the K_i value for this inhibitor determined from the Dixon plot (Fig. 9) was 1 μM independently of the Q_2 concentrations used (0.6 and 2 K_m, respectively). The same pure non-competitive inhibition of the enzyme by thenoyltrifluoroacetone was found with the K_i value of 8 μM. More complex than the simple hyperbolic saturation behaviour was observed when Triton X-100 was present in the assay mixture.

Reconstitutive properties of the enzyme

The reconstitution has proved to be the most reliable test for the activity of isolated components of the respiratory chain [46]. It was of interest to compare the properties of our preparation with those of soluble succinate dehydrogenase in the reconstitution tests. As shown in Table VI succinate-cytochrome *c* reductase can be reconstituted by admixing of succinate-dehydrogenase and the b-c_1 complex. An unexpected finding documented in the table is that succinate-cytochrome *c* reductase appears after mixing the succinate-ubiquinone reductase and the b-c_1 complex, i.e., from the complexes which both contain the ubiquinone reactivity conferring protein (QP_s). It should be emphasized that the observed succinate-cytochrome *c* reductase activity does not result from the independent operation of quinone-reducing and quinol-oxidizing systems, since no Q_2 was added to the assay mixture and Q_6 used for 'activation' of the b-c_1 complex does not act as an electron acceptor for succinate-ubiquinone reductase (Table II). Further evidence for the structural association of the complexes was

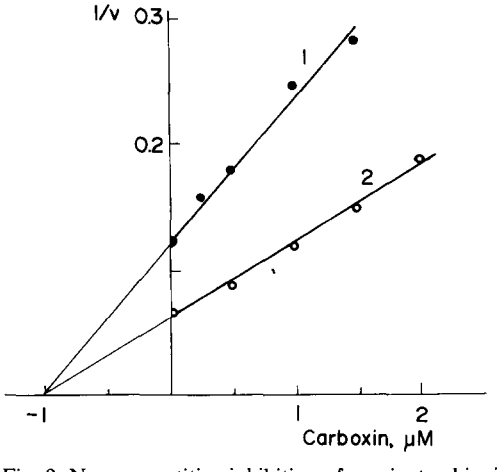

Fig. 9. Non-competitive inhibition of succinate-ubiquinone reductase by carboxin. The activities were determined as indicated in Fig. 5 in the presence of 0.3 μM (line 1) and 1.0 μM (line 2) Q_2.

TABLE IV

RECONSTITUTION OF SUCCINATE CYTOCHROME *c* REDUCTASE FROMT HE PURIFIED COMPONENTS

The reconstitution was started by the addition of the succinate dehydrogenase-containing preparations (limiting amounts). The final concentrations of the enzyme preparations in the reconstitution mixture (50 mM phosphate/20 mM succinate, pH 7.8) were: b-c_1 complex and NEM (*N*-ethylmaleimide)-treated succinate cytochrome *c* reductase, 5 mg/ml, succinate-ubiquinone reductase, 0.15 mg/ml, succinate dehydrogenase, 0.3 mg/ml. After 30 min incubation at 25°C the reconstituted systems were diluted 4-times, cooled and stored in ice. The proper amount of the samples were withdrawn and assayed in the standard mixture containing 33 μM cytochrome *c*. All the activities were antimycin A-sensitive.

Preparations	Activity (per mg limiting component)
1 b-c_1	0
2 Succinate dehydrogenase	0
3 b-c_1 + succinate dehydrogenase	8.7
4 Succinate-ubiquinone reductase	0
5 b-c_1 + succinate-ubiquinone reductase	16.7
6 NEM-treated succinate-cytochrome *c* reductase	0
7 NEM-treated succinate-cytochrome *c* reductase + succinate dehydrogenase	0
8 NEM-treated succinate-cytochrome *c* reductase + succinate-ubiquinone reductase	7.8

obtained when the reconstituted system (Table IV, sample No. 5) was sedimented at $20000 \times g$ and both the succinate-ubiquinone reductase and succinate cytochrome *c* reductase activities were quantitatively recovered in the precipitated material. The results obtained suggest there exist a specific protein-protein interaction between the succinate-ubiquinone and ubiquinol-cytochrome *c* reductases. Additional evidence for different types of interaction between the components within the complexes formed from either soluble succinate dehyrogenase or soluble succinate-ubiquinone reductase and the b-c_1 complex was obtained in the experiments with succinate-cytochrome *c* reductase , in which succinate dehydrogenase was specifically inactivated by *N*-ethylmaleimide (Table IV, samples 7 and 8). No reconstitution was observed when this system was used in combination with soluble succinate dehydrogenase, whereas an active cytochrome *c* reductase was formed when the *N*-ethylmaleimide-treated cytochrome *c* reductase and succinate-ubiquinone reductase were mixed.

Discussion

The described method of purification of the succinate-ubiquinone reductase from mitochondrial membranes has been developed with several aims in mind. Since the early 1960's, complex II, prepared as originally described [1] or with some minor modifications [2,13], has been the only succinate-ubiquinone reductase purified from the mammalian mitochondrial membranes. The procedure of complex II isolation is rather complex, time-consuming and the yield of the final preparation is not high. The reduction of DCIP by succinate in the absence of exogenous quinone [1] catalyzed by complex II indicates the presence of residual functionally active ubiquinone in the preparation. This hampers the kinetic analysis of an acceptor site of the enzyme. Thus it appeared to be desirable to work out the simpler procedure for isolation of succinate-ubiquinone reductase that would be suitable for kinetic and spectroscopic analyses. Another reason for the development of new procedures for fragmentation of the respiratory chain is more general, and may briefly be illustrated by the structure-function relationship in the succinate-cytochrome *c* reductase region of the mammalian respiratory chain. An active succinate-cytochrome *c* reductase has been reconstituted from complexes II and III [47] as well as from the soluble succinate dehydrogenase and b-c_1 complex [22], but not from the succinate dehydrogenase and complex III. It may thus be deduced that the components which is responsible for the reactivity of succinate dehydrogenase towards ubiquinone remains bound to either succinate dehydrogenase or to b-c_1 complex dependent on the procedure used for the resolution. Such a consideration emphasizes the ambiquity of the term 'complex', which somehow corresponds to the isolation procedure rather than to the minimal structure unit of the respiratory chain capable of some definite natural functions.

The procedure described in this paper has several advantages, e.g., simplicity (and uninitiated person can comfortably make the preparation for 10 h), use of a simple and inexpensive equipment, and high yield in terms of the flavin content and catalytic activity. The preparations obtained do not reduce DCIP or WB in the absence of exogenous Q_2, which facilitates the kinetic studies of an ubiquinone reactive site. An obvious disadvantage of the method is the use of Triton X-100 as a solubilizing agent, since this detergent is not easy to remove or exchange for the other. It should also be mentioned that although an active cytochrome oxidase can be prepared from the material remaining after Triton X-100 extraction, the b-c_1 complex is lost during the succinate-ubiquinone reductase purification (our unpublished observations).

A comparison of some properties of the enzyme prepared by our method with the other succinate-ubiquinone reductases described in the literature is given in Table V. A considerable similarity of several properties is evident. Small variations in M_r of the major components are apparently due to the differences in electrophoretic procedures used by the authors. Two larger peptides undoubtedly belong to succinate dehydrogenase [10] as evident from the isolation of the enzyme from the complex (Fig. 1). No definite conclusions can be drawn about the functions of two low molecular weight components of the enzyme. An active succinate-ubiquinone reductase has been reconstituted from

the soluble succinate dehydrogenase and a single peptide which contains only a trace amount of heme *b* (QP_s prepared by the method 1) [48]. On the other hand, a simple 1 : 1 stoichiometry of flavin to cytochrome *b* was found in complex II and, as evident from the reconstitution and reisolation experiments [6] *b*-560 species is an intrinsic part of the succinate-ubiquinone reductase. Our preparation which shows the molecular activity close to that in the parent particles also exhibits at least two bands in the low molecular weight region, although the stoichiometry of flavin : heme *b* is far less than unity. It may be thus concluded that heme *b* is not an obligatory component of the succinate : ubiquinone reductase in the mammalian respiratory chain. It is of interest that simple 1 : 1 stoichiometry of flavin and cytochrome *b* was found in the enzyme from *Neurospora crassa*, and

TABLE V

THE PROPERTIES OF SUCCINATE-UBIQUINONE REDUCTASE PREPARATIONS

Parameters	Complex II from bovine heart [1–3,13,14]	Succinate-ubiquinone reductase from *Neurospora crassa* [49]	Succinate-ubiquinone reductase from bovine heart [15]	Succinate ubiquinone reductase from bovine heart (this paper)
Polypeptide composition				
Number of protein bands on SDS-electrophoresis	10	3	4–5	4–5
M_r of the major bands (kDa)	73; 24; 13.7; 12.5	72; 28; 14	not specified	74; 26; 15.8; 14.9
Redox components (nmol or ngatoms per mg protein)				
Covalently bound flavin	4.6–5.0	6.0	5.8	5.9–6.2
Nonheme iron	36–38	35–40	48	52–54
Acid-labile sulfur	32–38			48–50
Cytochrome *b* heme	4.5–4.8	5.0	1.2	1.2–2.5
Cytochrome c_1 heme	1.5	0		0
Ubiquinone		2	2.0	2–3
Phospholipids (mg per mg of protein)	0.2		0.38	
ESR-detectable components (electron equivalents per mole of flavin)				
Succinate-reduced enzyme iron-sulfur center ($g = 1.94$)	0.85–1.03			0.83
Semiquinone (g-2.00)	0.08–0.13			0.1
Oxidized enzyme Iron-sulfur center ($g = 2.01$)	1.0–1.2			0.97
The specific activity (μmol succinate oxidized per min per mg of protein)	50–55 (38°C)	11.5 (30°C)	17.5 (23°C)	28 (25°C) 40 (30°C) 54 (38°C)
Turnover number (min^{-1}, calculated per flavin content)	11000 (38°C)	1900 (30°C)	3000 (23°C)	5000 (25°C) 9000 (38°C)

only a single low molecular weight peptide was detected in SDS-electrophoresis gel [49] (see also Ref. 6, and references cited therein, for more detailed discussion of cytochrome *b* function in the succinate : ubiquinone reductase).

The turnover number of the enzyme calculated on the basis of acid non-extractable flavin content is about $5 \cdot 10^3$ per min at 25°C, i.e., the same as that in the parent particles. Our preparation contains no functionally active ubiquinone and being solubilized by Triton X-100, certainly exists in the artificial environment. Thus it appears that neither ubiquinone [50] nor the natural environment [51] influences molecular activity of succinate dehydrogenase, and the differences in the succinate dehydrogenase turnover number which is seen in different enzyme preparations [51] are apparently due to inadequate use of artificial electron acceptors rather than to the properties of the enzyme. This conclusion is in accord with our earlier findings obtained using quite different experimental approaches [52].

An apparent K_m value for Q_2 (0.5 μM) is close to that previously calculated for less pure preparations (0.3 μM [45]). The possibility that in the kinetic experiments exogenous quinone interact with the tightly bound ubiquinone and the artificial dyes such as DCIP or WB are not able to accept electrons from the bound coenzyme seems hardly likely because Q_2H_2 was found to compete kinetically with Q_2 for the acceptor site [45]. Another evidence for the binding of Q_2 at the specific site of the enzyme is our recent finding that added quinone protects succinate-ubiquinone reductase against dissociation induced by the lysine-specific reagent [18].

If the simple Michaelis-Menten kinetics of the reaction are due to the saturation of the specific quinone binding site, then this system seems very convenient for the studies on the kinetic mechanism of the enzyme and the specific inhibitors effects. Carboxin [53,54] and thenoyltrifluoroacetone [55] are the specific inhibitors of succinate oxidation which inhibit the succinate-ubiquinone reductase reaction by the same mechanism. Kinetic studies demonstrated that these inhibitors prevent oxidation of the succinate dehydrogenase S-3 center by the ubiquinone pool [5]. Although the effects of carboxin and thenoyltrifluoroacetone on succinate dehydrogenating systems have been extensively studied (Refs. 5, 56 and 57 and references cited therein), the mode of their action still remains obscure. Most of the recent proposals on the mechanism of carboxin and thenoyltrifluoroacetone suggest explicitly [58,59] or implicitly [56,57] the competition between ubiquinone (bulk phase or bound in a semiquinone form) and the inhibitors. It has been actually reported that thenoyltrifluoroacetone inhibition of succinate-cytochrome *c* reductase is competitively reversed by exogenous ubiquinone [60]. An apparent competition between thenoyltrifluoroacetone and Q_2 has also been reported for the reconstituted succinate-ubiquinone reductase [61]. On the other hand, it has been reported [9] that the apparent K_i for carboxin in the reconstituted succinate-ubiquinone reductase does not depend on the amount of a fraction containing QP_s and endogenous ubiquinone. The latter finding hardly fits to the proposals on the simple competition between the inhibitor and ubiquinone. The results obtained in this study showed that kinetically carboxin acts as a simple non-competitive inhibitor (Fig. 8 and 9). This finding agrees with our earlier results [9] and with the experimentally supported proposal [44] that the inhibitor act as a clip, thus preventing the mutual conformation mobility of succinate dehydrogenase and ubiquinone reactivity conferring peptides.

At the moment it is difficult to rationalize an unusual behaviour of the enzyme-detergent complex in well-defined terms. Whatever the mechanism of the dissociation is, the phenomenon reported here seems to be of great importance, since it may be expected to exist for other enzyme-detergent complexes. Indeed, the qualitatively similar phenomenon was observed in our laboratory with succinate-cytochrome *c* reductase prepared as described in Ref. 22 and dissolved in 0.1% Triton X-100.

An active succinate-cytochrome *c* reductase can be reconstituted by simply admixing of succinate-ubiquinone reductase and the b-c_1 complex. Data from many laboratories suggest that the individual components of the respiratory chain are not assembled in a simple 1 : 1 stoichiometry [20,62,63]. This is particularly true for succinate dehydrogenase, whose content in the mitochondria or in

submitochondrial particles is much less than that of cytochrome *b*. Since succinate dehydrogenase is water soluble and no specific interaction between the phospholipid membrane and the enzyme was demonstrated, it is reasonable to assume that the specific hydrophobic peptide(s) of complex II serve as the stoichiometric binding site for succinate dehydrogenase. In this simple model the assembled succinate-ubiquinone reductase operates in the membrane independently of the b-c_1 complex, and the electron transfer between two complexes is mediated by the mobile ubiquinone pool [64,65]. The data presented in this paper make this simple model doubtful and point the specific protein-protein (or lipid) interaction between some components of the complexes II and III. In fact, the high affinity of the peptides which are responsible for the ubiquinone reactivity of succinate dehydrogenase to the b-c_1 complex is strongly supported by their apparently stoichiometric presence in some preparations of the latter [22]. It should be emphasized that the appearance of the antimycin-sensitive succinate-cytochrome *c* reductase in our experiments (Table V) is not resulted from the redistribution of succinate dehydrogenase between succinate-ubiquinone reductase and b-c_1 complex, since the active cytochrome *c* reductase can be reconstituted from succinate-ubiquinone reductase and NEM-treated succinate-cytochrome *c* reductase in which the binding sites for dehydrogenase are certainly occupied by the enzyme. Thus it appears that complexes II and III interact specifically and the structure-function relationship in this segment of the respiratory chain is quite similar to that for the specific interaction between complexes I and III [66,67].

Acknowledgements

The search for a new procedure for isolation of succinate-ubiquinone reductase was started by one of us (A.D.V.) in 1979 in the Laboratory of Bioenergetics, Department of Chemistry, New York State University at Albany (U.S.A.). The author is grateful to Professor Tsoo E. King for his continued encouragement at the initial stages of this project.

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