STUDIES ON THE TERMINAL ELECTRON TRANSPORT SYSTEM

VIII. CONVERSION OF SUCCINIC DEHYDROGENASE COMPLEX TO SOLUBLE SUCCINIC DEHYDROGENASE

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Papers I and VII of this series[1,2] have dealt with the succinic dehydrogenase complex (SDC). This particulate entity catalyzes the oxidation of succinate by electron acceptors such as ferricyanide, indophenol, and phenazine methosulfate as well as by cytochrome *c*. The SDC contains flavin, cytochromes *b* and c_1 and non-heme iron.

In the present communication, a method for extracting a soluble succinic dehydrogenase from SDC, based on the initial observations of SINGER AND KEARNEY[3], is described. The dehydrogenase, a ferro flavoprotein, catalyzes the oxidation of succinate by ferricyanide and phenazine methosulfate but not cytochrome *c*, and the reduction of fumarate by reduced flavin mononucleotide ($FMNH_2$). The enzyme is compared with the succinic dehydrogenase prepared from beef heart mitochondria by SINGER AND KEARNEY *et al.*[3-11].

ANALYTICAL METHODS

Protein, flavin, and total iron were determined as described previously[1].

Assay of enzymic activity

Succinic dehydrogenase activity was measured with ferricyanide or phenazine methosulfate as electron acceptors as described previously[1].

Fumaric reductase activity was measured according to the method of SINGER AND KEARNEY[10]. $FMNH_2$** is used as the electron donor and its rate of reoxidation is measured spectrophotometrically at 450 mμ. Activity is determined at several levels of $FMNH_2$ concentration, and the maximal rate is obtained by extrapolation of the data to infinite $FMNH_2$ concentration by a double reciprocal plot of rate *vs.* reactant concentration.

The molecular extinction coefficient of FMN minus $FMNH_2$ is taken to be $11.3 \cdot 10^6$ cm²/mole (based on the ε value for the oxidized form assigned by WHITBY[12]).

Preparation of an acetone powder of SDC

A sucrose suspension of SDC prepared from mitochondria as described in Papers I or VII[1,2] or from the electron transfer particle (ETP)[13] is diluted with an equal volume of 0.12 *M* KCl and adjusted to pH 6 with acetic acid. The suspension is centrifuged in the No. 30 or 40 rotor of the Spinco preparative centrifuge for 5 to 10 minutes at 30,000 or 40,000 r.p.m. respectively. The sedimented SDC is taken up in a minimum amount of 0.12 *M* KCl and poured into at least 10 volumes of acetone at —10°. The particles are allowed to settle and the acetone supernatant is decanted. One third to one half the original volume of fresh acetone is added and the suspension is stirred.

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** FMN was a Hoffman-LaRoche product obtained from Dr. H. BEINERT.

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The suspension is allowed to settle again and is then filtered through No. 50 Whatman paper in a Büchner funnel, with suction. A small amount of ether is poured on the funnel as soon as all the acetone has been filtered, and the partially defatted SDC is then dried to a light pink powder.

Extraction of enzyme from acetone powder

Enough water is added to the acetone powder of the SDC to make a 6 to 10% suspension by weight. The mixture is homogenized in a ground glass Potter-Elvehjem type homogenizer and the resulting suspension adjusted to pH[8] with 0.1 *N* KOH. The suspension is centrifuged in the No. 40 rotor of the Spinco preparative centrifuge at 40,000 r.p.m. for 10 minutes. The supernatant solution (Extract 1), which contains protein but little succinic dehydrogenase activity is poured off and the residue is resuspended in the original volume of water. After homogenization, the suspension is adjusted to pH 10 by the slow addition of 0.1 *N* KOH. The alkaline suspension is centrifuged in the same rotor at 40,000 r.p.m. for 20 minutes and the yellow-brown supernatant solution, Extract 2, is separated from the residue with a pipette. The alkaline extraction is repeated to obtain Extract 3. The residue is resuspended in water or 0.25 *M* sucrose for enzymic assay. The extracts and suspension of the final residue are immediately neutralized by the addition of a few γ of molar phosphate buffer at pH 7.

The succinic dehydrogenase activities as measured with ferricyanide as the electron acceptor, as well as the recovery of units in a typical extraction procedure are shown in Table I. About 60% of the dehydrogenase contained in the acetone powder of the SDC can be extracted, of which 70% is obtained in a highly purified form by a single extraction. The extract has no activity with DPNH as substrate.

TABLE I

EXTRACTION OF SUCCINIC DEHYDROGENASE

	Protein mg	*Specific activity**	*Units***
Original APSDC****	1260	20	25,200
Extract I	110	17.7	1,950
Extract II	110	100	11,000
Extract III	40.5	63	2,552
Residue	744	7.8	5,803

* Ferricyanide assay, μmoles CO_2/5 min/mg protein.
** Specific activity × total mg protein.
*** APSDC = Acetone powder of the SDC.

The enzyme may be concentrated by precipitation with ammoniacal ammonium sulfate (5 ml concentrated NH_4OH, 95 ml saturated ammonium sulfate) without loss of activity, but an increase in activity over the initial extract has not been achieved by fractional precipitation with ammonium sulfate.

The activity declines 20 to 40% upon storage in phosphate buffer overnight (whether at 0° or —20°), then slowly declines upon prolonged storage. Addition of succinate delays somewhat, but does not completely prevent, the decline in succinic dehydrogenase activity. The addition of sulfhydryl compounds (BAL, cysteine, glu-

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tathione) to the enzyme or to the assay mixture does not restore the lost activity of stored preparations, as has already been shown by SINGER *et al.*[4].

The enzymic activity is unaffected by the addition of ferrous, ferric, manganous, magnesium or molybdate ions either alone or in conjunction with flavin adenine dinucleotide.

The enzyme can be adsorbed on and eluted from calcium phosphate gel, but here again the specific activity of the preparation is not increased over the initial extract.

Absorption spectrum

The absorption spectra of the oxidized and reduced forms of the enzyme are shown in Fig. 1 as well as the difference spectrum (oxidized minus reduced). The absorption spectrum is essentially the same as that reported by KEARNEY AND SINGER[7] for the enzyme with two atoms of iron per mole extracted directly from mitochondria.

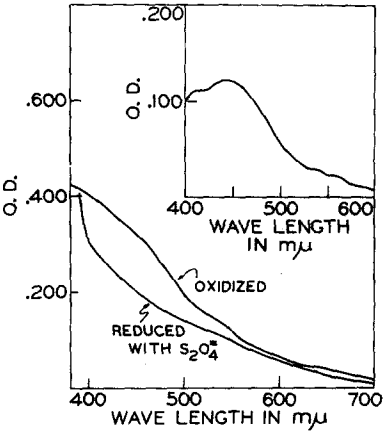

Fig. 1. The absorption spectrum of succinic dehydrogenase. The cuvette contained 0.85 mg of enzyme protein and 50 μmoles of phosphate (pH 7.4.) in a total volume of 0.5 ml. The inset shows the difference spectrum (oxidized minus reduced).

Flavin and iron content

The soluble dehydrogenase contains $3–5 \cdot 10^{-3}$ μmoles of flavin/mg protein and $8–10 \cdot 10^{-3}$ μg atoms Fe/mg protein, and hence is comparable to the 1 flavin-2 iron enzyme of SINGER *et al.*[4,7] rather than the more active 1 flavin-4 iron enzyme[8]. Flavin is released from the enzyme only after tryptic digestion[1].

Catalytic activities

The dehydrogenase catalyzes both the dehydrogenation of succinate and the hydrogenation of fumarate as has been shown by SINGER *et al.*[6].

A comparison of the enzyme extracted from SDC with the SINGER-KEARNEY enzyme is shown in Table II. The main difference in the two preparations appears to be the lower reactivity of the SINGER-KEARNEY enzyme with fericyanide as the electron acceptor, since their enzyme is only 30 to 40% as active with ferricyanide as with phenazine methosulfate[5], whereas the enzyme described in the present communication is 70% as active with ferricyanide as with phenazine methosulfate.

The enzyme is completely devoid of cytochrome *c* reductase activity. In fact, when SDC is adjusted to pH 10 (the condition for optimal splitting of the soluble dehydrogenase from the particle), the cytochrome reductase activity is immediately destroyed.

DISCUSSION

No significant purification of the enzyme over the first soluble extract from SDC has been achieved. It was hoped that a purification could be shown by following the fumaric hydrogenase activity, since SINGER AND MASSEY (private communication)

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TABLE II

COMPARISON OF SDC-SUCCINIC DEHYDROGENASE AND SINGER-KEARNEY ENZYMES

	Enzyme isolated from SDC	*Enzyme isolated by* SINGER *et al.*	
		1 Flavin : 2 Fe[11]	*1 Flavin : 4 Fe*[8,11]
Activity			
Ferricyanide as electron acceptor μmoles succinate/5 min/mg protein	50	"22–30"	"44–60"
Q_{O_2}	6700	"3000–4000"	"6000–8000"
Phenazine methosulfate as acceptor μmoles succinate/5 min/mg protein	73	"75"	"149"
Q_{O_2}	9800	10,000	20,000
Composition			
Flavin – μmole/mg protein	$3–5 \cdot 10^{-3}$	$4–5 \cdot 10^{-3}$	$4–5 \cdot 10^{-3}$
Iron – μg-atom/mg protein	$8–10 \cdot 10^{-3}$	$10 \cdot 10^{-3}$	$20 \cdot 10^{-3}$

The Q_{O_2} listed under ferricyanide assay is an apparent Q_{O_2} and is a conversion of μmoles CO_2/5 min/mg protein to μliters O_2/hour/mg protein by multiplication by (1/4)(22.4)(12) = 67.2. The figures in quotation marks are calculated from data of SINGER *et al.*[8,11].

TABLE III

COMPARISON OF ACTIVITIES

	*Succinic dehydrogenase**	*Fumaric hydrogenase***
	μmole succinate/ min/mg protein	*μmole fumarate/ min/mg protein*
SDC	4.9	0.24
APSDC***	1.8	0.12
Extract-APSDC		
FR I§	5.3	0.42
FR II	5.8	0.59
FR III	2.3	0.10

* ½ μmole CO_2/min/mg protein in the ferricyanide assay (38°) = μmole succinate/min/mg protein.

** μmole $FMNH_2$/min/mg oxidized at 38° = μmole fumarate/min/mg protein.

*** APSDC = Acetone powder of the SDC.

§ Ammoniacal ammonium sulfate fractions; FR I 0–33%, FR II 33–43%, FR III, 43–57%.

had shown that the enzyme is more stable when assayed in the reverse direction. However, in our hands (see Table III) although the activity did not decline rapidly in storage, little purification could consistently be achieved by ammoniacal ammonium sulfate fractionation. The apparent separation of hydrogenase and dehydrogenase activities in Fraction III may be due to differential inactivation of the enzyme with respect to one activity and not with respect to the other.

The fact that a soluble succinic dehydrogenase with a purity comparable to the 1 flavin-2 iron enzyme of SINGER AND KEARNEY can be extracted from the SDC in a single extraction, without subsequent purification, gives added evidence for the purity of the SDC.

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SUMMARY

A method for the extraction of soluble succinic dehydrogenase from the particulate succinic dehydrogenase complex (SDC) is described.

The enzyme, as extracted from SDC, catalyzes the dehydrogenation at 38° of 10 μmoles of succinate per min per mg with ferricyanide as the electron acceptor, or 14 μmoles of succinate per min per mg with phenazine methosulfate as electron acceptor. It contains $3 - 5 \cdot 10^{-3}$ μmoles of flavin as well as $5 - 10 \cdot 10^{-3}$ μg atoms of non-heme iron per mg of protein.

REFERENCES

1 D. E. GREEN, S. MII AND P. M. KOHOUT, *J. Biol. Chem.*, 217 (1955) 551.
2 R. E. BASFORD, H. D. TISDALE, J. L. GLENN AND D. E. GREEN, *Biochim. Biophys. Acta*, 24 (1957) 107.
3 T. P. SINGER AND E. B. KEARNEY, *Biochim. Biophys. Acta*, 15 (1954) 151.
4 T. P. SINGER, E. B. KEARNEY AND N. ZASTROW, *Biochim. Biophys. Acta*, 17 (1955) 154.
5 E. B. KEARNEY AND T. P. SINGER, *J. Biol. Chem.*, 219 (1956) 963.
6 T. P. SINGER, V. MASSEY AND E. B. KEARNEY, *Biochim. Biophys. Acta*, 19 (1956) 200.
7 E. B. KEARNEY AND T. P. SINGER, *Biochim. Biophys. Acta*, 17 (1955) 596.
8 T. P. SINGER, E. B. KEARNEY AND V. MASSEY, *Arch. Biochem. Biophys.*, 60 (1956) 255.
9 T. P. SINGER, E. B. KEARNEY AND V. MASSEY, in O. H. GAEBLER, *Enzymes: Units of Biological Structure and Function*, Academic Press, New York, 1956.
10 T. P. SINGER AND E. B. KEARNEY, in D. E. GLICK, *Methods of Biochemical Analysis*, Vol. IV, Interscience Publishers, Inc., New York, (in the press).
11 T. P. SINGER, E. B. KEARNEY AND P. BERNATH, *J. Biol. Chem.*, (in the press).
12 L. G. WHITBY, *Biochem. J.*, 54 (1953) 437.
13 F. L. CRANE AND L. J. GLENN, in preparation.

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STUDIES ON THE ELECTRON TRANSPORT SYSTEM

IX. FRAGMENTATION OF DPNH OXIDASE*

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In a previous communication[1] we have described the effects of deoxycholate on the DPNH oxidase (reduced diphosphopyridine nucleotide oxidase)—a derivative form of the electron transport particle which has lost succinic dehydrogenase activity. DPNH oxidase is prepared in a "closed" state in which external cytochrome *c* cannot interact with the heme chain either as electron acceptor or donor. After exposure to deoxycholate the oxidase is converted to an open state in which cytochrome *c* can react maximally with the heme chain, whereas DPNH oxidase in its closed form cata-

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