A lipid-soluble form of cytochrome *c* from the electron transport particle of beef-heart mitochondria*

The electron transport chain of ETP[1] isolated from beef-heart mitochondria shows no requirement for cytochrome *c* in the oxidation of DPNH** or succinate, and concentrated salt solutions do not extract cytochrome *c* from the particle. On the other hand, a cytochrome component with an α-band maximum at 550 mμ has been observed in this particle by low-temperature spectroscopy[2].

We have recently isolated from beef-heart mitochondria and from ETP a cytochrome which is insoluble in water, but which is soluble in certain lipid solvents. This cytochrome shows an absorption spectrum in the visible region identical with that of cytochrome *c*. This compound will be referred to as lipid cytochrome *c*.

This lipid-soluble form of cytochrome *c* can be extracted into heptane after addition of dithionite and an equal volume of ethanol to the mitochondria. It can also be formed by mixing an extract containing mitochondrial phospholipids with a solution of reduced cytochrome *c* and then by extracting the mixture with heptane. Preliminary studies indicate that the cytochrome-complexing or combining factor is most concentrated in the cephalin fraction from a silica-gel column, but full recovery of complexing activity has not been achieved. Lipid cytochrome *c* is soluble in heptane, iso-octane, petroleum ether (30° to 60°), and ethyl ether, but is insoluble in water and is destroyed by treatment with ethanol or acetone. This cytochrome *c* complex may be similar to that described by MICHELAZZI[3].

When ETP is extracted with iso-octane for 1 h at 0° the DPNH oxidase activity[1] is lost, whereas the DPNH ferricyanide[1] activity is unchanged and DPNH-cytochrome *c* reductase[1] activity emerges. After one extraction of ETP the DPNH oxidase activity is fully restored by the addition of either aqueous or lipid-soluble forms of cytochrome *c*. On further isooctane extraction the ability of aqueous cytochrome *c* to restore activity is gradually lost, whereas the lipid cytochrome *c* is still capable of restoring full activity. The phospholipid fraction referred to above does not restore activity in the absence of cytochrome *c*, while cholesterol, α-tocopherol[4] and mitochondrial carotene do not restore activity either in the absence or presence of cytochrome *c* The quinone (Q_{275}) which we have recently isolated from beef heart mitochondria[5], and which stimulates succinate oxidation in iso-octane-extracted ETP does not have any effect on the ability of cytochrome *c* to restore DPNH oxidase activity in the extracted ETP. The effect of iso-octane extraction on the enzymic activity of ETP as well as the restoration of activity by lipid cytochrome *c* are shown in Table I.

TABLE I

THE RESTORATION OF DPNH OXIDASE ACTIVITY IN ISO-OCTANE–EXTRACTED ETP

Number of extractions	*DPNH oxidase*				*DPNH cyt. c reductase*	*DPNH ferricyanide*
	no addition	*+cyt. c*	*+ lipid alone*	*+ lipid cyt. c*		
none	1.2	1.3	1.3	1.2	0.10	2.5
one	0.2	1.8	0.1	2.0	0.35	3.9
three	0.0	0.35	0.0	1.9	0.50	2.5

0.3 mg phospholipid fraction in ethanol or 0.02 ml 1% cytochrome *c* added as indicated. DPNH oxidase assay without versene. All activities expressed as μmoles DPNH oxidized/min/mg protein.

Iso-octane extraction also reduces succinate oxidase activity[1], and this activity is restored by the addition of aqueous cytochrome *c*. In contrast to the effect of repeated iso-octane extraction on DPNH oxidase activity, even after several iso-octane extractions aqueous cytochrome *c* is capable of restoring succinoxidase activity, and it is worthy of note that the activity of extracted ETP in presence of cytochrome *c* is more than that of the original ETP. Q_{275} and lipid cytochrome *c* are capable of partial restoration of activity when added separately, but together restore the original activity of ETP. The effects of lipid cytochrome *c*, Q_{275} and aqueous cytochrome *c* on the succinnate oxidase activity of intact and iso-octane-extracted particles are shown in Table II.

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** Abbreviations used include DPNH, reduced diphosphopyridine nucleotide; iso-octane 2, 2, 4-trimethylpentane; ETP, electron transport particle.

TABLE II

THE RESTORATION OF SUCCINNATE OXIDASE ACTIVITY IN ISO-OCTANE-EXTRACTED ETP

Number of extractions	Succinoxidase (μmoles succ./min/mg)*				
	no addition	+ Q_{275}	+ lipid cyt. c	+ lipid cyt. c + Q_{275}	+ aqueous cyt. c
none	0.46	0.43	0.46	0.42	0.48
one	0.05	0.08	0.26	0.30	0.52
two	0.00	0.04	0.20	0.36	0.68

* Assay as described previously[1]. 0.2 mg Q_{275} in 0.05 ml ethanol, 0.1 ml 0.1% lipid cytochrome *c* in ethyl ether (followed by evaporation of the ether at 0°) and 0.1 ml 1% cytochrome *c* added as indicated. Gas phase is air.

TABLE III

DISTRIBUTION OF LIPID CYTOCHROME *c* DURING ISO-OCTANE EXTRACTION OF ETP

Number of extractions	Lipid cytrochrome c (μmole · 10^{-3}/mg original protein)*		
	ETP	sucrose supernatant	iso-octane extract
none	0.28	—	—
one	0.07	0.18	0.00
two	0.02	0.05	0.00
three	0.00	0.01	0.00

* Values based on the assumption that the extinction of lipid cytochrome *c* is the same as that of the aqueous form.

After extraction with iso-octane, ETP no longer shows the presence of lipid cytochrome *c*, and insoluble particles associated with cytochrome *c* are found in the 0.25 *M* sucrose supernatant obtained after ETP is separated from iso-octane by centrifugation. The amount of lipid-soluble cytochrome *c* in each of the fractions obtained after iso-octane extraction is shown in Table III.

In previous studies at this laboratory it was found that treatment of ETP with deoxycholate induces a requirement for cytochrome *c* in the oxidation of DPNH and succinate by molecular oxygen[6,7]. A small amount of cytochrome *c* was found in the deoxycholate extract, but all attempts to show that this extracted cytochrome *c* could replace lipid cytochrome *c* were unsuccessful. We now find that after extensive dialysis of the deoxycholate extract against distilled water the cytochrome *c* reverts to a water-insoluble form which is capable of restoring DPNH oxidase activity in the deoxycholate-extracted ETP.

These results imply that the endogenous form of cytochrome *c* in ETP and in the KEILIN-HARTREE preparation[8,9] is the lipid-bound cytochrome *c* described above. Further studies to establish this relationship with endogenous (bound) cytochrome *c* and to determine the nature of the lipid component are under way.

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1 F. L. CRANE, J. L. GLENN AND D. E. GREEN, *Biochim. Biophys. Acta*, 22 (1956) 475.
2 R. ESTABROOK AND B. MACKLER, *J. Biol. Chem.*, 224 (1957) 637.
3 L. MICHELAZZI, *Experientia*, 11 (1955) 389.
4 A. NASON AND I. R. LEHMAN, *J. Biol. Chem.*, 222 (1956) 511.
5 F. L. CRANE, Y. HATEFI, R. L. LESTER AND C. WIDMER, *Biochim. Biophys. Acta*, 25 (1957) 220.
6 F. L. CRANE AND J. L. GLENN, *Biochim. Biophys. Acta*, 24 (1957) 100.
7 B. MACKLER AND D. E. GREEN, *Biochim. Biophys. Acta*, 21 (1956) 1.
8 D. KEILIN AND E. F. HARTREE, *Nature*, 176 (1955) 200.
9 E. G. BALL AND O. COOPER, *J. Biol. Chem.*, 226 (1957) 755.

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