The Effect of Cytochrome *c* and its 'Dimer' on Electron Transfer and Energy Transformation

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A preparation that contained cytochrome *c*, mainly in the form of its 'dimer', was studied and compared with native cytochrome *c* with respect to its ability to support electron transfer and energy transformation in cytochrome *c*-depleted rat liver mitochondria. When the depleted mitochondria were titrated with either cytochrome *c* or the 'dimer', the extent of coupling between respiration and phosphorylation was enhanced, as manifested by an increase in the P/O ratio. The 'dimer' was relatively ineffective as an electron carrier in the respiratory system, but it was as effective as cytochrome *c* in reconstitution of oxidative phosphorylation in depleted mitochondria. Addition of 'dimer' to the depleted mitochondria, in the presence of a low, non-saturating concentration of cytochrome *c*, increased the P/O ratio without concomitant stimulation of respiration. Both cytochrome *c* and the 'dimer' stimulated spontaneous swelling and electron transport-driven proton translocation in depleted mitochondria. The pattern of action of cytochrome *c* and its 'dimer' is in accord with the assumption that they affect an early step in energy conservation.

According to the chemical hypothesis of oxidative phosphorylation (Slater, 1953) a respiratory carrier plays a role in both electron transfer and energy transformation. One approach to assessing the direct role of a respiratory carrier in energy transformation is to show that, under certain conditions, it affects the transfer of electrons and energy differentially. Experiments along this line were performed with cytochrome *c* (Penniston *et al.*, 1966; MacLennan *et al.*, 1966; Lenaz & MacLennan, 1966; Streichman & Avi-Dor, 1967; Avi-Dor & Streichman, 1969) which can be removed and re-bound (Jacobs & Sanadi, 1960) without excessive damage to the mitochondria. In the above studies it was demonstrated that mitochondria depleted of cytochrome *c* show low P/O ratios in oxidative phosphorylation and perform certain energy-dependent reactions with low efficiency. Re-binding of cytochrome *c* was shown to restore the damaged functions to a value close to that observed in intact mitochondria. It was, however, rather difficult to find conditions (Avi-Dor & Streichman, 1969) under which the effect of cytochrome *c*, on electron transfer and on energy transfer, could be unequivocally resolved. We hoped to obtain a better separation of the two, presumably different, functions of cytochrome *c* in the restoration of cytochrome *c*-depleted mitochondria by substituting for the native cytochrome *c* a modified form, which is known to be ineffective as an electron carrier in the respiratory chain. A derivative fulfilling the above conditions is

the oligomeric (mainly dimeric) form of cytochrome *c*. This compound was isolated and characterized by Margoliash and co-workers (Margoliash, 1954; Margoliash *et al.*, 1959; Margoliash & Lustgarten, 1961; Margoliash, 1962; Margoliash & Lustgarten, 1962*a,b*). It differs from the native form not only in the extent of aggregation, but also in its tertiary structure (Schejter *et al.*, 1963) and probably also in the displacement of methionine-80 as a ligand of the haem-iron (Streenathan & Taylor, 1971). This form of cytochrome *c* will be referred to hereafter as the 'dimer'.

Experimental

Materials

ADP, ATP, horse heart cytochrome *c*, oligomycin, antimycin A and hexokinase from yeast, type III, were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Catalase (ox liver) A grade (39000 units/mg of protein) was purchased from Calbiochem, Los Angeles, Calif., U.S.A. Rotenone was from K & K Laboratories, Plainview, N.Y., U.S.A. Amberlite CG-50 (200–400 mesh) was a product of Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A. ^{32}P was a product of The Radiochemical Centre, Amersham, Bucks., U.K. [^{32}P]ATP, labelled in the γ -phosphate group, was a generous

gift from Dr. H. Carmeli, Tel-Aviv University. Other chemicals were of analytical grade. All solutions and reaction mixtures were prepared with double-distilled water.

Methods

Rat liver mitochondria. Mitochondria were isolated from liver homogenates prepared in 0.25M-sucrose, by differential centrifugation, as described by Ernster & Löw (1955). The mitochondrial pellet was finally suspended in 0.25M-sucrose in a concentration of approx. 30mg protein/ml.

Cytochrome *c* depleted mitochondria. Unless otherwise indicated, the following procedure for the extraction of cytochrome *c* from mitochondria was used. Mitochondria were exposed to hypo-osmotic conditions by diluting the stock suspension (in 0.25M-sucrose) in 5 vol. of ice-cold water. Five parts of the diluted mitochondria were immediately added to 1 part of ice-cold Amberlite CG-50 (200–400 mesh) suspension, prepared by the method of Margoliash & Walasek (1967), and stirred gently for 10min in an ice bath. The mixture was then centrifuged for 1min at 600g in a Sorvall model RC-2 refrigerated centrifuge. The supernatant containing the cytochrome *c*-depleted mitochondria was collected and the Amberlite pellet was discarded. In some experiments the depleted mitochondria were prepared by a modified procedure of Jacobs & Sanadi (1960), as described by Streichman & Avi-Dor (1967). It was found that by both procedures 75% of the total content of cytochrome *c* in intact mitochondria could be removed.

Preparation of cytochrome *c* 'dimer'. Cytochrome *c* was polymerized by ethanol precipitation, as described by Margoliash & Lustgarten (1962b). Fractionation of the product on a column of Sephadex G-75, by the method of Margoliash & Lustgarten (1962b), revealed that the dimeric form of cytochrome *c* was the major constituent, with small amounts of higher oligomers and less than 10% of cytochrome *c* monomers. The E_{695} , which is known to be characteristic for native cytochrome *c* in its oxidized form (Schejter *et al.*, 1963), could not be detected in the product, when measured in a Cary model 15 recording spectrophotometer.

Quantitative determination of cytochrome *c* in mitochondria. Cytochrome *c* was measured from the difference spectrum (reduced–oxidized) of the mitochondrial sample as described by Williams (1964, 1968). Measurements were done in a Cary model 15 recording spectrophotometer.

Respiration and phosphorylation. Respiration was measured polarographically with a Clark oxygen electrode attached to a Gilson oxygraph (type KM). After anaerobiosis had been reached the reaction was

stopped by adding 0.2ml of 50% (w/v) trichloroacetic acid. After centrifugation 0.6ml of the supernatant was removed and the $^{32}\text{P}_i$ was determined by the method of Avron (1960). For the calculation of the P/O ratio, the amount of P_i esterified in the time interval between the addition of the mitochondria and anaerobiosis, was divided by the oxygen uptake in the same time-interval. The standard assay medium contained 4mM-tris-HCl buffer (pH 7.4), 10mM-potassium phosphate buffer (pH 7.4) containing $^{32}\text{P}_i$ (1.5×10^5 – 2.5×10^5 c.p.m./ μmol), 60mM-KCl, 8mM-MgCl₂, 0.1mM-EDTA neutralized by tris (pH 7.4), 20mM-glucose, 0.5mg of hexokinase/ml and 1mM-ADP. The substrates, cytochrome *c* and cytochrome *c* 'dimer' were added in concentrations as indicated. The experiment was initiated by the addition of cytochrome *c*-depleted mitochondria equivalent to a concentration of approx. 3.0mg of protein/ml.

Measurement of volume changes. Mitochondrial swelling was studied by measuring changes in E_{520} in a Gilford model 2400 spectrophotometer in cuvettes of 1 cm light-path against water as reference.

Respiration-driven proton translocation. Respiration was measured polarographically with a Clark oxygen electrode. Proton translocation was estimated by monitoring the pH changes, in the same reaction vessel, with a glass electrode connected to a Radiometer-26 pH meter. Both oxygen uptake and pH changes were recorded with a Göerz model 520 double-channel recorder. The pH changes were converted into changes in proton concentration per atom of oxygen. For calibration, 5 or 10 μl of 0.1M-NaOH was injected into the reaction mixture and the deflexion of the recorder was measured.

Assay of adenosine triphosphatase activity. The reaction mixture consisted of 50mM-tris-HCl buffer (pH 7.4), 5mM-MgCl₂ and a mitochondrial preparation containing about 1.4mg of protein, in a total volume of 1ml. Where indicated, 2,4-dinitrophenol was also added to a final concentration of 20 μM . The reaction was started by adding 1.2mM- ^{32}P ATP (specific radioactivity in the range 1×10^5 – 2×10^5 c.p.m./ μmol) to the mixture. Incubation was for 10min at 30°C and the reaction was stopped with 0.5ml of 50% (w/v) trichloroacetic acid. After centrifugation, 0.2ml of the supernatant was removed and the $^{32}\text{P}_i$ that was liberated was determined (Avron, 1960). Controls, in which trichloroacetic acid was added to the reaction mixture before ATP, yielded corrections of less than 10% for the counts contributed by $^{32}\text{P}_i$ present in the ^{32}P ATP preparation and liberated non-enzymically.

Radioactivity determination. Radioactivity in the samples was measured with a Packard Tri-Carb model 3320 liquid-scintillation spectrometer.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951).

Results

Effect of cytochrome c and the 'dimer' on respiration and phosphorylation

The rates of oxidation in cytochrome *c*-depleted mitochondria of β -hydroxybutyrate, succinate and ascorbate+*NNN'**N'*-tetramethyl-*p*-phenylenediamine were found to be approx. 20% of the rates measured in intact mitochondria (Figs. 1 and 2). The P/O ratios for oxidative phosphorylation were about

1 for β -hydroxybutyrate, and less than 1 for succinate. No P_i uptake could be demonstrated when ascorbate+*NNN'**N'*-tetramethyl-*p*-phenylenediamine was the electron donor (Figs. 1 and 2). Thus the treatment causing removal of cytochrome *c* affected more than one phosphorylation site in the respiratory chain. When cytochrome *c*-depleted mitochondria were titrated with either cytochrome *c* (Fig. 1) or the 'dimer' (Fig. 2), the rate of phosphorylation was stimulated more than the rate of respiration, and as a

Fig. 1. *Effect of cytochrome c on respiration and respiration-coupled phosphorylation in cytochrome c-depleted mitochondria*

To the standard assay medium (see the Experimental section) which also contained depleted mitochondria (3.0mg of protein/ml) the following additions were made: $\circ$, $\bullet$, 5mM-DL- β -hydroxybutyrate; Δ , $\blacktriangle$, 5mM-sodium succinate and 0.1 μ M-rotenone; $\square$, $\blacksquare$, 6.6mM-sodium ascorbate, 0.13mM-*NNN'**N'*-tetramethyl-*p*-phenylenediamine and 0.25ng/ml of antimycin A. Cytochrome *c* was added as indicated. Open symbols, rate of respiration; closed symbols, P/O ratio. Total volume 2.0ml. Temp. 28°C. Other conditions were as described in the Experimental section.

Fig. 2. Effect of the 'dimer' on respiration and on the respiration-coupled phosphorylation in cytochrome *c*-depleted mitochondria

For details see the legend of Fig. 1. All titrations were done with the 'dimer' in place of cytochrome *c*. The concentration of the 'dimer' was expressed in equivalents of monomeric units.

corollary there was a continuous rise in the P/O ratio. In particular the P/O ratio continued to rise even after respiration had reached its maximum rate. This phenomenon was especially pronounced in the case of the 'dimer' (Fig. 2). As an electron carrier the 'dimer' was a poor substitute for cytochrome *c*, yielding maximum increments in the rates of respiration that were less than 20% of the increments obtained by reconstituting cytochrome *c*-depleted mitochondria with cytochrome *c* (compare Fig. 2 with Fig. 1). However, the 'dimer' replaced cytochrome *c* effectively in reconstitution of phosphorylation in cytochrome *c*-depleted mitochondria. The maximum P/O ratios obtained by adding 'dimer' to depleted mitochondria were 2.4 for β -hydroxybuty-

rate, 1.8 for succinate and 0.65 for ascorbate + *NNN'*-tetramethyl-*p*-phenylenediamine. In Figs. 1 and 2 it can also be seen that cytochrome *c* and the 'dimer' saturated the respiratory and the phosphorylating systems at approximately equal concentrations. Although extensive binding studies have not yet been done, the results of preliminary experiments show that under identical incubation conditions, with equal concentrations of either cytochrome *c* or the 'dimer' in solution, about 10–12 times more dimer than cytochrome *c* is bound to cytochrome *c*-depleted mitochondria. In spite of such excessive binding of the 'dimer' to depleted mitochondria, it could be shown (Table 1) that addition of cytochrome *c* to the reaction mixture, which already contained

Table 1. *Effect of cytochrome c on respiration and phosphorylation of cytochrome c-depleted mitochondria in the presence of 1 μ M-'dimer'*

To the standard reaction medium were added 5 mM-sodium succinate and 0.1 μ M-rotenone. All other conditions are as described in Fig. 1.

'Dimer' (μ M)	Cytochrome <i>c</i> (μ M)	Oxygen consumed (ng-atom/min)	P _i esterified (nmol/min)	P/O ratio
0.00	0.00	300	238	0.79
1.00	0.00	345	588	1.70
	0.25	685	960	1.40
	1.00	615	1170	1.90
	2.50	631	1200	1.90

Table 2. *Effect of cytochrome c 'dimer' on respiration and phosphorylation of cytochrome c-depleted mitochondria in the presence of 0.5 μ M-cytochrome c*

To the standard reaction mixture 5 mM-sodium succinate and 0.1 μ M-rotenone were added. All other conditions were as in Fig. 1.

Cytochrome <i>c</i> (μ M)	'Dimer' (μ M)	Oxygen consumed (ng-atom/min)	P _i esterified (nmol/min)	P/O ratio
0.00	0.00	330	200	0.60
0.5	0.00	850	450	0.52
	0.35	850	780	0.91
	0.70	850	1040	1.22
	1.40	780	1120	1.43
	2.10	760	1150	1.56

1 μ M-'dimer', further accelerated the respiratory rate and that the P/O ratio for this latter increment remained unchanged. Thus either a considerable fraction of the binding sites for cytochrome *c* remained vacant after binding of the 'dimer' or, alternatively, cytochrome *c* was able to remove the bound 'dimer' by competition.

In an experiment complementary to the one described in Table 1, cytochrome *c* was added to depleted mitochondria in an amount (0.5 μ M) sufficient to support the oxidation of succinate at a near maximum rate, but not adequate for efficient coupling of the respiration to phosphorylation (Table 2). This system was then titrated with the 'dimer'. It can be seen from Table 2 that the 'dimer' slightly depressed the rate of succinate oxidation but increased the P/O ratio from 0.52 to 1.56. The two roles assigned to cytochrome *c* were thus separated clearly: the native cytochrome *c* acted mainly as a respiratory carrier, whereas the 'dimer' acted solely on the coupling system.

Effect of cytochrome c and the 'dimer' on spontaneous mitochondrial swelling

Streichman & Avi-Dor (1967) showed that the addition of cytochrome *c* to cytochrome *c*-depleted

mitochondria stimulates spontaneous swelling without affecting the endogenous respiration that is the driving force for the swelling. The above findings have now been extended for the 'dimer' (Table 3). The swelling induced by the 'dimer', like that promoted by cytochrome *c*, displayed the features characteristic of energy-dependent swelling driven by electron transport, i.e. it was suppressed by respiratory inhibitors and by dinitrophenol and was not affected by oligomycin. The possibility was considered that cytochrome *c* or the 'dimer' may affect swelling by serving as terminal electron acceptors. (Accelerated electron flow through the 'shunt' created by cytochrome *c* or the 'dimer' would not be revealed by measuring oxygen uptake). However, since the swelling induced by the haemoproteins was inhibited by sodium azide, the above possibility can be excluded and thus the effect must be on energy conservation itself.

Effect of cytochrome c and the 'dimer' on respiration-driven proton translocation

Mitchell & Moyle (1967) have shown that when an anaerobic suspension of rat liver mitochondria is pulsed with oxygen in the presence of a respiratory substrate, a transient acidification results. The

Table 3. *Effect of cytochrome c and the 'dimer' on spontaneous swelling in cytochrome c-depleted mitochondria*

The assay medium consisted of 125mM-tris-HCl buffer (pH7.4) and depleted mitochondria in an amount yielding an initial extinction of 0.4–0.5 at 520nm (approx. 0.14mg of protein/ml). Temp. 28°C. Total volume 1.0ml.

Preparation	Cytochrome <i>c</i> (μM)	'Dimer' (μM)	Additions	Extent of swelling (ΔE_{250})	Respiration (ng-atom of O/min)
Intact mitochondria	—	—	—	0.095	3.5
Depleted mito- chondria	—	—	—	0.008	3.3
	5.5	—	—	0.110	2.5
	—	5.5	—	0.085	3.2
	5.5	—	NaN ₃ (1 mM)	0.013	—
	—	5.5	—	0.010	—
	5.5	—	Antimycin A (20ng/ml)	0.021	—
	—	5.5	—	0.012	—
	5.5	—	Oligomycin (50nmol)	0.100	—
	—	5.5	—	0.080	—
	5.5	—	Dinitrophenol (20 μM)	0.010	—
	—	5.5	—	0.011	—

Fig. 3. *Effect of cytochrome c and the 'dimer' on respiration-driven proton translocation in cytochrome c-depleted mitochondria*

The assay medium contained 150mM-sucrose, 10mM-KCl, 3mM-sodium succinate, 0.1 μM -rotenone, 40 units of ox liver catalase and depleted mitochondria (1.0mg of protein/ml) prepared by the method of Streichman & Avi-Dor (1967). The reaction medium was adjusted to pH7.4. The oxygen pulse was generated by injecting 2 μmol of H₂O₂. Temp. 28°C. Total volume 4.0ml. Cytochrome *c* (○) or the dimer (●) were added as indicated. ▲, H/O ratio in intact mitochondria.

magnitude of the H/O ratio strongly depends on the integrity of the coupling membrane and other factors in energy conservation. In the experiment shown in Fig. 3 we investigated the effect of cytochrome *c* and

the 'dimer' on proton translocation in cytochrome *c*-depleted mitochondria. Fig. 3 shows that in depleted mitochondria the H/O ratio is only a fraction of that in intact mitochondria under identical conditions. Both cytochrome *c* and the 'dimer' increased the H/O ratio although the latter was somewhat less effective in this respect.

Effect of cytochrome c and the 'dimer' on adenosine triphosphatase activity

The results of the experiments on swelling and proton movement showed that cytochrome *c* and the 'dimer' affected an early step in energy conservation. We also tested the effect of these haemoproteins on mitochondrial ATPase (adenosine triphosphatase) (Table 4), which is the final step leading to ATP synthesis. The results show that activation of the latent ATPase occurred in the step that leads to the removal of cytochrome *c* from the mitochondria. ATPase activity in depleted mitochondria was as high as the dinitrophenol-activated ATPase in intact mitochondria, or osmotically shocked mitochondria, and could not be further activated by uncoupler. Addition of cytochrome *c* or the 'dimer' did not change the activity of ATPase in depleted mitochondria. Thus, the changes that lead to the release of the latent ATPase activity during the removal of endogenous cytochrome *c* apparently could not be reversed when cytochrome *c* or the 'dimer' was bound to cytochrome *c*-depleted mitochondria.

Discussion

The pattern of action of cytochrome *c* 'dimer' described here accords with the view that it plays a direct role in energy conservation. A similar claim

Table 4. *ATPase activities of different mitochondrial preparations*

The experiments were done as described in the Experimental section.

Preparation	Addition	ATP hydrolysed (nmol/10 min per mg of protein)	
		No dinitrophenol	With dinitrophenol (20 μ M)
Intact mitochondria	None	52.9	224.1
Osmotically shocked mitochondria	None	38.2	224.1
Depleted mitochondria	None	211.1	235.9
	Cytochrome <i>c</i> (5.0 μ M)	187.4	226.4
	'Dimer' (5.5 μ M)	209.4	233.3

was made for cytochrome *c* (Avi-Dor & Streichman, 1969). However, separation of the roles in electron transfer and in energy transformation was easier in the case of the 'dimer', since it proved to be relatively ineffective as a substitute for cytochrome *c* in electron transport, but nearly as effective as the native cytochrome *c* in the restoration of energy-linked functions in cytochrome *c*-depleted mitochondria. The low efficiency of the 'dimer' as an electron carrier in a cytochrome *c*-depleted Keilin-Hartree preparation has already been demonstrated by Margoliash & Lustgarten (1962*b*). The direct effect of the 'dimer' on energy conservation is especially apparent from the experiment (Table 2) in which the addition of the 'dimer' increased the P/O ratio threefold without further stimulation of the respiratory rate in depleted mitochondria, to which most of the respiratory activity had been restored by a low concentration of cytochrome *c*. The stimulation of the electron transport-driven swelling of cytochrome *c*-depleted mitochondria by the 'dimer' or by cytochrome *c* without an effect on the respiratory rate is also in accord with their assumed direct role in energy conservation. The restoration of respiration-dependent proton translocation to depleted mitochondria by the 'dimer' indicates an effect on the first step of energy conservation, as suggested by Mitchell and his school (c.f. Greville, 1969). However, Mitchell's view in this respect is still disputed by some workers (cf. Greville, 1969). The effect of the 'dimer' and cytochrome *c* on energy conservation, in addition to their effect as electron carriers, recalls the findings of Racker & Horstman (1967) and of Schatz *et al.* (1967) on the catalytic and structural function of coupling factor F_1 in energy transformation. The binding of cytochrome *c* or the 'dimer' may alter the structure of a segment in the membrane in such a way that it becomes more efficient in energy

transformation. It is relevant to discuss some other suggestions that have been made in connexion with the effect of cytochrome *c* on energy transformation. (a) Tsou & Van Dam (1969) supplemented mitochondria with a low concentration of uncoupler and demonstrated that the P/O ratio increased with the rate of respiration. Such an observation is not compatible with the pattern of action of cytochrome *c* and the 'dimer' described in the present paper, since an increase in P/O ratio, in our case, was observed under conditions in which the respiratory rate remained constant. It is also not likely that cytochrome *c* and the 'dimer' increased the P/O ratio by virtue of their ability to remove an endogenous uncoupler in cytochrome *c*-depleted mitochondria, since haemoglobin and basic proteins such as lysozyme or polylysine were found to suppress rather than improve energy transformation (Streichman, 1969). (b) Exchange of exogenous adenine nucleoside phosphates for endogenous ones is an essential step both in ATP synthesis and hydrolysis in mitochondria (Klingenberg & Pfaff, 1966). Margoliash *et al.* (1970) suggested that cytochrome *c* may play a role in this exchange. However, we have found that ATPase activity in cytochrome *c*-depleted mitochondria is higher than in intact mitochondria and is as active as dinitrophenol-activated ATPase in native preparations. Moreover, neither cytochrome *c* nor the 'dimer' had an effect on the rate of ATP hydrolysis in depleted mitochondria whereas they did improve energy-linked functions under similar conditions. As the lack of ATP-ADP exchange does not limit ATPase activity in depleted mitochondria (cf. Mitchell & Moyle, 1971) it is also not likely to be the rate-limiting step in ATP synthesis.

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