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CONDITIONS FOR OPTIMAL ELECTRON TRANSFER ACTIVITY OF CYTOCHROME c OXIDASE ISOLATED FROM BEEF HEART MITOCHONDRIA

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Summary

The electron transfer activity of cytochrome c oxidase in disrupted but unfractionated beef heart mitochondria has been measured as close to 400 sec^{-1}. Published reports of the activity of the isolated enzyme have been very variable and consistently much less than this value (i.e. 30-300 sec^{-1}). Here we show that the apparent loss of activity upon isolation of cytochrome c oxidase is because less than optimal conditions have been used for assaying the enzyme. We describe an assay procedure which gives cytochrome c oxidase activities as high as those seen in unfractionated mitochondria. This involves dispersing the enzyme in Triton X-100 before assaying in mixed micelles of asolectin and lysophosphatidylcholine-1-oleoyl.

Introduction

Cytochrome c oxidase, the terminal member of the mitochondrial respiratory chain, transfers electrons from reduced cytochrome c to molecular oxygen (for reviews see 1,2). The energy released by this reaction is conserved for ATP synthesis, probably through a proton gradient generated across the inner mitochondrial membrane (3,4). Cytochrome c oxidase is difficult to study in intact mitochondria or in submitochondrial particles because many other proteins are present in these preparations. For this reason, most of the current work on the structure and function of cytochrome c oxidase utilizes the isolated enzyme.

It is generally assumed that cytochrome c oxidase is unaltered by its removal from the inner mitochondrial membrane, although there is no definitive evidence for this. On the contrary, the electron transfer activity reported for the isolated enzyme is invariably much lower than that of cytochrome c oxidase in detergent-disrupted but unfractionated mitochondria (5,6). This has led Vanneste et al (5) to suggest that the available fractionation procedures irreversibly alter the structure of the enzyme.

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In this communication we present evidence that the low activities reported for cytochrome c oxidase are because less than optimal conditions have been used in assaying the enzyme. We detail an assay procedure in which the purified enzyme is as active as cytochrome c oxidase in unfractionated mitochondria.

Materials and Methods

Materials. Cytochrome c (Type VI), Triton X-100, Tween 80, ascorbic acid, and lysophatidylcholine-1-oleoyl were obtained from Sigma. Asolectin was obtained from Associated Concentrates. Cholic acid and deoxycholic acid were obtained from Aldrich. Cholic acid and deoxycholic acid were recrystallized from 70% ethanol and solubilized with sodium hydroxide. Asolectin was solubilized in 1% cholate, 0.05 M sodium phosphate (pH 7.4) at a final concentration of 0.5%. Ascorbic acid was adjusted to pH 5 with Tris base and brought to a final concentration of 0.6 M in 1 mM EDTA.

Methods. Cytochrome c oxidase was isolated from beef heart mitochondria by the method of Capaldi and Hayashi (7). It was resuspended in 0.02 M Tris-HCl, 0.09 M NaCl (pH 7.4) to a protein concentration of 50-100 mg/ml and stored in small aliquots at -20°C.

For some experiments enzyme (10 mg in 0.3 ml) was dialyzed against 1 l of 0.05 M sodium phosphate (pH 7.0) at 4°C for 4 hours and with one change of buffer to remove residual cholate. Protein concentration was estimated by the method of Lowry et al (8) using bovine serum albumin as a standard. Cytochrome c concentration was measured by using a $\Delta\varepsilon_{mM}$ (reduced minus oxidized) of 21 $mM^{-1}cm^{-1}$ at 550 nm (9). Cytochrome c oxidase concentration was measured according to Van Gelder (10).

Mitochondria were treated with deoxycholate according to the method of Smith and Camerino (11). Cytochrome c oxidase activity was measured with a Clark-type oxygen electrode in a 2 ml water-jacketed chamber maintained at 25°C. The assay buffer included 0.05 M sodium phosphate (pH 7.4), 30 mM ascorbate, 40 μM cytochrome c, and 2-5 nM cytochrome c oxidase. Where indicated, 0.5% Tween 80 or 0.02% lysophosphatidylcholine were added. The high concentration of cytochrome c used gave maximal rates of electron transfer. Electron transfer activity was measured as moles oxygen per mole cytochrome c oxidase per sec (MA.) by multiplying by 4.

Results and Discussion

The overall rate of electron transfer in coupled mitochondria is low (around 10 sec^{-1}), even under the state 3 conditions of excess ADP and phosphate (12). However, cytochrome c oxidase is not involved in the rate limiting step and activities as high as 120 sec^{-1} have been obtained for this enzyme in mitochondrial suspensions by adding excess cytochrome c and ascorbate (11). Smith and Camerino (11) were able to further increase the activity of cytochrome c oxidase in mitochondria by adding sufficient deoxycholate to disrupt the inner membrane and obtained a maximal activity of 350 sec^{-1}. Similar values for the optimal activity of the enzyme have been obtained by others (5,13,14). We have

TABLE I
Maximal Activity of Cytochrome c Oxidase
in Deoxycholate Solubilised Mitochondria

Report	Method	pH	Temp	MA*
Smith and Camerino (11)	Polarographically	7.0	25°	350
Nicholls (14)	"	7.4	30°	450
			(25°)	(360)†
Vanneste et. al. (5)	spectrophotometric	7.0	25°	530
this work	polarographically	7.4	25°	415

* μmoles of cytochrome c oxidase per sec/μmole heme a_3
† estimate using Q_{10} (25-35°C) = 1.58 (13)

measured this activity and obtained a value of 415 sec^{-1} (Table I). The values obtained in the different studies are in reasonable agreement considering that slightly different assay conditions were used and different methods of estimating the enzyme concentration were employed.

The electron transfer activity of isolated cytochrome c oxidase has been measured in many different laboratories (15-21). The values reported are highly variable (in the range 30-300 sec^{-1}, average 100-150 sec^{-1}) and are in all cases considerably lower than that observed in unfractionated mitochondria. This low activity could be due to some denaturation of the enzyme during isolation, as proposed by Vanneste et al (5), or it could reflect the fact that the conditions employed to assay the purified enzyme did not support full activity. In order to decide between these possibilities, we have examined several factors which may affect the activity of the isolated enzyme.

Our first consideration was the state of aggregation of cytochrome c oxidase under the assay conditions. After some isolation procedures, the enzyme is dialyzed against buffer alone (18,19) or buffer containing Tween 80 (22) before assay. Commonly used assay procedures involve dilution of the enzyme into detergent-free buffer (23), dilution into detergents such as Tween 80 or Emasol 1130 (20,24), or mixing the enzyme with phospholipids (21,24). In our experience, both the dialysis step and dilution in the poorly dispersing amphiphiles listed above leads to aggregation of the enzyme. Similarly, there are problems of aggregation when phospholipids are used in the assay procedure. Aggregation of cytochrome c oxidase might be expected to lower the activity be-

cause of the problem of accessibility of substrate cytochrome c to the enzyme. In support of this conclusion, our cytochrome c oxidase preparation assayed under conventional procedures was only 75-150 sec^{-1} (Table II gives the data for a typical preparation), but this activity could be dramatically increased by incubating the enzyme in Triton X-100. Our previous studies (2) have shown that cytochrome c oxidase is monodisperse and a dimer (345,000 daltons) in solutions of this detergent. For the assay, Triton X-100 was used in the ratio 2 mg detergent per mg protein and the dispersed enzyme was diluted by a factor of more than 1000 with buffer containing Tween 80 for assay. This diluted out the Triton X-100, which is itself an inhibitor of cytochrome c oxidase (20,24, 25), but should not allow reassociation of the enzyme into higher aggregates because of the low final concentration of protein (1-2 μg/ml). Cytochrome c oxidase, treated with Triton X-100 and assayed in buffer containing Tween 80 consistently showed activities in the range 200-250 sec^{-1} (see Table II).

A second factor we considered was which amphiphile would provide the optimal environment for cytochrome c oxidase activity. Reassociation of the enzyme with phospholipids would provide the most physiological conditions for assay, but there is the problem of inaccessible enzyme molecules in phospholipid vesicles. The alternative to date has been to use Tween 80 or Emasol 1130 to replace the lipids around the protein and to keep the enzyme soluble. We have

TABLE II
Activity of Cytochrome c Oxidase Under Different Assay Conditions

Assay Conditions	MA†
Enzyme dialysed to remove cholate (see text) then assayed in 0.5% Tween 80	117*
Enzyme dialysed to remove cholate then assayed in the presence of asolectin according to [21]	132
Enzyme dispersed with Triton X-100 then assayed in 0.5% Tween 80	220
Enzyme dispersed with Triton X-100 then assayed in the presence of asolectin	285
Enzyme dispersed with Triton X-100 then assayed in 1-oleoyl-lysolecithin	380
Enzyme dispersed in Triton X-100 then assayed in asolectin-lysolipid mixed micelles	420

† μmoles of cytochrome c oxidase per sec/μmole heme a_3 as in Table I.
* all values reported are for one cytochrome c oxidase preparation - the range of values with different preparations is given in the text.

examined several other detergents for use in assaying cytochrome c oxidase. The one which supported the highest activity was lysophosphatidylcholine-1-oleoyl. Samples of cytochrome c oxidase treated with Triton X-100 and diluted into solutions of this lysolipid showed activities between 340-380 sec^{-1}. Tween 80 and lysophosphatidylcholine-1-oleoyl contain the same fatty acid, namely oleic acid, as the hydrophobic portion. Therefore, the increased activity seen with lysophosphatidylcholine cannot be due to its fatty acid composition, but must be due to the structure of its polar portion. It could be that these two detergents have different micellar properties or that a direct interaction of cytochrome c oxidase with the phosphoglycerol backbone of phospholipids is important for maximal activity.

Cytochrome c oxidase activity of enzyme dispersed with Triton X-100 and assayed with lysophosphatidylcholine as the activating detergent was higher than reported previously for the isolated enzyme, but the values obtained were still lower than those seen with unfractionated mitochondria. We therefore examined the possibility that some diacyl lipids in addition to the lysolipids were necessary for full activity. Thus, asolectin, solubilized with cholate, was added to cytochrome c oxidase that had been dispersed with Triton X-100. After a one hour incubation at $4^{o}C$, this sample of enzyme was assayed in the presence of a large excess of lysophosphatidylcholine. The lysolipid maintained the solubility of the enzyme in the presence of phospholipid during the assay. This addition of asolectin effected a further increase in electron transfer activity. Values of 380-450 sec^{-1} were now obtained. These values are as high as those seen for cytochrome c oxidase in unfractionated mitochondria. If the dispersion step with Triton X-100 was omitted and the enzyme was diluted into asolectin plus lysolipid directly, the activities obtained were only 200-250 sec^{-1} and therefore this step could not be omitted if full activity was to be obtained. Figure 1 then, summarizes the steps of the assay procedure which are essential for full enzymic activity.

In summary, an assay procedure is described in which isolated cytochrome c oxidase shows an activity as high as that seen in unfractionated mitochondria.

Step 1. An aliquot (1 mg) was mixed with Triton X-100 (2mg) from a 20% solution made up in the same buffer as the protein and this mixture was incubated on ice for 15 min.

Step 2. The Triton X-100-dispersed protein (0.2 mg) was added to a solution (2ml final volume) containing asolectin (1 mg) in 0.1% cholate, 50 mM sodium phosphate pH 7.4 and this mixture was incubated on ice for 1 hour.

Step 3. An aliquot of the protein-asolectin mixture (2µg of protein) was diluted to 2 ml in a solution containing 0.02% 1-oleoyl lysolecithin, 50 mM sodium phosphate pH 7.4 and cytochrome c oxidase was then assayed polarographically.

FIGURE 1. ASSAY PROCEDURE

Stock enzyme (50-100 mg/ml) was stored in 90 mM NaCl, 20 mM Tris HCl pH 7.4 (soluble in residual cholate)

This assay procedure involves dissolving the enzyme in Triton X-100 and then exchanging this dispersing detergent for mixed micelles of phospholipid and lysophosphatidylcholine, which in turn "activate" the enzyme. Thus the enzyme is not irreversibly denatured during purification. Under the conditions described above cytochrome c oxidase is not only fully active, but also optically clear and therefore ideally suited for spectroscopic as well as other studies of the structure and functioning of this complex enzyme.

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