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Isolation of Totally Inverted Submitochondrial Particles by Sonication of Beef Heart Mitochondria

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Abstract

A novel procedure for isolating totally inverted preparations of submitochondrial particles by sonication of beef heart mitochondria is described. The procedure involves only differential centrifugation in 0.25 M sucrose containing 0.15 M KCl. The submitochondrial particles have 96% of their cytoplasmic face cytochrome *c*-binding sites sequestered within the particles. Mild sonication exposes cytochrome *c*-binding sites to the medium. The oligomycin-sensitive ATPase of sonic-derived submitochondrial particles, like that of electron transport particles, is inhibited 98% by exogenous isolated ATPase inhibitor protein. NADH oxidase activity in these particles is inhibited by oligomycin. The respiratory control index (uncoupled rate/oligomycin-inhibited rate) is approximately 3.4 and can be increased by washing the particles with medium containing bovine serum albumin.

Key Words: Submitochondrial particles; ATPase; cytochrome *c*.

Introduction

Crane *et al.* (1956), Harmon and Crane (1974, 1976), and Harmon *et al.* (1974) have previously illustrated that electron transport particles isolated by alkaline homogenization of mitochondria using a Potter–Evelhjem glass–Teflon homogenizer are approximately 95% inside-out compared to intact mitochondria. These electron transport particles (ETP),[2] however, do not exhibit any coupled activity; the P/O ratio is zero and the respiratory control index (using oligomycin and uncouplers, Lee *et al.* (1969) is one. The vesicles not exhibit stimulation of respiration upon addition of exogenous cytochrome

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[2]SMP, submitochondrial particles; ETP, electron transport particles; RCI, respiratory control index; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid.

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c nor do they exhibit inhibition of succinate oxidase activity by addition of protamine or polylysine (Harmon *et al.*, 1974; Smith and Conrad, 1956).

In the early 1960's the technique of sonicating mitochondria to yield submitochondrial particles (SMP)[2] was introduced. The benefits of such a procedure are that submitochondrial particles could be isolated in a few hours (isolation of ETP requires 6–8 hr) and that SMP are coupled. However, numerous publications have noted that the SMP preparations isolated by sonication are not a population of homogeneously oriented vesicles (Harmon *et al.*, 1974; Malviya *et al.*, 1968; Huang *et al.*, 1973; Smith *et al.*, 1980). Under normal conditions, the SMP preparation has approximately 75% of its cytochrome *c*-binding sites sequestered within the SMP vesicle (compared to 95% of ETP); addition of exogeneous cytochrome *c* stimulates respiration approximately 33%. Such a preparation is not homogeneous but useful in the study of energy-linked functions (Huang *et al.*, 1973).

Recent studies have indicated that homogeneously oriented (90–95%) populations of SMP can be isolated from sonicated mitochondria by affinity chromatography over sepharose–cytochrome *c* columns (Godinot and Gautheron, 1979; Lötscher *et al.*, 1979) or by precipitation of mitochondria (or "right-side-out vesicles") from the SMP mixture (nonhomogeneous) using wheat germ agglutinin and anti-wheat germ agglutinin antibodies (D'Souza and Lindsay, 1981). These techniques, while effective, are expensive either in affinity chromatography material or time required to isolate antibodies. In this publication a technique will be described to isolated coupled SMP with greater than 95% inversion (cytochrome *c* site sequestered) from beef heart mitochondria via sonication and differential centrifugation.

Materials and Methods

Mitochondria are isolated from fresh bovine heart trimmed of all connective tissue. All subsequent steps are performed on ice or in a 7°C cold room. The meat is then passed through a cold sieve of 1/8 inch-holes of a commercial meat grinder. Approximately 800 ml of ground meat, 2 liters of 8.5% (w/v) cane sugar, and 75 ml of 1 M Tris base (unbuffered) are blended for 45 sec at low speed in a 1 gallon Waring commercial blender. The pH is adjusted to 7.0–7.2 using pH paper with either Tris base or concentrated acetic acid. The mixture is then centrifuged at 500 × *g* for 20 min in four 1-liter bottles in a swinging bucket rotor. The supernatant solution is filtered through four layers of cheesecloth and centrifuged at 27,000 × *g* for 15 min. The dark mitochondrial pellet is resuspended in 0.25 M sucrose and centrifuged at 27,000 × g for 15 min. The heavy and light mitochondrial fraction

layers are resuspended separately at approximately 55 mg protein/ml in 0.25 M sucrose and stored at −40°C in 10-ml aliquots until used.

To isolate submitochondrial particles (cf. Fig. 1), a 5–10-ml aliquot of mitochondria is thawed, homogenized, and resuspended in 0.25 M sucrose to a volume of 30 ml. Following centrifugation at 27,000 × *g* for 15 min, the dark mitochondrial pellet is resuspended at a protein concentration of 30 mg/ml in 0.25 M sucrose containing 10 mM sodium phosphate, pH 7.4 (Sorensen type). The colorless center "button" of the pellet is *not* resuspended. The mitochondria are then sonicated for 30 sec in a 30-ml stainless steel vessel at saturated full power using a Model 185 Branson sonifier with 1/2-inch diameter tip. The sonicated mixture is then centrifuged at 23,500 × *g* for 10 min; the

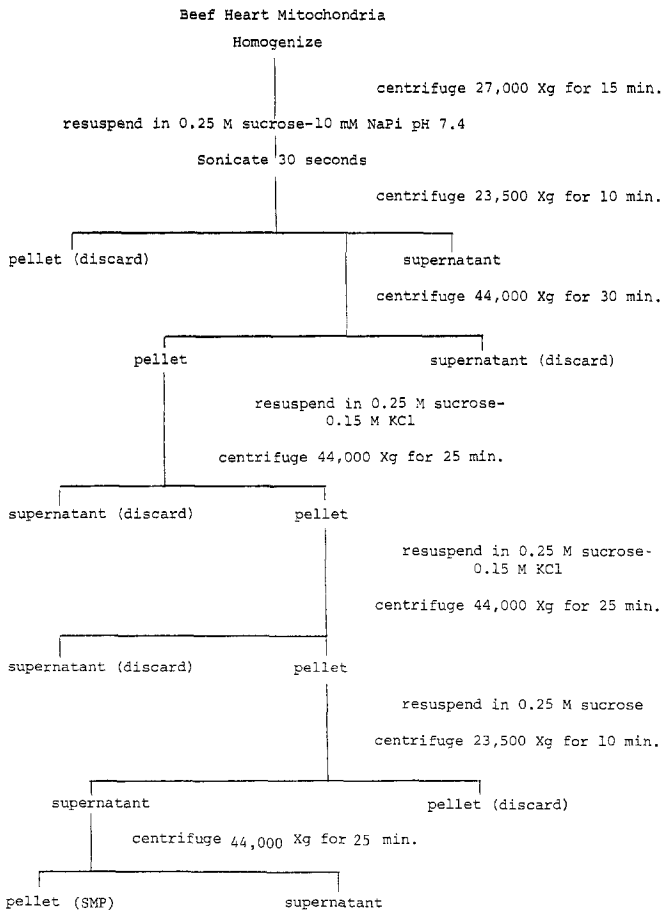

Fig. 1. Flow chart of isolation procedure.

supernatant (cloudy and slightly opalescent) is removed with a pipette and bulb. Care must be exercised to not remove any portion of the dark, dense pellet material. The supernatant solution is centrifuged at 27,000 rpm (44,000 × *g*) in a type 50 Spinco rotor for 30 min. The pellets are resuspended and washed twice in 30 ml of 0.25 M sucrose containing 0.15 M KCl. The pellets are resuspended in 0.25 M sucrose and centrifuged at 23,500 × *g* for 10 min. The supernatant solution was then centrifuged at 44,000 × *g* for 25 min in a type 50 Spinco rotor. The pellets were resuspended in 0.25 M sucrose and stored on ice until used; 20–30% of starting protein is usually present in the SMP fractions.

NADH oxidase activity was measured at 25°C in a 1.8-ml glass water-jacketed chamber fitted with a Clark electrode. The buffer medium contained 83 mM sodium phosphate (Sorensen) and 83 μM EDTA (pH 7.4). Between 50 and 150 μg of submitochondrial particles were used in each assay. NADH (0.7 mM) is used as substrate and 200 μg cytochrome *c* (type III, Sigma) was added where indicated.

Respiratory control indices were determined by measuring the rate of NADH oxidase in the presence of 1 μg oligomycin and in the presence of 2.67 μM CCCP (Huang *et al.*, 1973). Oxygen uptake was measured in a 1.8-ml reaction mixture containing 0.25 M sucrose–40 mM Tris-SO_4, pH 7.4.

ATPase activity was determined by the procedure of Adolfsen and Moudrianakis (1971) in the presence of 4.8 μM CCCP. Oligomycin sensitivity was measured by addition of 1 μg oligomycin. ATPase inhibitor protein was isolated from beef heart mitochondria by the procedure of Horstman and Racker (1970).

Protein concentrations were determined using either the Biuret (Yonetani, 1961) or Lowry (Lowry *et al.*, 1951) method with bovine serum albumin as standard.

Difference spectra were recorded using a Johnson Research Foundation (University of Pennsylvania) double-beam scanning spectrophotometer (model DBS-3). Dithionite was used as reductant. Cytochrome content was calculated using millimolar Δ extinction coefficients (reduced *minus* oxidized) of cytochromes *c*, *b*, and *a* of 19.5, 28, and 24, respectively.

Electron transport particles were isolated by the alkaline procedure of Crane *et al.*, (1956).

Results

Electron transport particles derived by alkaline homogenization of mitochondria (Crane *et al.*, 1956) are routinely 94–95% inverted as determined by both the lack of stimulation of NADH and succinate oxidase

Table I. Effect of Exogenous Cytochrome *c* on Respiration of Submitochrondrial Particles and Electron Transport Particles

Particle	Oxygen consumption (μmole O_2/min/mg protein)	Percent exposure of unoccupied cyt *c* binding sites
SMP[a]		
− cyt c	1.34	
+ cyt c[b]	1.38	3
ETP		
− cyt c	0.96	
+ cyt c[b]	0.99	4

[a] 2.4 μM carbonyl cyanide *m*-chlorophenylhydrazone present.
[b] 200 μg cyt c added.

activity by cytochrome *c* addition or by inhibition of succinate oxidase activity by protamine (with cytochrome *c* present) (Harmon and Crane, 1974, 1976; Harmon *et al.*, 1974). After washing with 0.15 M KCl, the stimulation of succinate oxidase activity by exogenous cytochrome *c* is still approximately 4% as shown by the typical data in Table I.

Submitochondrial particles isolated by the sonication/centrifugation treatment described here exhibit less than 4% stimulation of NADH oxidase activity as shown in Table I. Of 32 isolations, respiration in the absence of exogenous cytochrome *c* was 96 ± 2.8% that in the presence of cytochrome *c*. This indicates that 96% of the cytochrome *c*-binding sites are not exposed to the medium. Following sonication of SMP for 30–45 sec at full power with microtip, the respiration in the absence of exogenous cytochrome *c* was 74 ± 6.5% that in the presence of exogenous cytochrome *c* (eight separate determinations), indicating that nonoccupied cytochrome *c*-binding sites previously sequestered within the vesicle are now exposed.

Addition of the isolated endogenous ATPase inhibitor protein to ETP isolated by alkaline homogenization (Crane *et al.*, 1956) routinely inhibits 97–98% of the membrane-bound oligomycin-sensitive uncoupler-stimulated ATPase activity (Table II). Addition of ATPase inhibitor protein to SMP inhibits 98% of the oligomycin-sensitive uncoupler-stimulated ATPase activity also. Sonication of SMP does not cause an increase in ATPase activity, indicating that all ATPase molecules are exposed to the medium.

That inhibition of ATPase activity by exogenous inhibitor protein is a reliable indicator of membrane orientation in submitochondrial particles is shown by the agreement of the extent of inhibition of ATPase (exposure of ATPase) with the lack of exposure of cytochrome *c*-binding sites. Because the ATPase complex and cytochrome *c* sites are known to be located on opposite surfaces of the membrane, exposure of one marker should correspond to the lack of exposure of the other. In SMP isolated by the technique of Huang *et al.*

Table II. Effect of Exogenous ATPase Inhibitor Protein on ATPase Activities of Submitochondrial Particles and Electron Transport Particles

Particle	Additions[b]	ATPase activity[a]	Percent inhibition[c]
ETP (100 μg)	CCCP	1.0	
	oligomycin	0.26	
	3 μg inhibitor + CCCP	0.28	97%
SMP (44.8 μg)	CCCP	3.1	
	oligomycin	0.54	
	6 μg inhibitor + CCCP	0.60	98%

[a] μmol P_i released/mg protein/10 min.
[b] 1 μg oligomycin added where indicated; 4.8 μM CCCP added where indicated.
[c] Percent inhibition calculated by the formula (CCCP) − (Inhibitor + CCCP) rate/(CCCP) − (Oligomycin) rate.

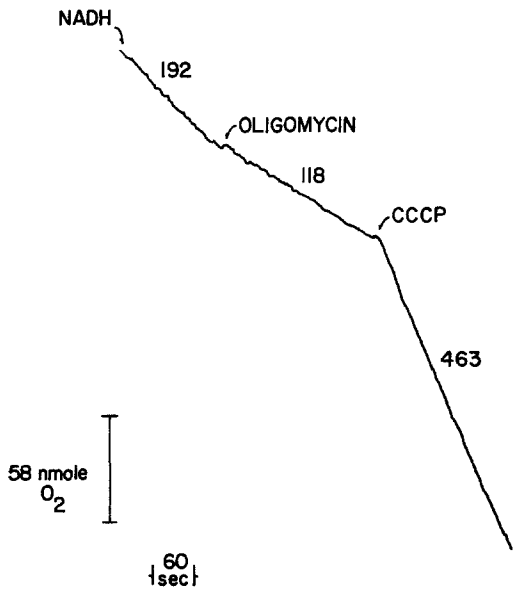

Fig. 2. Respiratory control in submitochondrial particles. 89 μg beef heart submitochondrial particles were suspended in 0.25 M sucrose–40 mM Tris-SO_4 (pH 7.4) at 25°C. The reaction was initiated by addition of NADH (0.7 mM final concentration) Where indicated, 10 μg oligomycin or 2.67 μM CCCP were added. Numbers beside the drawings indicate nmol O_2 consumed/min/mg protein.

(1973) 25% stimulation of respiration in the presence of exogenous cytochrome *c* (80% of cytochrome *c* sites sequestered within the vesicle) and 80% inhibition of ATPase activity by exogenous inhibitor protein have been reproducibly observed here.

While alkaline-isolated ETP routinely show no inhibition of respiration upon addition of oligomycin, SMP isolated by sonication exhibit inhibition of respiration by oligomycin and a "release" of the inhibition upon addition of uncouplers such as CCCP. The ratio of respiratory rates in the presence of uncoupler and oligomycin (RCI, Huang and Lee, 1975) in Fig. 2 is 3.9 using NADH as substrate. The RCI can be increased up to 5–6 if the SMP are washed once in 3% bovine serum albumin and then washed twice in 0.25 M sucrose (Lee, 1979). The RCI of these preparations is similar to that of other investigators using beef heart mitochondria as starting material (Huang *et al.*, 1973; Huang and Lee, 1975; Lee, 1979).

The calculated values of cytochrome content of submitochondrial particles are 0.63, 0.54, and 0.63 nmol/mg protein for cytochromes $a + a_3$, b, and $c + c_1$, respectively (Fig. 3). These values are similar to those of ETP (Crane and Glenn, 1957) and SMP (Huang, *et al.*, 1973).

Discussion

From the data in Table I it is apparent that submitochondrial particles that are greater than 95% inverted can be isolated from beef heart mitochondria by sonication. The lack of stimulation of respiration by exogenous

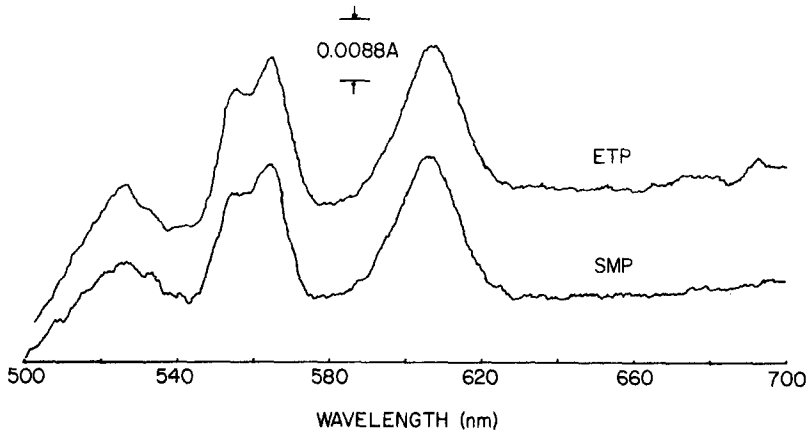

Fig. 3. Difference spectra of submitochondrial particles and electron transport particles. 1.29 mg of SMP and 1.2 mg of ETP were suspended in 0.25 M sucrose–50 mM sodium phosphate buffer (pH 7.4).

cytochrome *c* following washing with medium containing 0.15 M KCl indicates that the cytochrome *c*-binding site is note exposed to the medium. Since the cytochrome *c*-binding site has been shown previously to be on the cytoplasmic face only (see Harmon *et al.*, 1974, for a review), the data indicate that SMP are 97% inverted. In this aspect sonication-isolated SMP are extremely similar to ETP derived by alkaline treatment (Crane *et al.*, 1956).

The isolation procedure described here involves two washes with 0.15 M KCl-containing medium to remove any exposed cytochrome *c* that is bound to the membrane (Harmon and Crane, 1974; Harmon *et al.*, 1974; Jacobs and Sanadi, 1960). As a result, respiratory rates of particles in the absence of exogenous cytochrome *c* reflect the activity of only those particles that are both inverted and contain cytochrome *c*; respiratory rates obtained in the presence of cytochrome *c* reflect the activity of inverted and noninverted membranes. Since respiration is limited by the presence of cytochrome *c*, the ratio of rates in the absence and presence of exogenous cytochrome *c* is an indication of the exposure of cytochrome *c*-binding sites.

That SMP are inverted and have the cytochrome *c*-binding site sequestered inside the vesicle is illustrated by the fact that sonication of 97% inverted vesicles "opens" the vesicles; the result is that exogenous cytochrome *c* stimulates respiration 26%. Brief sonication destroys the permeability barrier to cytochrome *c* and allows cytochrome *c* to bind to exposed vacant binding sites and stimulate respiration, as was observed by Mackler and Green (1956) and by D'Souza and Lindsay (1981) for rat liver SMP.

That the SMP are of an inverted nature is substantiated by the extensive inhibition of ATPase activity by exogenous inhibitor protein. If the particles were inverted, they would exhibit ATPase inhibition to an extent similar to the value of nonexposure of cytochrome *c*. Both SMP and ETP exhibit approximately 97% inhibition of oligomycin-sensitive ATPase and 95% nonexposure of cytochrome *c*; both preparations of particles are similarly oriented, although sonic-derived SMP exhibit higher ATPase activity. For comparison, addition of exogenous ATPase inhibitor protein to freshly isolated rabbit heart mitochondria [using the procedure described by Chance and Hagihara (1963) for pigeons] results in only 8% inhibition of oligomycin-sensitive ATPase activity.

ATPase inhibitor protein has a molecular weight of approximately 10,000–11,000 daltons and is soluble in water (Horstman and Racker, 1970); because of its size and obvious polar nature, it is not expected to cross the membrane and will inhibit only exposed ATPase molecules. Like oligomycin (Racker, 1963), the inhibitor protein is far more effective in inhibiting membrane-bound ATPase than soluble ATPase (Horstman and Racker, 1970); addition of ATPase inhibitor (6 μg as in Table II) inhibits 10% of the

ogligomycin-insensitive ATPase activity of soluble ATPase isolated following sonication of submitochondrial particles. That both oligomycin and the inhibitor protein inhibit activity to the same extent is consistent with their acting on membrane-bound ATPase. We can assume that all oligomycin-sensitive ATPase is exposed to the medium and that ATPase activity not inhibited by oligomycin or the inhibitor protein is due to soluble non-membrane-bound ATPase.

Procedures for the isolation of coupled SMP from beef heart mitochondria by sonication have been described previously (Huang *et al.*, 1973; Huang and Lee, 1975; Lee, 1979). However, not all preparations are 95–100% inverted (Harmon *et al.*, 1974; Malviya, *et al.*, 1968; Huang *et al.*, 1973; Smith *et al.*, 1980; and Results section). Procedures to isolate SMP by using a Parr bomb or other similar procedures to isolat SMP by using a Parr bomb or other similar pressure systems (Fleischer *et al.*, 1974) have also been described. These procedures all seem to yield 95–100% inverted vesicles on the basis of lack of stimulation of respiration by exogenous cytochrome *c*. However, examination of the procedures and the data indicates that treatment of the particles with high-ionic-strength media is not included. SMP preparations could contain noninverted membranes that have endogenous cytochrome *c* bound to the exposed sites; addition of cytochrome *c* would have little effect on respiration since the exposed sites are already occupied.

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