Preparation and Some Properties of Submitochondrial Particles from Tightly Coupled Mung Bean Mitochondria

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ABSTRACT

Osmotic shock was found to be better than freezing and thawing, a French press, or sonic oscillation for the preparation of submitochondrial particles from mung bean (Phaseolus aureus) hypocotyl mitochondria. Particles prepared by osmotic shock rapidly oxidize reduced nicotinamide adenine dinucleotide and succinate, but they oxidize malate slowly. NADH oxidation was slightly stimulated by cytochrome c, ATP, and ADP; succinate oxidation was markedly increased by ATP, slightly by ADP and cytochrome c; and malate oxidation required the addition of NADH. NADH oxidation is inhibited weakly by amylal, completely by antimycin A and KCN, but not by rotenone. Chlorosuccinate, malonate, antimycin A, and KCN inhibit succinate oxidation. The action of antimycin A and KCN is incomplete, while chlorosuccinate and malonate were competitive inhibitors. Antimycin A combined stoichiometrically with particle protein in the ratio of 0.23 millimicromole per milligram of protein.

Oligomycin and bis(hexafluoroacetonitril) acetone, a potent uncoupler of oxidative phosphorylation, were without effect on oxygen uptake but did influence the ATP-stimulated onset of respiration when succinate was substrate. Fresh particles were markedly inhibited by oxylguanidine, indicating energy conservation, but this inhibition decreased on storage of the particles.

Spectra show the presence of cytochrome components the same as those of the intact mung bean mitochondrion, but present at higher concentrations. The molar concentrations of the particle cytochromes were two to three times those of the intact mitochondrion and the molar ratios were calculated as 0.9:1.0:1.0:2.8 for cytochromes a:b:c:flavoprotein, respectively.

Submitochondrial particles prepared from animal mitochondria have been found to show many of the properties and functions of the intact mitochondria and have been extensively studied in elucidating processes of electron transport and phosphorylation (cf. 14, 16).

Ikuma and Bonner (8) have described a procedure for the production of tightly coupled mitochondria with reproducible characteristics from dark-grown mung bean hypocotyls (Phaseolus aureus var. Jumbo). These mitochondria have been characterized with respect to malate, NADH, and succinate as substrates and to a range of uncouplers and inhibitors of electron transport and phosphorylation (8-10, 17). Studies of the spectral properties of the electron transfer components have been reported (11).

This paper compares four methods of producing submitted mitochondrial particles and describes a simple method which converts mung bean mitochondria to submitochondrial particles with reasonable yield. The particles produced have a relatively high activity for the oxidation of substrates and can be stored at −15° C and still retain much of their activity. The apparent Michaelis constant (Km) for malate, succinate, and NADH have been measured together with the inhibition by examples of each of the four classes of inhibitors previously characterized with intact mung bean mitochondria. Malonate and chlorosuccinate were used as inhibitors of succinoxidase, amylal, and rotenone to inhibit NADH-cytochrome C reductase. Antimycin A was used as an inhibitor of electron transport between cytochromes b and c, and cyanide as an inhibitor of cytochrome oxidase.

Oxytylguanidine was used as an inhibitor of energy transfer acting close to the respiratory chain (15, 8), 1799 as an uncoupler of energy transfer from electron transport, and oligomycin as an inhibitor of phosphorylation.

Accompanying spectra show that the submitochondrial particles have electron transfer components similar to those of the intact mung bean mitochondria.

METHODS

Initially mitochondria were isolated from dark-grown mung bean hypocotyls 5 to 6 days old as described earlier (8). Later, increased yields of mitochondria were obtained by disrupting the tissue for 5 sec with a Polytron mixer instead of hand grinding.

For a comparison of preparative methods, submitochondrial particles were made in a disruption medium containing 0.25 M sucrose, 5 mM ATP, 1 mM EDTA. All operations were carried out at 4° C with freshly isolated, tightly coupled mitochondria.

Five cycles of freezing and thawing were carried out by freezing the mitochondria suspended in the sucrose medium with liquid nitrogen, then thawing in cold water.

Two treatments with a French Press at 8000 p.s.i. were performed with an Aminco French pressure cell.

Ultrasonic treatment for 1.5 min at 4 amp was given at 30-sec bursts with a 1-min break between each burst with a Branson Sonifier model LS-75.

The osmotic shock treatment consisted of slowly adding cold glycerol to a suspension of the mitochondria until the final glycerol concentration was about 80%. After the mitochondria were allowed to soak in this medium for 30 min, the suspension was injected with a hypodermic syringe fitted with a No. 18

1 Abbreviations: 1799: bis(hexafluoroacetonitril) acetone; BSA: bovine serum albumin.
needle into 20 volumes of a rapidly stirred disruption medium.

In the improved osmotic shock method, subsequently adopted as routine for preparing submitochondrial particles, the sucrose disruption medium was replaced by 0.1% BSA and 1 mM EDTA adjusted to pH 7.2 with 0.1 M KOH. The particles produced appeared similar to those prepared in the sucrose medium.

After disruption of the mitochondria by one of the above methods, any remaining intact mitochondria were removed by centrifugation at 10,000g for 15 min, and the submitochondrial particles were isolated at 100,000g for 60 min. The isolated particles were washed by resuspension in wash medium (0.3 M mannitol, 0.1% BSA, 1 mM EDTA, pH 7.2) and resedimented at 100,000g. Further particles could be recovered by resuspending the mitochondrial residue in wash medium and centrifuging at 10,000g and 100,000g. The isolated particles were either used for experiments or stored at -15°C until required.

Table 1 summarizes the results obtained from the different methods used for mitochondrial rupture, and Figure 1 shows the flow sheet for the improved osmotic shock method. Protein contents were determined by the Lowry procedure (13) with BSA as the standard.

Oxygen uptake was measured by the polarographic method with a Clark-type oxygen electrode (Yellow Springs Instrument Co.) in Lucite chambers of 1- or 3-ml capacity stirred by a magnetic stirrer. Oxygen tension was recorded with a linear recorder, and respiratory rates were calculated from the traces on the basis of 240 μM oxygen in the aerated medium.

The Michaelis constant, Km, for succinate, malate, and NADH was determined from the rate of oxygen consumption after successive small additions of the substrates to the reaction mixture given below. NADH solutions were standardized spectrophotometrically at 340 nm with 6.22 as mm extinction coefficient (3). Because of the low malate dehydrogenase activity, only succinate and NADH were used as primary substrates to study the effects of inhibitors. Inhibition by malonate, chlorosuccinate, amytal, rotenone, antimony A, KCN, and octylguanidine was measured by following the oxygen uptake for at least 1 min after successive small additions of the inhibitor (dissolved in water or ethanol and adjusted to approximately pH 7.2). The inhibitor constant, Ki, was calculated according to the method of Dixon (6). Molecular weights of 153, 250, 394, and 500 were assumed for chlorosuccinate, amytal, rotenone, and antimony A, respectively.

The basic reaction medium contained 0.3 M mannitol; 10 mM KCl; 10 mM potassium phosphate buffer, pH 7.2; 5 mM MgCl2. After the addition of submitochondrial particles, 10 mM succinate or 1 mM NADH, 0.2 mM ATP, and 2 mM cytochrome c were added to initiate respiration. When malate was used as substrate, the basic mixture and submitochondrial particles were supplemented with 20 mM malate, and 2 mM NADH.

Difference spectra were recorded at liquid nitrogen temperatures with a split beam spectrophotometer (3). When necessary, spectra were replotted on a flat base line with isobestic points at 615, 530, and 450 nm (5). Concentrations of respiratory components were calculated by the method of Chance and Williams (4). Wave length pairs were modified to take account of the fact that the absorption peaks of plant respiratory carriers do not coincide with those of animal carriers (11).

Chlorosuccinate was kindly donated by Dr. E. Racker of Cornell University, octylguanidine sulphate by Dr. B. Pressman of the Johnson Research Foundation, and 1799 by Dr. P. Heytler of DuPont de Nemours, Wilmington, Delaware.

### RESULTS

Critique of Production of Submitochondrial Particles. The methods are compared for their ability to produce a reasonable yield of particles with high rates of oxidation of succinate and NADH which could be retained after overnight storage at -15°C. The osmotic shock technique was superior in the specific activity of succinate and NADH oxidase and further developed to improve yield. Unlike the intact mitochondria, the particles could be stored at -15°C and still retain much of their activity. It was also possible to store the glycerol suspension of mitochondria for up to a month at -15°C and subsequently produce very active particles. The energy-linked functions which have been described elsewhere (18) were always rapidly lost on storage. Table 1 shows the ability of fresh and frozen submitochondrial particles, produced by the improved osmotic shock method, to oxidize NADH, malate, and succinate compared with the activities of fresh and frozen mung bean mitochondria.

### Substrate Requirements for the Oxidation of NADH, Succinate, and Malate.

Particles produced by the improved osmotic shock method could oxidize NADH and were further stimulated by added cytochrome c and ATP (Fig. 2A). There was no oxidation
addition of ATP produced a slow increase in respiration (Fig. 3), but not as large as that observed on the addition of ATP. It has been observed (9) that uncouplers exert their maximal effect on intact mitochondria only when the mitochondria have been exposed to ATP.

In agreement with the observations of Lardy et al. (12) with mammalian submitochondrial particles, oligomycin up to 4 \( \mu \text{g/ml} \) was without effect on oxygen consumption by mung bean submitochondrial particles when either NADH or succinate was substrate in the presence of ADP. Oligomycin prevented the rise in respiration occurring when ATP was added in the presence of succinate (Fig. 3).

The effects of octylguanidine on NADH and succinate oxidation of fresh particles and on succinate oxidation by particles stored 24 hr are shown in Figure 4. The half-maximal inhibition of fresh particles occurred at 0.64 mM for NADH oxidation and 1.12 mM for succinate oxidation compared with values of 0.36 and 0.68 for NADH and succinate oxidation, respectively, by intact mitochondria (17). Stored particles lost much of their octylguanidine sensitivity in a manner analogous to that observed for intact mitochondria.

Effects of Inhibitors of Electron Transfer. Both malonate and chlorsuccinate inhibited succinate oxidation by submitochondrial particles, the values of the inhibitor constant \( K_i \) for malonate being in agreement with that observed for intact mitochondria (10) (Table II). Chlorsuccinate, however, had a value of \( K_i \) higher for the particles than for the mitochondria. Unlike mammalian submitochondrial particles, mung bean submitochondrial particles did not oxidize chlorsuccinate.

Rotenone up to 90 \( \mu \text{M} \) was without effect on the submitochondrial particles oxidizing NADH. Amytal was also relatively ineffective (Fig. 5); the half-maximal inhibition occurring at 7 mm compared with 2 to 2.5 mM for intact mitochondria with malate as substrate. The inhibition by amytal, however, varied between preparations. Because of the poor response to these inhibitors when NADH was being oxidized, no attempt was made to study their effect on succinate oxidation.

![Fig. 2. Substrate requirements of respiration of fresh and aged (24 hr at -15 C) submitochondrial particles (SMP). Oxygen electrode traces showing: A: NADH oxidation; B: malate oxidation; C: succinate oxidation, fresh particles; D: succinate oxidation, particles aged 24 hr at -15 C.](image)

![Fig. 3. Oxygen electrode traces showing the effect of magnesium 1799, and oligomycin added to submitochondrial particles oxidizing succinate: A: without MgCl\textsubscript{2}; B: with 5 mM MgCl\textsubscript{2}, 2 \( \mu \text{g} \) of oligomycin added after 0.2 mM ATP and 0.2 mM ADP; C: with 5 mM MgCl\textsubscript{2}, 20 \( \mu \text{g} \) of 1799 added prior to adding 2.5 mM ATP; D: with 5 mM MgCl\textsubscript{2}, 2 \( \mu \text{g} \) of oligomycin added prior to adding 0.2 mM ATP.](image)

![Fig. 4. The effect of octylguanidine on the respiration of submitochondrial particles supplied with succinate or NADH as substrate. Particles fresh and aged (24 hr at -15 C).](image)

Table II. \( K_m \) and \( K_i \) Values for Mung Bean Submitochondrial Particles and Mitochondria

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<th>( K_m )</th>
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<td>( \times 10^{-4} )</td>
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<tr>
<td>Succinate</td>
<td>0.44</td>
<td>3.0</td>
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<tr>
<td>Malate</td>
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cytochromes \( a \) and \( a_3 \) (11), a large multiple band at 561 to 547 nm due to cytochromes \( b \) and \( c \) (11), and a large band at 515 nm with a Soret peak around 420 nm. Room temperature spectra show a shift of a few nanometers toward the red end of the spectrum compared with those produced at \(-190^\circ C\). Comparison of low temperature NADH-reduced-oxidized and dithionite-reduced-oxidized spectra (Fig. 9) shows that a high percentage of the various cytochrome components can be enzymically reduced compared with intact mitochondria, which contain a component which can only be reduced by dithionite (11). The proportion of the respiratory pigments which could be enzymically reduced varied depending on the age and condition of the particles. From room temperature spectra the concentrations of the respiratory pigments were calculated to be 0.3, 0.37, 0.32, and 0.97 \( \mu \)mole/mg protein corresponding to molar ratios of 0.9:1.0:1.0:2.8 for cytochromes \( a + a_3, b, c \), and flavoproteins, respectively. The concentrations are approximately two to three times those reported earlier for intact mung bean mitochondria (11), a feature

Figure 6 shows that the effect of antimycin A on the submitochondrial particles varied somewhat with batches, but was similar to that found for intact mitochondria, the inhibition being dependent on the concentration of submitochondrial particles. The slope of a plot of half-maximal inhibition concentration against protein concentration was found to be 0.23 \( \mu \)mole/mg of protein, similar to that observed for intact mitochondria. Values for the half-maximal inhibition were identical for both succinate and NADH, indicating that a similar antimycin A site is utilized for the oxidation of both inhibitors. Succinate oxidation, however, is incompletely inhibited by antimycin A.

Cyanide inhibited NADH oxidation almost completely, but the half-maximal concentration varied somewhat between batches. For the example used in Figure 7, this was 11 \( \mu \)M NADH, close to that observed for the intact mitochondria. Succinate oxidation was also variable in its response to KCN but was always less sensitive to KCN inhibition (half-maximal 19 \( \mu \)M), with 15 to 20\% of the respiration being insensitive to high concentrations of the inhibitor.

**Spectral Properties of Submitochondrial Particles.** Submitochondrial particles prepared from mung bean mitochondria possess properties similar to those found in the intact mitochondria. Low temperature spectra show considerable enhancement and improved resolution compared with those at room temperature (Fig. 8). Reduced-oxidized spectra measured at low temperature, \(-190^\circ C\), have a sharp peak at 598 nm attributable to
in agreement with the correspondingly high NADH oxidase activity of the particles. Similar observations have been reported for submitochondrial particles prepared from beef heart mitochondria (7). The molar ratios, however, indicate relatively less of cytochrome c and flavoprotein, both of which are easily removed from mitochondria. Unlike the intact organelle, pyridine nucleotides do not appear to be present in submitochondrial particles since externally added NADH is required to initiate malate oxidation.

Further resolution of the cytochrome components was achieved by the use of antimycin A or cyanide as inhibitor (Fig. 10). In the presence of antimycin A, NADH-reduced-oxidized spectra show only the cytochrome b components. These can be observed as a double peak at 553 and 557 nm with a shoulder at 561 nm, a weak b-band at 532 and 525 nm, and a single Soret band at 427 nm. Comparison of the spectrum with a similar treatment of intact mitochondria shows a smaller amount of the 561 nm b component in the particles. When antimycin A is replaced by KCN cytochrome oxidase and cytochrome c remain reduced. Cytochrome oxidase can be observed as a band at 597 nm and peaks at 443 and 436 nm in the Soret region. The sharp peak at 547 nm is the combined bands of cytochromes c with b-bands at 507 and 510 nm and a Soret band at 461 nm. The cytochromes b were not reduced in the presence of KCN. In the intact mitochondria the reduction of the b cytochromes is markedly energy-dependent.

**DISCUSSION**

The results reported in this paper clearly demonstrate that active, stable submitochondrial particles can be prepared from mung bean mitochondria. The particles produced have been characterized with respect to substrates and inhibitors sufficiently to allow their use in further studies. Although the particles were still active after prolonged storage, the variable sensitivities to inhibitors probably resulted from a slow deterioration, particularly of the energy-linked functions. Particles prepared by osmotic shock of mung bean mitochondria show many of the properties of the original mitochondria but are less sensitive than the original organelle to some inhibitors and uncouplers, a feature most marked in the NADH-cytochrome b segment of the electron transport chain, where sensitivity to amytal is greatly reduced. It has been reported earlier (10) that mung bean mitochondria are only poorly inhibited by rotenone and are insensitive in the absence of ADP (state 4), and the submitochondrial particles are unaffected by rotenone.

The respiration of the particles is unaffected by oligomycin and uncouplers, suggesting a loss of energy coupling resulting from the disruption of the structure of the mitochondrion. However, the particles are still inhibited by octylglycannide and are affected by ATP, ADP uncouplers, and oligomycin in the early stages of respiration, particularly when succinate is used as substrate. These observations indicate that some energy coupling is still retained in fairly fresh particles. In another paper of this series (18) the energy-linked functions of the submitochondrial particles will be examined in greater detail.

Disruption of the mitochondrial structure results in greater accessibility of substrate to the inner membrane fragments and also to a loss of easily solubilized components. Thus, there is a loss of mitochondrial protein which results in a high specific activity of NADH oxidase and an increased concentration, on a protein basis, of respiratory pigments. In the fragmentation process pyridine nucleotide, flavoprotein, and cytochrome c are also lost. Spectral evidence shows that large amounts of enzymically reducible cytochrome c remain in the mung bean submitochondrial particles, but a large portion of this absorption band may be contributed by the second, firmly membrane-bound c-type cytochrome (11).

Osmotic shock has proved to be a relatively gentle technique for the disruption of cells and organelles (1). When particles are prepared from mammalian mitochondria by sonic oscillation, fusion of the broken membranes gives rise to spherical submitochondrial particles which are inside-out compared with the intact mitochondrion. No evidence is at present available to indicate the situation when plant mitochondria are disrupted by osmotic shock. Refusion of the broken membranes must occur, but whether the vesicles are inside-out or retain the same configuration as in the intact mitochondrion remains unknown.

When succinate was used as substrate, antimycin A and KCN did not completely inhibit oxygen consumption by the particles. The proportion of respiration insensitive to the two inhibitors varied between preparations, but was much greater than that for NADH as substrate. Differential effects of antimycin and KCN have been observed elsewhere (10) for mung bean mitochondria utilizing malate or succinate. Submitochondrial particles were 95 to 100% sensitive to antimycin A and KCN when NADH was
supplied as substrate, but 15 to 30% insensitive to the inhibitors when succinate was substrate.

LITERATURE CITED