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Mitochondrial Heterogeneity in *Aspergillus nidulans*: Evidence of *in vivo* Transformation Among Different Mitochondrial Populations

J. Montoya and M. J. López-Pérez

Departamento de Bioquímica
Facultad de Farmacia
Universidad Complutense
Madrid-3

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The distribution of mitochondria from *Aspergillus nidulans* on a dextran-sucrose gradient resulted in the fractionation of three bands. The lightest band presented the highest respiratory and cytochrome c oxidase activities and was also the most active in the incorporation of tritiated glycerol. Pulse-chase experiments with ^{3}H-glycerol suggested that mitochondria from the light band was transformed to denser organelles as the organism grew.

The separation of different mitochondrial populations by means of a sucrose gradient has been repeatedly described in animals (2, 4, 13-15) and fungi (1, 10). More recently, isotonic gradients generated by macromolecules, such as Ficoll or Dextran T-40, have been used to study this heterogeneity (12, 18, 19, 21, 22).

STORRIE and ATTARDI (19) suggested three main possibilities to explain mitochondrial heterogeneity: *a*) an asymetric mitochondrial fission resulting in segregation of two types of mitochondria, *b*) a continuous spectrum in a slow dynamic equilibrium representing diferent development stages of the mitochondria, and *c*) the existence of cell heterogeneity with respect to their own mitochondrial populations. However, mitochondrial heterogeneity has been repeatedly explained as different stages of differentiation of the organelles towards fully developed organelles (5, 13-15, 17, 18).

This paper shows the existence of heterogeneity *Aspergillus nidulans* mitochondria sedimenting in a dextran-sucrose gradient, and how tritiated glycerol is selectively incorporated into light population, thus making it possible to determine by pulse-chase experiments whether this population was transformed into denser mitochondria during cell growth.

Materials and Methods

Chemicals. Peptone Bacteriological Technical, Bactoagar and Malt Extracts were purchased from Difco; NADH and cytochrome c (type IV from beef heart) from Sigma; Dextran T-40 from Pharmacia Fine Chemicals; 2(n)-^{3}H-glycerol (143 mCi/mmol) was obtained from the Radiochemical Centre (Amersham). All other chemicals were purchased from Merck.

Organism. The strain of *Aspergillus nidulans* used in this work was the R-21 paba A_1, y A_2 (a p-aminobenzoic acid auxotroph), kindly supplied to us by Dr. Turner of the University of Bristol. Conidia and mycelium were grown in the medium previously described (16), with glucose as the source of carbon. The strain was cultured in 1,500 ml of minimal medium in a conical flask in an orbital incubator (Gallenkamp) at 180 rev/min and at 37° C for 20 hours. The media contained the required auxotrophic supplements and the concentration of conidia added was 10^6/ml.

Preparation and fractionation of mitochondria. Mitochondria were extracted as previously described by TURNER (20). The 10,000 × *g* pellet was resuspended in 1 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 7.0 containing 0.3 M sucrose and layered on the top of a 0-20 % (w/w) dextran T-40 gradient in 0.1 mM Tris-HCl, pH 7.0 with 0.45 M sucrose, and generated over a 0.8 ml cushion of 1.7 M sucrose in 10 mM Tris-HCl, pH 7.0. This gradient is similar to that of STORRIE and ATTARDI (19), except for the sucrose concentration. The gradient was centrifuged at 22,500 r.p.m. for 150 min in a SW 27.1 rotor of a L 5-50 Beckman ultracentrifuge. All operations were carried out at 4° C.

After centrifugation, the gradient was fractionated by pumping and the refraction index of each fraction was measured in a Abbe's refractometer at 20° C. Densities were calculated from a graph which correlated the refraction index and the density of the solutions containing 0, 5, 10, 15, and 20 % (w/w) dextran T-40 in 0.45 M sucrose and 0.1 mM Tris-HCl, pH 7.0.

Cytochrome c oxidase activity. Cytochrome c oxidase was tested by following reoxidation of cytochrome c, reduced with sodium dithionite, as previously described by TURNER (20). Results are expresed in μmoles of cytochrome c oxidized × min^{-1}, × ml^{-1}, using the extinction coefficient of MASSEY (9).

Respiratory activity. Respiration of mitochondria was measured polarographically, using a Rank Oxygen electrode, in a 2 ml reaction mixture containing 0.5 M sucrose, 10 mM KCl, 10 mM KH_2PO_4, 15 mM $MgCl_2$, pH 7.0 with 0.16 mM NADH and 0.2 ml of the gradient fractions, at 25° C. Results are expressed in nmoles O_2 consumed × min^{-1} × ml^{-1} and calculated according to the method of ESTABROOK (3).

Counting of radioactivity. 0.5 ml of each gradient fraction were precipitated with 0.5 ml of 20 % trichloroacetic acid, filtered on Whatman GF/C filters and washed with 5 ml of 5 % trichloroacetic acid. Dried samples were counted in 2 ml of scintillation liquid (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzol 0.2 g, 2,5-diphenyloxazole 10 g, toluene 2 l and Triton X-100 1 l) in a Packard Tri-Carb mod 2425 scintillation counter with a 63 % efficiency.

Results

Distribution of mitocondria in a dextran-sucrose gradient. Figure 1 shows the distribution of cytochrome c oxidase and respiratory activity of *Aspergillus nidulans* mitochondria sedimented in a dextran-sucrose gradient. The enzymatic activity

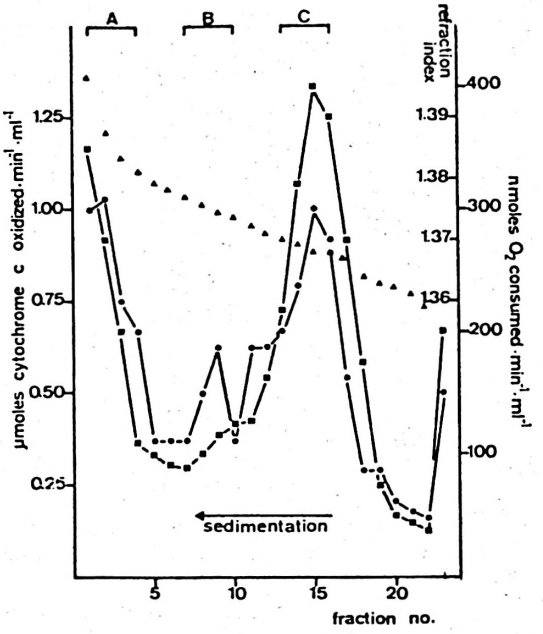

Fig. 1. *Centrifugation on a dextran-sucrose gradient of* Aspergillus nidulans *mitochondria.* Cytochrome c oxidase (●—●) is expressed as μmol × min^{-1} × ml^{-1} and respiratory activity as nmol × min^{-1} × ml^{-1} (■—■). Refraction index (▲—▲).

appeared in three different positions. A high activity was located near the bottom of the tube on the 1.7 M sucrose cushion (zone A). A minor peak was found at the middle of the gradient (zone B) and another band with the highest proportion of cytochrome c oxidase activity occurred at a lower density (zone C). Respiration presented a similar pattern, except for the middle band which had a low respiratory activity.

The densities of the bands were 1.08 and 1.07 g/ml for the middle and upper bands respectively. These levels are much lower than that of *Aspergillus nidulans* mitochondria banded on a sucrose gradient which show a density of 1.19 g/ml (20).

Glycerol labelling experiments. Figure 2 *a* shows a pulse labelling experiment with 0.07 μCi/ml of ^{3}H-glycerol for 20 min. The highest incorporation of the isotope occurred in the light density band. When this fraction was extracted twice with an equal volume of diethyl ether, 75-80 % of the total radioactivity was found in the organic solvent, thus indicating that the radioactivity of the ^{3}H-glycerol was preferentially located in the lipidic material of the membrane, possibly integrated in the phospholipids.

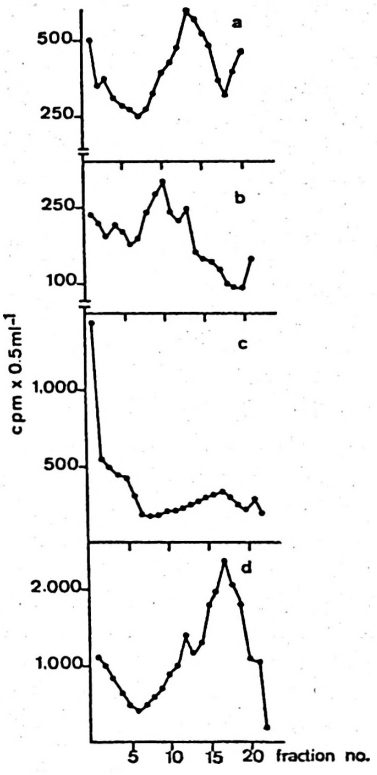

Fig. 2. *Centrifugation on a dextran-sucrose gradient of* Aspergillus nidulans *mitochondria. a*) previously labelled for 20 min with 0.07 μCi/ml of ^{3}H-glycerol; *b*) incubated for 20 min with 0.07 μCi/ml of ^{3}H-glycerol, washed out, resuspended in an equal volume of medium without tritiated glycerol and further incubated for 4 h; *c*) incubated for 60 min with 0.07 μCi/ml of ^{3}H-glycerol and chased by the same procedure after 7 h, and *d*) pulse of 0.07 μCi/ml of ^{3}H-glycerol and chased after 10 h.

After a 4 hour chase the radioactivity was preferentially found in the middle of the gradient (1.08 g/ml). After a 7 h chase, the highest counting was mostly presented on the heavy side, appearing again at the lightest band after a 10 chase (figure 2 *b*. 2 *c* and 2 *d*, respectively).

The chases of 7 and 10 h were carried out after a 60 min pulse, instead of 20 min as in the two previous experiments, in order to avoid difficulties in counting by isotopic dilution. A previous pulse labelling experiment with ^{3}H-glycerol for 90 min also showed that during this period most of the counting still appeared at the light density band. In every case these experiments were carried out in such a way that the mycelia were collected after the chase period with a final time growth of 20 h, except in the longest chase (fig. 2 *d*) where the growth time was 22 h. In the 7 and 10 h chase experiments counting was higher than in the other cases because of the longer pulse time.

Discussion

The results presented here are similar to those described for the mitochondria from Hela cells (19) and pea cotiledons (18) sedimenting in a dextran-sucrose gradient, in which light mitochondria present the highest cytochrome c oxidase and respiratory activities. However, some disagreement may be observed in the buoyant densities (1.07 and 1.08 g/ml) found in *Aspergillus nidulans* in our experiments which are appreciably smaller than the mitochondria from Hela cells (1.10 and 1.11 g/ml). We cannot determine whether these data reflect a real difference in the density of the mitochondria of both organism, or are caused by the differences in the sucrose concentration (0.45 M in our experiments and 0.55 M in those of Hela cells mitochondria) (19).

STORRIE and ATTARDI found a band sedimenting at the bottom of the tube, with similar characteristics to ours and made up of mitochondria and membranes (19). A similar band with a density of 1.17 g/ml and contaminated with disrupted mitochondria was also described by POLLAK and MUNN (15) in rat liver mitochondria. These facts suggest the presence of contaminated membranes in the band sedimenting at the bottom of the tube.

The incorporation of ^{3}H-glycerol suggested that the light mitochondria were the most active in integrating phospholipids in the mitochondrial membranes. SATAV *et al.* (17) showed that light and heavy mitochondria from rat liver presented different rates of ^{3}H-glycerol incorporation into phospholipids.

The 4 and 7 hours chase experiments (fig. 2 *b* and 2 *c*) cannot be interpreted as a molecular turnover of labelled glycerol by which these molecules after degradation of mitochondrial membranes were integrated into new organelles. If this molecular turnover occurred during these chase periods, the radioactivity might appear again at the light densities of the gradient where the mitochondria sedimenting there incorporated the isotope more actively in the pulse experiment (fig. 2 *a*). However, in both cases radioactivity appeared at the middle and densest fractions respectively, which strongly suggests a real development of light mitochondria during the chase time.

In the longest chase experiment (fig. 2 *c*), radioactivity appeared again at the light fraction of the gradient. In this case we cannot conclude whether the recycling of glycerol occurs by means of a molecular turnover, as explained above, or by a dividing of mitochondria through an unstable stage represented by the organelles of the heavy band, yielding after fission light mitochondria in a similar way to that proposed by POLLAK in the rat's liver (13) or LUCK in *Neurospora crassa* (7, 8). More recently, several biochemical evidences have been obtained which strongly

support the existence of a mitochondrial fission (6, 11).

At any rate the pulse-chase experiments suggest that mitochondria from the light band are transformed into denser organelles as cell growth proceeded. These result bring to light new evidence of a relationship between heterogeneity and different biogenetic stages of mitochondria, as discussed by several authors (5, 13-15, 17, 18, 21).

Resumen

Mitocondrias de *Aspergillus nidulans* se distribuyen en un gradiente de sacarosa-dextrano en tres bandas. La banda de menor densidad es la que presenta mayores actividades citocromo c oxidasa y respiratoria y es la más activa en la incorporación de H^3-glicerol. Los experimentos de pulso-caza con H^3-glicerol sugieren que las mitocondrias de la banda más ligera se transforman en orgánulos más densos a medida que crece el organismo.

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