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Identification by flow cytometry of two distinct rhodamine-123-stained mitochondrial populations in rat liver

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Isolated rat liver mitochondria were split into three fractions of increasing density when applied to a Percoll gradient. NADH-ubiquinone oxidoreductase, succinate dehydrogenase and cytochrome-*c* oxidase but not F_1-ATPase activities increased with density as well as respiratory rate in state 3 and the respiratory control index. Flow cytometry of mitochondrial density fractions stained with rhodamine-123 revealed the occurrence in each density fraction of two distinct mitochondrial populations with different fluorescence intensity. The high fluorescence population was minor and its proportion decreased with density. The extent of high fluorescence population staining depended on the deenergized state of the mitochondria suggesting that this population represents an immature form of the mitochondria which may develop into a fully functional organelle by the incorporation of structural and/or functional proteins.

Mitochondrion; Rhodamine-123; Flow cytometry

1. INTRODUCTION

In recent years there has been increasing interest in rhodamine-123 as a specific staining for mitochondria [1,2]. Interest has focused on this dye because its uptake by mitochondria seems to be associated with membrane potential, and this might be used for the measurement of membrane potential in situ [3,4]. Thus, rhodamine-123 inhibits the import of mitochondrial enzymes, a process dependent on membrane potential [5]. In addition, the mitochondria of tumor cells are strongly stained by rhodamine-123 as compared to non-metastatic cells [6], a fact that may be related to the potent cytostatic effect displayed by the dye [7]. Regardless of the biological effects of the dye, staining with rhodamine-123 has been shown to be a useful tool for the study of mitochondria by flow cytometry both in whole cells [8] and in isolated mitochondria [9,10].

In the present work we studied the enzyme activities, respiratory behavior and rh-123 staining of three mitochondrial fractions separated by a Percoll gradient. Our results suggest that the three fractions may represent three states of increasing mitochondrial development with decreasing proportions of a strongly rh-123-stained mitochondrial population as a putative immature precursor of the fully functional organelle.

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Abbreviations: rh-123, rhodamine-123; WMF, whole mitochondrial fraction; M_1, M_2, and M_3, purified mitochondrial fractions; LFP, low fluorescence population; HFP, high fluorescence population; SSC, side scatter (90° angle light scatter); FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone

2. MATERIALS AND METHODS

2.1. *Isolation of mitochondrial populations*

The whole mitochondria fraction (WMF) was isolated from Wistar rat (250 g body wt) liver by a discontinuous Percoll gradient technique (fig.1) essentially based on the method describ-

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ed by Reinhart et al. [11] except that the isolation medium contained 250 mM sucrose, 1 mM EDTA and 10 mM Hepes (pH 7.4). WMF was layered onto a second gradient with decreased Percoll proportions (2 ml of 7%, 3 ml of 19%, 3 ml of 31% and 3 ml of 42% from top to bottom; v/v) and split into three fractions of increasing density. The fractions were collected, washed and separately layered onto a third indentical gradient (fig.1); the last operation was repeated and the purified M_1, M_2 and M_3 fractions were collected and used for all experimental purposes. In agreement with previous reports [12,13], isolation with Percoll did not interfere with mitochondria viability. The density of the mitochondrial fractions (table 1) was measured by coloured density-marker beads (Pharmacia LKB, Uppsala, Sweden). The M_1, M_2 and M_3 fractions showed negligible catalase activity [14] and no detectable acid phosphatase [15], 5'-nucleotidase [16] and glucose-6-phosphatase [17] activities.

2.2. *Assay of enzyme activity*

Mitochondrial enzyme activities were measured in the mitochondrial fractions by the methods described for NADH-ubiquinone-oxidoreductase [18], succinate dehydrogenase [19], cytochrome-*c* oxidase [20] and F_1-ATPase [21]. Proteins were measured by the method of Lowry et al. [22].

2.3. *Respiratory measurements*

Respiratory measurements were carried out in respiratory medium containing: 225 mM sucrose, 10 mM succinate, 10 mM ClK, 5 mM Cl_2Mg, 10 mM PO_4H_2K, 1 mM EDTA and 10 mM Tris (pH 7.4). 200 nmol of ADP were added to the respirometer (Oxymeter, Gilson Medical Electronics, France) cell (2 ml) to measure respiratory rate in state 3, 10 μg oligomycin to inhibit phosphorylation and 0.22 μg FCCP as uncoupler.

2.4. *Flow cytometry of mitochondrial populations*

Isolated mitochondrial fractions were preincubated in isolation medium, respiratory medium, respiratory medium plus ADP (3 mM) or respiratory medium plus ADP and FCCP (1.18 μM) or valinomycin (0.54 μM) at 30°C for 1.5 min before staining. Mitochondria were stained by incubation with rh-123 (10 μg/ml) at 37°C for 15 min in the dark; the unbound dye was removed by centrifugation and the pellet washed twice with isolation medium.

Mitochondrial fluorescence and light scatter (light reflected at 90° angle) were measured for at least 10 000 events in the Facstar flow cytometer using the Consort 30 software programme (Becton/Dickinson, Mountain View, CA, USA). The data on rh-123 binding were obtained using an argon ion laser tuned at 488 nm and 250 mW and the green fluorescence was measured through a 530 ± 15 nm band pass filter. Graphics were plotted by means of the Lysys software programme (Becton/Dickinson, Mountain View, CA, USA).

3. RESULTS

3.1. *Isolation of mitochondrial populations*

The whole mitochondrial fraction (WMF) isolated by the Percoll gradient (fig.1) according to Reinhart et al. [11], was split into three fractions when layered onto a second Percoll gradient as described in section 2. These fractions were purified by two additional identical gradients, resulting in three mitochondrial populations (M_1, M_2 and M_3) which accounted for 5.1%, 14.0% and 80.9% of the WMF, respectively. The densities, the activities of some relevant mitochondrial enzymes and respiratory parameters of these three fractions are shown in table 1. Except for the F_1-ATPase activity, the other enzyme activities measured gradually increased with density, showing significant differences between the M_1 and M_3 fractions. This suggests that the three fractions may be functionally different and presumably result from different stages of mitochondrial development. No significant differences were observed in the total or in the oligomycin-insensitive state-4-respiratory rate among the three mitochondrial fractions (table 1), suggesting that passive proton permeability through the inner mitochondrial membrane is similar in all three mitochondrial fractions. However, state-3 respiratory rate increased significantly as far as density, resulting in a parallel enhancement of the respiratory control index. This suggests the occurrence of a more coupled state in heavy mitochondria when compared with those of the light fractions. The FCCP-uncoupled respiratory rate increased with density, suggesting the occurrence of an improved electron transport in the heavier mitochondrial fractions.

3.2. *Flow cytometric analysis of mitochondrial fractions*

Fig.1 shows the flow cytometric analysis of the rh-123-stained whole mitochondrial fraction (WMF) and purified mitochondrial fractions (M_1, M_2 and M_3) according to a 90° angle light scatter (SSC) and the green fluorescence parameters. It

Fig.1. Density gradients and flow cytometry of isolated mitochondrial populations of rat liver. (*x* axis) Green fluorescence (log scale); (*y* axis) number of events; (*z* axis) 90° angle light scatter ('side scatter'; SSC) (linear scale). (a) Whole mitochondrial fraction (WMF); (b) low density fraction (M_1); (c) medium density fraction (M_2); (d) high density fraction (M_3). Figures in squares are densities (g/ml). Percentages of LFP/HFP are expressed in the lower right hand corner of each panel.

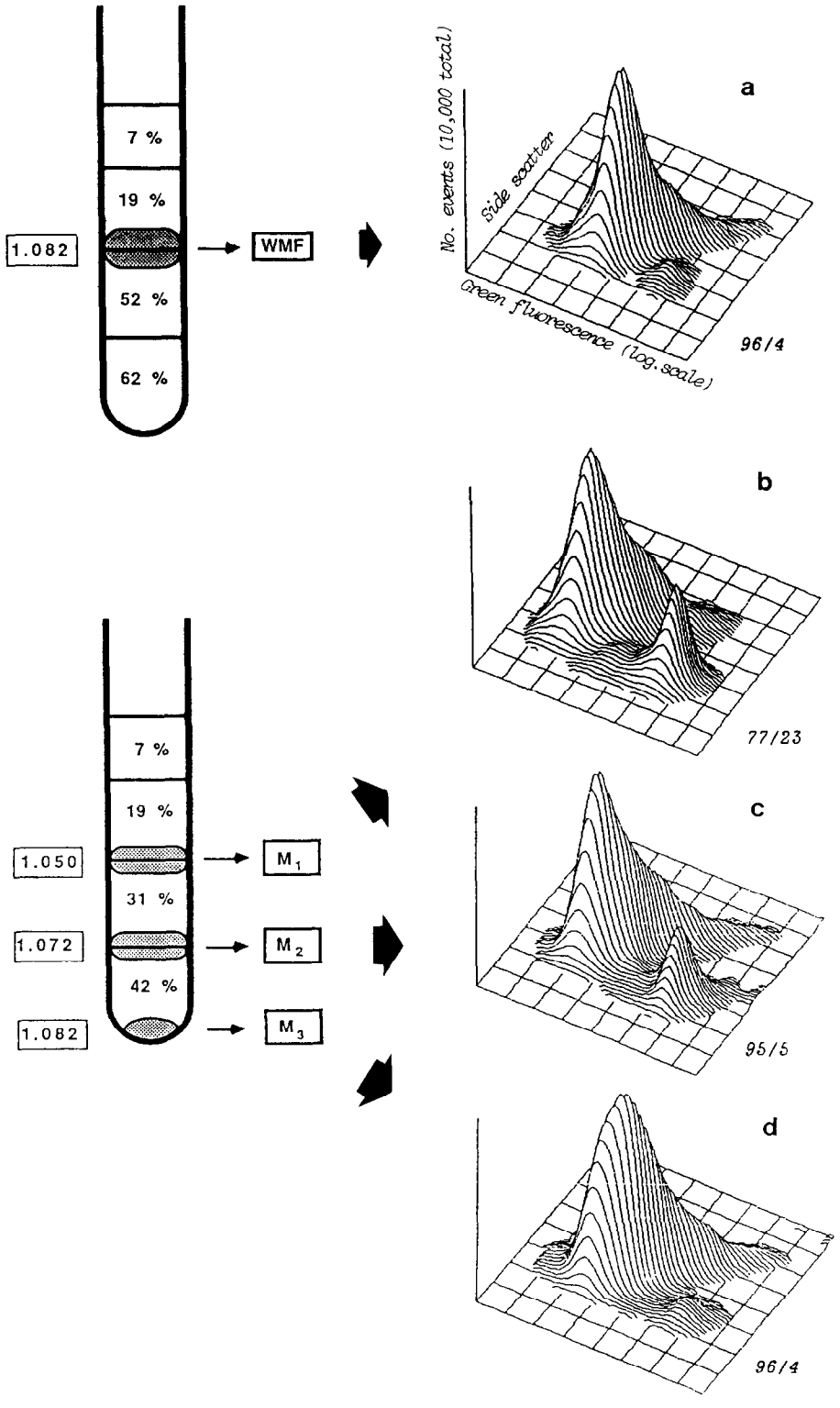
7 %
19 %
1.082
WMF
52 %
62 %
No. events (10,000 total)
Side scatter
Green fluorescence (log. scale)
a
96/4
b
77/23
7 %
19 %
1.050
M1
31 %
1.072
M2
42 %
1.082
M3
c
95/5
d
96/4

Table 1
Densities, enzyme activities and respiratory parameters of rat liver mitochondrial fractions

Fraction	Density (g/ml)	Enzyme activities (mU/mg protein)				Respiratory parameters (nmol O_2/min per mg protein)				
		NADH UQ-ox.	Succinate DH	Cytochrome-*c* oxidase	F_1-ATPase	E4	E4oin	E3	FCCP	RCI
M_1	1.050	74 ± 2	76 ± 3	240 ± 6	66 ± 2	15.5 ± 1.1	9.9 ± 1.0	68.8 ± 1.2	68.2 ± 3.2	4.44 ± 0.30
M_2	1.072	83 ± 4	82 ± 5	243 ± 5	63 ± 5	14.5 ± 0.8	8.9 ± 0.5	88.5 ± 4.6*	93.6 ± 6.6*	6.10 ± 0.40*
M_3	1.082	95 ± 6*	112 ± 5**	293 ± 16**	69 ± 2	15.9 ± 0.9	10.1 ± 1.0	103.6 ± 0.8**	110.1 ± 7.7**	6.51 ± 0.37**

Results are means ± SE ($n = 4$–5). Results significantly different from M_1 are expressed as *$p < 0.05$; **$p < 0.005$. E4, respiratory rate in the state 4; E4oin, respiratory rate in state 4 in the presence of oligomycin; E3, respiratory rate in state 3; FCCP, respiratory rate in the state 4 in presence of FCCP; RCI, respiratory control index

should be mentioned that SSC increases with the rugosity of the mitochondrial membrane while green fluorescence intensity is an index of the extent of rh-123 staining. WMF (fig.1a) showed two distinct fluorescence peaks, i.e. a low fluorescence population (LFP) accounting for 96% of the total, and a high fluorescence population (HFP) accounting for 4% of the total mitochondrial fraction. Although the tail of the LFP peak of increasing SSC is concurrent with HFP regarding the intensity of fluorescence, both populations are well delimited according to their SSC. In addition, the mean of the intensity ('channel') of fluorescence of both populations is substantially different (note the log scale) suggesting that both populations can be distinguished by the different rugosity and rh-123-affinity of their membranes. The proportion of HFP clearly increased in the M_1 and M_2 fractions (to 23% and 20% of the total, respectively) (figs 1b and c), but was very low (6%) in M_3 (fig.1d). These results suggest that the low density fractions are enriched in the HFP which decreases as density increases. However, HFP and

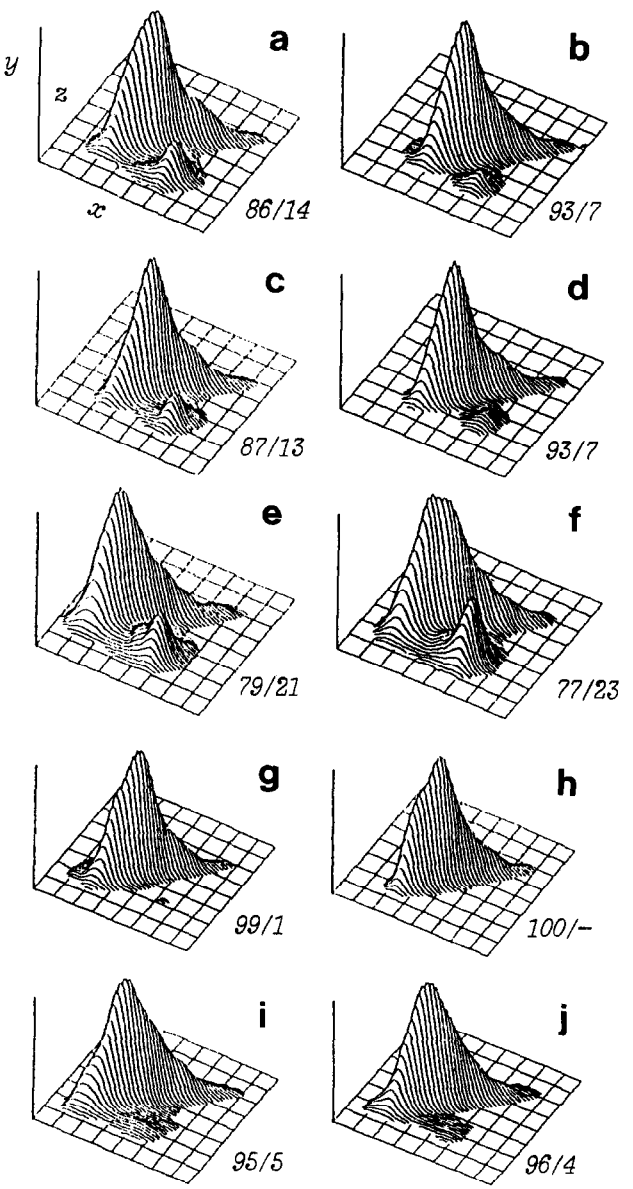

Fig.2. Flow cytometry of isolated mitochondrial populations of rat liver under different experimental conditions. For *x*, *y* and *z* axes, see fig.1. (a) M_1 fraction preincubated with respiratory medium (state 4); (b) M_1 fraction preincubated with respiratory medium + ADP (state 3); (c) M_2 fraction preincubated with respiratory medium (state 4); (d) M_2 fraction preincubated with respiratory medium + ADP (state 3); (e) M_2 fraction preincubated with respiratory medium + ADP + FCCP; (f) M_2 fraction preincubated with respiratory medium + ADP + valinomycin; (g) M_3 fraction preincubated with respiratory medium; (h) M_3 fraction preincubated with respiratory medium + ADP; (i) M_3 fraction preincubated with respiratory medium + ADP + FCCP; (j) M_3 fraction preincubated with respiratory medium + ADP + valinomycin. Percentages of LFP/HFP are expressed in the lower right hand corner of each panel.

LFP of all M_1, M_2 and M_3 fractions showed approximately the same fluorescence and SSC intensity (channel) means as in the WMF fraction. This suggests that HFP and LFP are identical in all density fractions.

3.3. *Sensitivity of the high-fluorescence mitochondrial population (HFP) to the energy status of mitochondria*

The proportion of the HFP significantly decreased (to 14%) when the M_1 fraction was preincubated in respiratory medium (state 4) before rh-123-staining (fig.2a); this phenomenon was enhanced (HFP decreased to 7%) in the presence of ADP (state 3; fig.2b). Similar results were obtained when the M_2 fraction was preincubated with respiratory medium (fig.2c) or respiratory medium plus ADP (fig.2d). By contrast, the presence of FCCP (fig.2e) or valinomycin (fig.2f) in state 3 significantly increased the proportion of HFP (to 21–23%), suggesting that the dissipation of either ΔpH or $\Delta\phi$ increases the HFP. The same experiment carried out with the M_3 fraction (fig.2) resulted in a negligible HFP in states 4 and 3 ($<1\%$) (fig.2g and h) and in a slight increase of HFP (to about 5%) in the presence of FCCP or valinomycin (fig.2i and j). It should be mentioned that in all three fractions, the decrease of HFP caused by energization of mitochondria was concurrent with a shift of the whole LFP and HFP to higher fluorescence intensity (channel) means. Conversely, FCCP and valinomycin decreased the mean fluorescence intensities of the LFP and HFP. This fact is in agreement with the notion that rh-123 staining of mitochondria depends on membrane potential [3,4,10,23].

4. DISCUSSION

Heterogenity of mitochondrial population has been shown in adult [24] and fetal [25] rat liver by sucrose gradient technique and in potato tuber by concanavalin A binding-fluorescence cytometry [9]. Our results show that adult rat liver mitochondria split into three fractions of increasing density. These fractions showed different enzyme activities and respiratory behavior, suggesting the occurrence of a development of mitochondrial functionality associated with an increase in density (table 1). The three fractions isolated by Percoll gradient may represent three states of development of liver mitochondria in which low and high density populations would represent immature and mature mitochondria, respectively, while those of medium density could account for an intermediate state of development. Likewise, the proportions of HFP decreased with density suggesting that HFP may be associated with immaturity. Actually, the HFP was present at high proportions in fetal rat liver representing more than 80% and 40% of the total in the M_1 and M_2 fractions, respectively (Lopéz-Mediavilla, C., Orfao, A. and Medina, J.M., unpublished). Energization of mitochondria causes a significant shift in the fluorescence mean channels of both HFP and LFP, a fact consistent with the results of O'Connor et al. [10] who reported that mitochondrial staining with rh-123 is enhanced under experimental conditions in which an increase of membrane potential is expected. Moreover, our results show that the staining of HFP depends on the energy status of mitochondria, allowing us to visualize the heterogeneity of the three density fractions. Thus, a minor population of mitochondria (HFP) changes its sensitivity to rh-123 staining according to its energy status. This suggests the occurrence of two types of mitochondria in rat liver with different binding sites for rh-123, i.e., the LFP whose binding sites for rh-123 are freely accessible whatever the energy status of the mitochondria and the HFP whose sites for rh-123 are hidden in the energized state and freely accessible in the deenergized form of the mitochondria. It should be noted that each population showed different fluorescence intensities; this can be the result of a different association between the dye and its target molecules. Consequently, it can be suggested that the binding sites for rh-123 in HFP and LFP are chemically different, presumably resulting in putative abducts with different fluorescence intensities. In addition, the requirement of HFP staining for a deenergized state of mitochondria suggests that the dye requires a relaxed inner mitochondrial membrane in which the binding sites for the dye are uncovered. If so, the structure of HFP and LFP membranes must be different and presumably show different states of mitochondrial development. In fact, HFP is significantly present in low density fractions (fig.1b and c) in which the respiratory control index and enzyme activities are low (table 1). Consequently, it could be speculated

that a minor population of adult rat liver mitochondria is in a transition state in which the definitive assembly of the inner membrane is not yet accomplished. Whether this type of mitochondria represents an immature form of the organelle destined to turn into a fully functional mitochondria by later incorporation of structural and functional proteins remains to be elucidated. However, if the specific association of rh-123 to F_0F_1-ATPase is confirmed [26] one of these putative incorporations might be a component of F_0F_1-ATPase, which would diminish the fluorescence intensity of rh-123 when it is incorporated into the F_0F_1-ATPase complex.

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