ON THE NATURE OF THE DENSE MATRIX GRANULES OF NORMAL MITOCHONDRIA

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INTRODUCTION

A constant morphological feature of normal mitochondria, either isolated or in the intact tissue, is the presence of varying numbers of dense granules in the matrix. Isolated mitochondria contain relatively large amounts of Ca^{++} and Mg^{++} (9) and are also able to accumulate divalent cations (1–3). Peachey (6) suggested that the normal matrix granules prime the accumulation of divalent cations, a conclusion that agrees with earlier morphologic interpretations of Rouillier (7). However, Vasington and Greenawalt (11, 12) and Greenawalt and Carafoli (5) found that the normal matrix granules are not the exclusive sites for the deposition of Ca^{++} and Sr^{++} by isolated mitochondria. Thus, the real nature and functional significance of the dense matrix granules in normal mitochondria are still unknown.

It was previously shown (*a*) that most of the Ca^{++} in mitochondria freshly isolated from normal rats is released by uncouplers of oxidative phosphorylation (10) and (*b*) that 2,4-dinitrophenol (DNP) discharges the large electron-opaque granules formed when mitochondria accumulate Ca^{++} and inorganic phosphate in vitro (4). In the present study, an attempt has been made to correlate the content of Ca^{++} and Mg^{++} with the number of normal matrix granules in mitochondria incubated for varying times in the presence and absence of DNP. The kinetics of release of Ca^{++} and Mg^{++} and of the diminution in the numbers of matrix granules indicate that neither of these metals alone accounts for the presence or absence of matrix granules in normal mitochondria; the same is true for Mn^{++}. However, the possible participation of a fairly large fraction of mitochondrial Mg^{++} in the composition of the granules was not completely ruled out.

MATERIALS AND METHODS

Preparation of Mitochondria

Mitochondria were prepared from the livers of commercial stock albino rats, kept on a standard diet, and fasted 12 hr before sacrifice. 6 min. before sacrifice, the rats received an intraperitoneal injection of $^{45}CaCl_2$ (20 μc, equal to about 2 μg of calcium per rat). Mitochondria were isolated by the sucrose procedure of Schneider (8), and washed once.

Biochemical Experiments

Mitochondria were incubated at 25° in centrifuge tubes containing a medium of the following composition: 0.11 M NaCl, 0.01 M Na-succinate, 0.01 M Tris-Cl, pH 7.4; when present, 2,4-dinitrophenol (DNP) was 0.0001 M. Duplicate tubes were run for each time of incubation: one was used for the chemical analyses, the other for the electron microscopy. At various times of incubation, tubes were removed from the incubation bath, rapidly cooled at 0°, and centrifuged at 20,000 *g* for 4 min, at 0°. The pellets were resuspended in 10% trichloroacetic acid and the denatured proteins were discarded after centrifugation. Mg^{++} was determined on the trichloroacetic acid extracts with a Perkin-Elmer 303 Atomic Absorption Spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.). The $^{45}Ca^{++}$ in aliquots of the trichloroacetic acid extracts was counted in an end window Geiger counter with a low-background scaler. Occasionally, mitochondrial Ca^{++} was also determined by atomic absorption spectrophotometry. The average values for mitochondrial Ca^{++} and Mg^{++} under the conditions used in the present study were 11.2 and 35.5 nmoles per mg of protein, respectively. Protein was determined on the mitochondrial suspensions with a biuret reaction.

Electron Microscopy

The pellets were immediately fixed in 6.25% glutaraldehyde in 0.13 M phosphate buffer, pH 7.5, at 0°. After 6 hr, the pellets were repeatedly washed with ice-cold 0.13 M phosphate buffer in 0.2 M sucrose. They were then postfixed for 2 hr with 1% OsO_4 in the same sucrose-phosphate medium. The pellets were dehydrated in acetone, and embedded in Epon. Sections obtained in a Porter-Blum ultramicrotome were stained with uranyl acetate and lead citrate; they were observed in a Siemens Elmiskop I A double-condenser electron microscope, at 80 kv, with apertures of 50 μ. The magnification varied from 5,000 to 15,000. The counting of the dense granules

was carried out on prints of the same magnification, and only sections obtained from the same level of the pellets were compared. At least 2,000 mitochondrial profiles were examined for each experimental point.

RESULTS

Effect of DNP on Mitochondrial Ca^{++} and Mg^{++}

Fig. 1 shows that Ca^{++} in rat liver mitochondria is rather stable to incubation in the absence of DNP. After 30 min of incubation at 25°, only about 5% of the Ca^{++} originally present in mitochondria was released to the medium. Mg^{++} appeared less stable to incubation under these conditions. The endogenous Mg^{++} was released by mitochondria in an approximately linear fashion during 30 min of incubation at 25° in the absence of DNP. Independent experiments showed that the loss of Mg^{++} leveled off after 40–60 min. of incubation at 25° when about 40–50% of the endogenous Mg^{++} had been lost by the mitochondria. Apparently, a large part of the mitochondrial Mg^{++} is firmly bound to the organelles. Mitochondrial Mn^{++} measured in separate experiments was as firmly bound as Ca^{++}.

Fig. 1 also shows that 0.0001 M DNP, added to the incubation medium, had a dramatic effect on mitochondrial Ca^{++}; in 2 min, about 80% of Ca^{++} was discharged from mitochondria. No additional Ca^{++} was discharged when the incubation was prolonged up to 30 min. Clearly, two pools of Ca^{++} were present in isolated rat liver mitochondria: a large pool, comprising about 80% of the mitochondrial Ca^{++}, which was apparently labile, and accessible to DNP, and a smaller pool, which was not influenced by DNP.[1] These results are in agreement with those of a recent study of the subcellular distribution of Ca^{++} in rat liver (2). The addition of DNP to the incubation medium had no significant effect on the release of Mg^{++} from mitochondria.

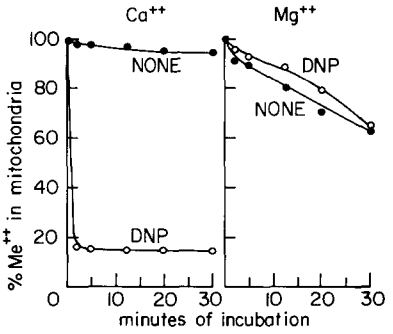

FIGURE 1 Effect of DNP on mitochondrial Ca^{++} and Mg^{++}. The treatment of the rats with $^{45}CaCl_2$, the composition of the incubation medium, and the determinations of Ca^{++} and Mg^{++} are described in Methods. The final volume was 2 ml, containing 10 mg of mitochondrial protein.

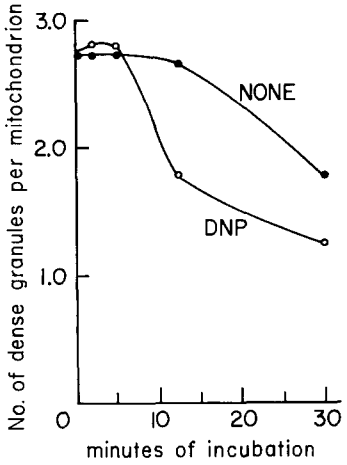

FIGURE 2 Effect of DNP on the dense granules of the mitochondrial matrix. Experimental conditions as described in Methods.

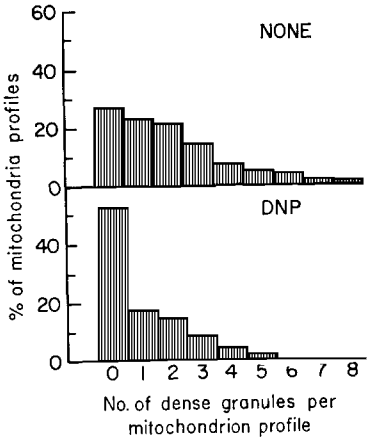

FIGURE 3 Number of dense granules in the mitochondrial matrix after 30 min of incubation in the absence and in the presence of DNP. Experimental conditions as described in Methods.

[1] When Ca^{++} was measured by atomic absorption spectrophotometry, the maximal release was between 60 and 70% of the total mitochondrial Ca^{++}. This finding suggests that a small fraction of the mitochondrial Ca^{++} did not equilibrate with the $^{45}Ca^{++}$ injected into the rats.

Effect of DNP on the Dense Granules of Isolated Rat Liver Mitochondria

The number of dense granules in isolated rat liver mitochondria has been found in our laboratories to depend on a number of variables, among them the age and possibly the strain of the rats, the isolation medium, and the number of the washings of the mitochondrial pellet. Under the conditions used in the present study, an average of slightly less than three granules were visible per each mitochondrial profile. Fig. 2 shows that the incubation at 25° in the absence of DNP had no significant effect on the number of dense granules present in mitochondria for the first 12.5 min. Then, their number began to decrease; after 30 min of incubation, fewer than 2 granules were visible per mitochondrial profile. Prolongation of the incubation to 60 min (independent experiments) did not result in a further decrease of the number of the granules. The inclusion of DNP in the incubation medium had no significant effect during the first 5 min. Then, the number of dense granules rapidly dropped to less than 2 per profile at 12.5 min, and to little over 1 per profile at 30 min. The results presented in Fig. 2 thus show that incubation in the presence of DNP for more than 5 min decreased the average number of dense granules per mitochondrion below the number found in the controls incubated in the absence of DNP.

The data presented in Fig. 3 analyze the results in more detail. Mitochondrial profiles were divided into percentage classes, according to the number of dense granules present in each profile. Incubation at 25° for 30 min in the presence of DNP increased very markedly the percentage of mitochondrial profiles that had no dense granules at all. Fig. 3 also shows that no profiles with more than 5 granules were visible after 30 min of incubation in the presence of DNP.

DISCUSSION

The experiments presented in this paper are directly relevant to the problem of the composition of the dense granules of the normal mitochondrial matrix. Ca^{++} was largely eliminated as a major contributor to the composition of the normal dense granules since incubation in the presence of DNP released 80% of the mitochondrial Ca^{++} in less then 2 min while, during the same period, no decrease in the number of dense granules per mitochondrial profile was observed. The experiments carried out in the absence of DNP also ruled out Ca^{++} as a component since an inverse correlation was observed between the number of matrix granules and the retention of Ca^{++}. Thus it would appear that only 20% of the total mitochondrial Ca^{++} could contribute directly to the structures recognized in the electron microscope as matrix granules. To contribute significantly to the electron opacity of these granules, this small amount of Ca^{++} would necessarily have to be highly concentrated. It is, of course, possible that Ca^{++} contributes indirectly to the stability of the granules and that only after Ca^{++} is released does the loss of granules become detectable.

The composition of normal matrix granules with respect to Mg^{++} is even less clear. After 12.5 min of incubation in dinitrophenol the Mg^{++} content and the number of dense granules decreased in a parallel fashion. However, the kinetics of release of Mg^{++} and of the loss of granules before 12.5 min in the absence of DNP were not completely parallel. Furthermore, the addition of DNP had practically no effect on the discharge of Mg^{++} whereas it induced an abrupt decrease in the number of dense granules per mitochondrial profile (a decrease of about 30% in about 5–10 min of incubation).

It thus seems clear that even if some Mg^{++} and/or Ca^{++} do participate in the formation of the normal matrix granules as morphological entities, either directly or indirectly, other still unidentified components, partially sensitive to DNP, must contribute to their morphology as well. In this regard, other workers (13, 14) have emphasized the osmiophilic qualitities of the normal dense granules and have suggested that they are lipid-containing bodies. Also, Thomas and Greenawalt (15) have shown that, in contrast to calcium phosphate granules formed in vitro, the dense matrix granules of normal mitochondria are completely volatilized by high temperature micro-incineration. These latter results indicate a marked difference in stability to high temperature but do not conclusively show an absence of minerals in the normal granules. To determine the nature of the still unidentified components of the normal matrix granules of mitochondria, further investigation will be required.

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