SUBMITOCHONDRIAL LOCALIZATION OF DNA POLYMERASE IN RAT LIVER TISSUE

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1. Introduction

The submitochondrial localization of rat liver mitochondrial DNA polymerase is not well established. A report of Schultz and Nass indicates that after treatment of mitochondria with deoxycholate, the specific activity of endogenously primed DNA polymerase is higher in the membrane components than in the whole mitochondria [1]. The results presented here show that mitochondrial DNA polymerase is fixed to the inner side of the granule inner membrane.

2. Methods and material

2.1. Tissue fractionation

Experiments were performed with male and female Buffalo rats weighing 200–300 g. The animals were killed by decapitation after 20 hr of fasting. Liver was immediately removed, chilled in ice-cold 0.25 M sucrose and homogenized in the same medium by means of a smooth-glass tube fitted with a Teflon pestle rotating at 3000 rpm. The suspension was brought to a volume of approx. 40 ml and spun for 10 min at 1700 rpm (600 *g* average) in an International PR_2 refrigerated centrifuge. The sediment was washed twice at 1500 rpm (470 *g* average). Supernatants obtained from the centrifugations were pooled to make the cytoplasmic extract. Fractionation of this extract was achieved with the technique of the Duve et al. [2]. A nuclear fraction (N), a heavy mitochondrial fraction (M), a light mitochondrial fraction (L), a microsomal fraction (P), and a soluble fraction (S) were isolated using the fractionation scheme described by these authors.

In density gradient centrifugation and activation experiments, a washed total mitochondrial fraction corresponding to the sum of the heavy and light mitochondrial fraction was used. Density gradient experiments were performed according to Beaufay et al. [3] with a Spinco model L2-65B ultracentrifuge.

2.2. Enzyme assays

Cytochrome oxidase, acid phosphatase and glucose-6-phosphatase were measured according to de Duve et al. [2], monoamine oxidase according to Schnaitman et al. [4], alkaline DNAase according to Beaufay et al. [5], sulfite cytochrome *c* reductase according to Wattiaux-De Coninck and Wattiaux [6], malate dehydrogenase according to Ochoa [7]. DNA polymerase was assayed in a volume of 0.2 ml containing 0.05 M Tris buffer pH 7.5, 0.05 M phosphate buffer pH 7.5, 20 mM succinate, 125 mM KCl, 8 mM $MgCl_2$, 0.5 mM ATP, 4 mM EDTA, 5 mM mercaptoethanol, 0.25 mg/ml heat denatured DNA, 0.25 M sucrose, 0.15% Triton X-100, 15 μM dCTP, dGTP, dATP, 2.5 μM tritiated dTTP. For the determination of free DNA polymerase activity, Triton X-100 was omitted from the reaction mixture. Incubation was performed at 37°. The incorporation of labeled deoxyribonucleotide into an acid-insoluble product was determined by pipetting 40 μl of incubation mixture after 0, 4, 8 and 12 min incubation onto discs of filter paper (Whatman n° 1). The discs were immersed in cold 5% trichloracetic acid containing 1% sodium pyrophosphate. After

washing successively with 5% trichloracetic acid, ethanol and ether [8], the discs were put into 10 ml of scintillation solution containing 10% naphthalene, 1% 2,5 -diphenyloxazole, 0.025% 2,2-*p*-phenylene bis-(phenyloxazole) and 20% (v/v) ethanol dissolved in dioxane and counted in a Nuclear Chicago liquid scintillation counter.

3. Results and discussion

3.1. Distribution of DNA polymerase after differential and isopycnic centrifugation

In rat liver, evidence has been presented that a DNA polymerase exists in the nuclei [10], the mitochondria [10–12] and the cytosol [13]. A prerequisite to the study of the submitochondrial localization of an enzyme is the determination of the amount of the activity recovered in the mitochondrial fraction which is truly associated with mitochondria. We present a balance sheet, showing the distribution of DNA polymerase activity after differential centrifugation of a rat liver homogenate and after density gradient centrifugation of a mitochondrial fraction. Fig. 1 shows the distribution pattern of DNA polymerase, cytochrome oxidase (mitochondria), acid phosphatase (lysosomes) and glucose-6-phosphatase (endoplasmic reticulum) after differential centrifugation according to de Duve et al. [2]. The distribution of the polymerase is quite similar to that of the mitochondrial reference enzyme, cytochrome oxidase. The activity recovered in the nuclear and microsomal fractions may be explained mainly by a contamination of these fractions by mitochondria. Moreover, as shown by fig. 2, the distribution of the enzyme after isopycnic centrifugation of a mitochondrial fraction resembles that of three mitochondrial enzymes, namely cytochrome oxidase, monoamine oxidase and malate dehydrogenase. Therefore, the rat liver DNA polymerase we measure is a true mitochondrial enzyme.

3.2. Structure linked latency of mitochondrial DNA polymerase

Mitochondrial DNA polymerase exhibits structure-linked latency: maximal activity is only obtained by

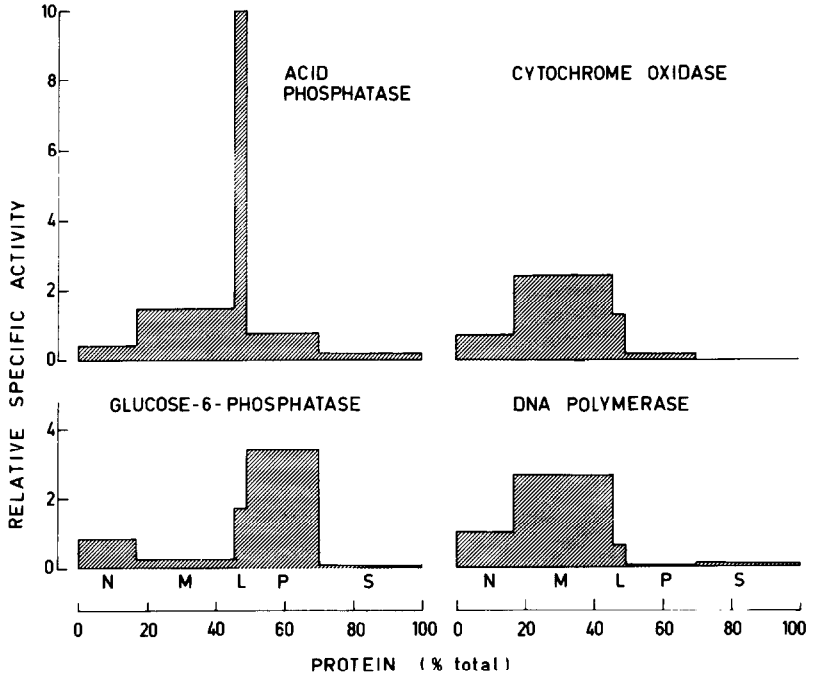

Fig. 1. Distribution patterns of enzymes. *Ordinate:* mean relative specific activity of fractions (percentage of total recovered activity/percentage of total recovered proteins); *abscissa:* relative protein content of fractions (cumulatively from left to right). Mean recoveries (3 fractionations) were 92.1% for cytochrome *c* oxidase, 100.6% for acid phosphatase, 97.0% for glucose-6-phosphatase and 105.6% for DNA polymerase. N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, soluble fraction.

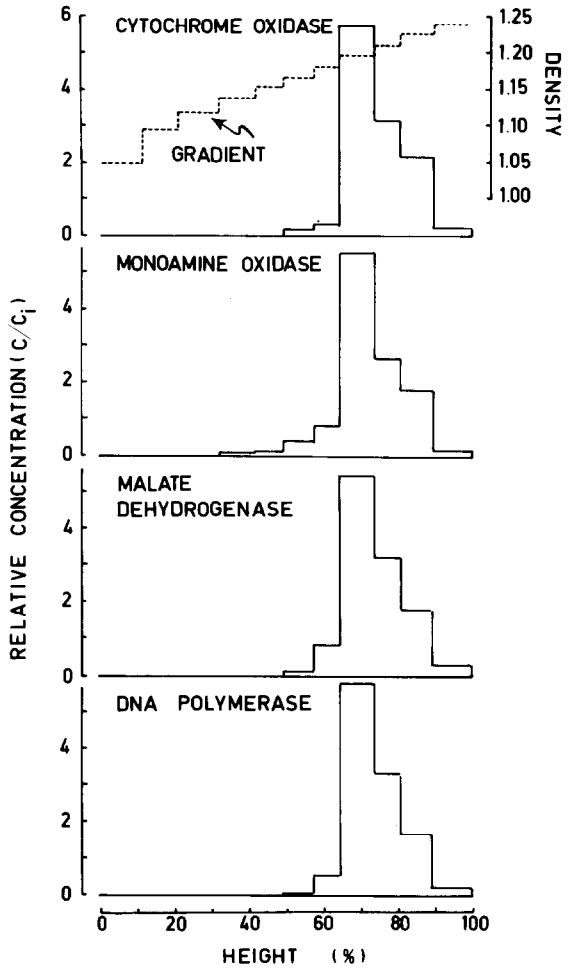

Fig. 2. Distribution of mitochondrial enzymes after isopycnic centrifugation in a sucrose gradient. Centrifugation was performed at 39,000 rpm ($\int w^2 dt = 144$ rad^2/nsec) in the Spinco rotor SW 65. The mitochondrial fraction was initially layered on the top of a sucrose gradient extending from 1.09 to 1.26 g/ml density. *Abscissa:* percentage of the height of the liquid column in tube; *ordinate:* relative concentration, i.e. the ratio of the observed activity to that which would have been found if the enzyme had been homogeneously distributed throughout the gradient. Recoveries were 86.5% for cytochrome oxidase, 113.9% for monoamine oxidase, 110.2% for malate dehydrogenase and 91.2% for DNA polymerase.

disrupting mitochondria to some extent, for example by adding Triton X-100 to granule preparations. Such results show that the enzyme is not localized at the external side of the outer membrane. It is possible to decide if a mitochondrial enzyme is in the intermembrane space or in the matrix compartment by compar-

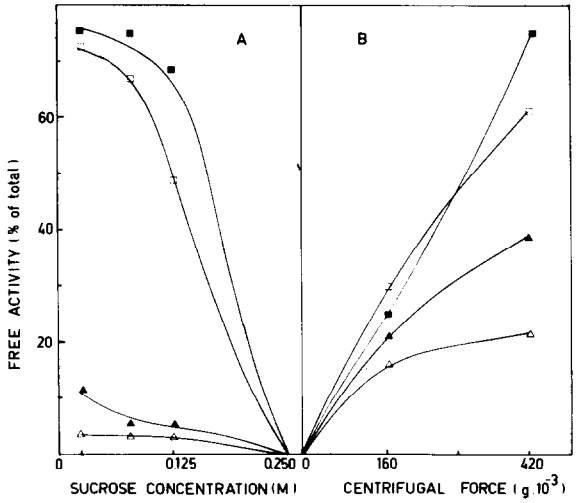

Fig. 3. Activation of sulfite cytochrome *c* reductase (■—■—■), alkaline DNAase (□—□—□), malate dehydrogenase (▲—▲—▲) and DNA polymerase (△—△—△), (A) by hypotonicity and (B) by high speed centrifugation. (A) Mitochondrial fraction (M + L) was exposed to the sucrose concentration indicated for 15 min at 0°. After this treatment, the sucrose concentration was readjusted to 0.25 M. (B) Mitochondrial fraction (M + L) was sedimented in 0.25 M sucrose (SW 65 Spinco Rotor) and maintained under the maximal centrifugal force indicated in abscissa during 45 min. After this treatment, granules were resuspended in 0.25 M sucrose. Free activity assays were performed in 0.25 M sucrose, total activity in presence of 0.15% Triton X-100. In order to make the comparison easier, the free activity before treatment has been subtracted from the free activity after treatment.

ing its behaviour in activation experiments with the behaviour of enzymes whose submitochondrial localization is known. Fig. 3 shows a comparison of the free activity of DNA polymerase, two intermembrane space enzymes: (sulfite cytochrome *c* reductase [6], alkaline DNAse [14]) and a soluble enzyme of the organelle matrix, malate dehydrogenase [15] when mitochondria are exposed to hypotonic media (fig. 3A) or subjected to high speed centrifugation in isoosmotic sucrose (fig. 3B). In both experiments, the unmasking of sulfite cytochrome *c* reductase proceeds together with the unmasking of alkaline DNAse. This is the result of the deterioration of the outer mitochondrial membrane leading to an increasing access of the enzymes to their external substrate. Both treatments are able only slightly to increase the free activity of DNA polymerase. Therefore deterioration of the outer membrane is not sufficient to allow external substrate to reach the poly-

merase. In the two experiments, malate dehydrogenase is more activated than DNA polymerase. This probably results from the fact that hypotonic treatment as well as high speed centrifugation leads to a certain deterioration of the inner mitochondrial membrane sufficient to increase its permeability to low molecular weight malate dehydrogenase substrate without allowing DNA primer to penetrate.

These experiments indicate that mitochondrial DNA polymerase is located at the inner side of the inner membrane or is free in the organelle matrix. To distinguish between these two possibilities, we have fractionated mitochondria by high speed centrifugation in a sucrose gradient.

3.3. Fractionation of mitochondria by high speed centrifugation in a sucrose gradient

When rat liver mitochondria are subjected to isopycnic centrifugation at 39 000 rpm (Spinco Rotor SW 65) in a sucrose gradient, the distributions of mitochondrial enzymes are quite similar, the equilibrium density being about 1.19 (fig. 2). When centrifugation is performed at 65 000 rpm, enzyme distributions become bi- or trimodal [16]. In that case, the distribution of an enzyme fixed to the mitochondrial inner or outer membrane chiefly differs from that of a soluble enzyme of the matrix by exhibiting a peak of activity in a region of low density of the gradient ($\approx$ 1.16 g/ml). This is caused by a disruption of the mitochondria, the matrix enzyme being released whilst mitochondrial ghosts equilibrate in a zone of lower density [16]. Fig. 4 illustrates the distribution of DNA polymerase, of cytochrome oxidase (inner membrane) and malate dehydrogenase (matrix) after centrifugation at 65 000 rpm (Spinco rotor SW 65) in a sucrose gradient of a rat liver mitochondrial fraction. Obviously, the distribution of DNA polymerase is similar to that of cytochrome oxidase and is markedly different from that of malate dehydrogenase. The distribution of the total activity of the enzyme is compatible with the possibility that the polymerase, normally free in the mitochondrial matrix, has adsorbed on the outer mitochondrial membrane after having been released in the external medium during disruption of the mitochondria. Indeed monoamine oxidase (outer membrane) exhibits a distribution similar to that of cytochrome oxidase in such an experiment [16]. For this

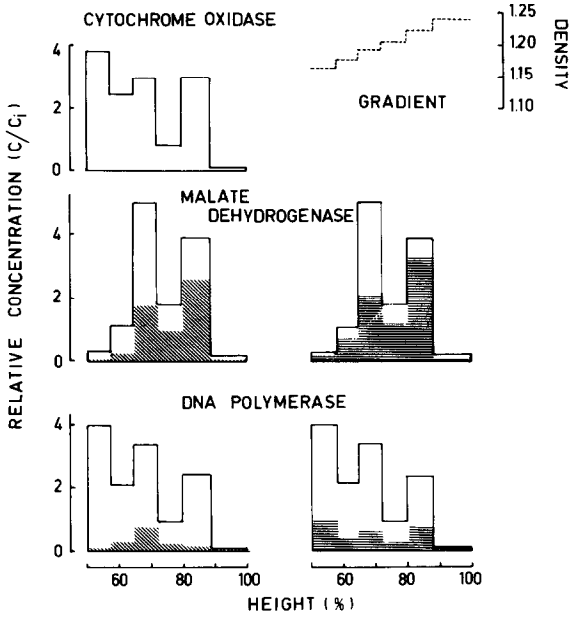

Fig. 4. Distribution of mitochondrial enzymes after isopycnic centrifugation in a sucrose gradient. Centrifugation was performed at 65 000 rpm ($\int w^2 dt$ = 144 rad^2/nsec) in the SW 65 Spinco rotor. The mitochondrial fraction was initially layered above a sucrose gradient extending for 1.09 to 1.26 g/ml density. Distribution of total activity of malate dehydrogenase and DNA polymerase are shown twice to allow a comparison with the percentage of soluble activity (▨) and free activity (▤) of the enzymes in each fraction of the gradient. The weak activity recovered beneath density 1.15 g/ml is not recorded on the graph. Recoveries were 102.5% for cytochrome oxidase, 96.3% for malate dehydrogenase and 94.5% for DNA polymerase.

reason we have measured also the free and soluble activity of DNA polymerase and malate dehydrogenase in the gradient fractions. Results are recorded in fig. 4; they show that DNA polymerase is masked to a large extent in each fraction. This can be explained only by supposing that the polymerase is fixed to the inner side of the inner membrane of mitochondria that have more or less lost their matrix content, as illustrated by the proportion of unsedimentable malate dehydrogenase recovered in the fraction. Preliminary results show that some DNA polymerase is released by treating mitochondrial membranes with salt solutions.

Acknowledgement

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