Biochimica et Biophysica Acta, 373 (1974) 10–17

BBA 76766

GLYCOPROTEIN BIOSYNTHESIS IN LIVER MITOCHONDRIA

II. MITOCHONDRIAL LOCALIZATION OF MANNOSYLTRANSFERASE

RENÉE MORELIS*, PIERRE BROQUET and PIERRE LOUISOT

University of Lyon, School of Medicine Lyon-Sud, Laboratory of Biochemistry, 69600 Oullins (France)

(Received May 10th, 1974)

SUMMARY

The preparation of liver mitochondria in the pure state, and submitochondrial fractionation in inner membrane, outer membrane and matrix, show that the mannosyltransferase of the mitochondria is localized in the inner membrane.

INTRODUCTION

In a preceding work [1, 2] we have shown in mitochondria, a mannosyltransferase capable of fixing the mannose onto an endogenous protein acceptor, from GDPmannose. In an in vitro acellular system, the enzyme functions optimally at 30 °C and pH 7.4. Its affinity constant for the substrate GDPmannose is equal to 110 nmoles. The enzyme is inhibited by ADP, GDP and GTP. Mn^{2+} and Mg^{2+} are activators, the maximum velocity of the reaction being at a concentration of Mn^{2+} equal to $5 \cdot 10^{-3}$ M. The enzyme is different from its microsomal homologue. Furthermore, it is independent of the mitochondrial energy functions.

This paper is devoted to the study of its submitochondrial localization.

MATERIALS AND METHODS

Preparation and purification of mitochondria

The mitochondria of rat liver are prepared by the method of Weinbach [3], conserved and controlled in previously described conditions [2]. In order to eliminate the contaminants, the mitochondria are treated with a (20 mg/ml) digitonin solution at the rate of 25 μg/mg of protein, for 10 min at +4 °C according to Colbeau et al. [4]. The action of the digitonin is stopped by the addition of 0.25 M, pH 7.4, sucrose (3:1, v/v). This last suspension is centrifuged at 9000 × *g*; the pellet obtained is washed once in 0.25 M sucrose to eliminate all traces of digitonin. The mitochondria are then replaced in suspension in the same 0.25 M, pH 7.4, sucrose solution, at the rate of 1 ml for 5 g of liver.

* Chargée de Recherches au C.N.R.S.

Another technique of purification is the sedimentation of the mitochondria in Ficoll discontinuous gradient 9, 12 and 16 % (w/v) in 0.33 M sucrose, 1 mM Tris–HCl (pH 7.4) according to Morgan et al. [5].

Submitochondrial fractionation

The outer membrane of the mitochondria is obtained through the action of digitonin at 100 μg/mg of protein, at 4 °C for 20 min, according to Schnaitman et al. [6]. The action of the digitonin is stopped by the addition of 3 vol. 0.25 M sucrose. A centrifugation at $10\,000 \times g$ allows the association inner membrane and matrix to sediment, whereas the outer membrane stays in the supernatant, where it is sedimented in 1 h at $144\,000 \times g$.

The inner membrane is prepared according to Parsons et al. [7] by the swelling of the inner membrane and matrix in a 20-mM, pH 7.2, phosphate buffer for 20 min, at 4 °C. Centrifugation at $10\,000 \times g$ allows the collection of the inner membrane in the pellet, whereas the matrix stays in the supernatant. This supernatant is concentrated by passing over a XM 50 Diaflo membrane, under N_2 pressure.

Purification of the different particulated submitochondrial fractions has been achieved either in discontinuous sucrose gradients (3 equal layers: 25, 37 and 51 % w/v; or in 12 layers, from 27 to 69 %) or in continuous sucrose gradients (0.8–1.6 M for the outer membrane; 0.8–2.4 M for the inner membrane) for 2 h at 24 000 rev./min, in an SW 25-1 Spinco rotor.

Control of the quality of the fractions obtained

The purity of the fractions obtained has been controlled by determinations of the activity of the enzymic systems taken as references.

The mitochondrial enrichment of the prepared subcellular fractions is followed by the quantitative determination of succinate dehydrogenase according to Devartanian and Veeger [8] and of the monoamine oxidase according to Tabor et al. [9].

The possible presence of non-mitochondrial contaminants is sought through the intermediary of their specific enzymes: 5'-nucleotidase (acid) according to Nachbaur et al. [10], for the lysosomes; glucose-6-phosphatase according to Schachter et al. [11] for the microsomes; 5'-nucleotidase (basic) according to Schachter et al. [11] for plasma membranes.

The specific feature of each submitochondrial fraction has been controlled by their characteristic enzymes: monoamine oxidase [9] for the outer external membrane, succinate dehydrogenase [8] for the inner mitochondrial membrane, and glutamate dehydrogenase according to Strecker [12], for the matrix. All the electrophoreses at high voltage have been achieved under 2500 V in a Pherograph apparatus, in 0.1 M pyridine · HCl, pH 6.5, buffer, on No. 3 M Whatman paper, for 2 h.

RESULTS AND COMMENTS

Purification of the mitochondrial fraction

The crude mitochondria are purified either by sparing treatment with digitonin or by sedimentation in Ficoll gradient. Table I shows an enrichment of the purified fractions. The sparing treatment with digitonin is the most effective for purification. The mannosyltransferase activity decreases with it. Figs 1a and 1b show that, if the

TABLE I

EVOLUTION OF THE SPECIFIC ENZYMATIC ACTIVITIES AFTER PURIFICATION OF THE MITOCHONDRIA

Enzymes	Crude mitochondria	Digitonin pretreatment	Ficoll gradient
Monoamine oxidase A ($10^2 \cdot min^{-1} \cdot mg^{-1}$)	60	74	50
Succinate dehydrogenase A ($10^2 \cdot min^{-1} \cdot mg^{-1}$)	13	18	16
Mannosyltransferase ($cpm \cdot mg^{-1}$)	546	147	170
5′-Nucleotidase acid (lysosomes) (arbitrary unit)	329	32	–
5′-Nucleotidase basic (plasma membrane) (arbitrary unit)	344	61	–
Glucose-6-phosphatase (microsomes) (arbitrary unit)	142	31	–

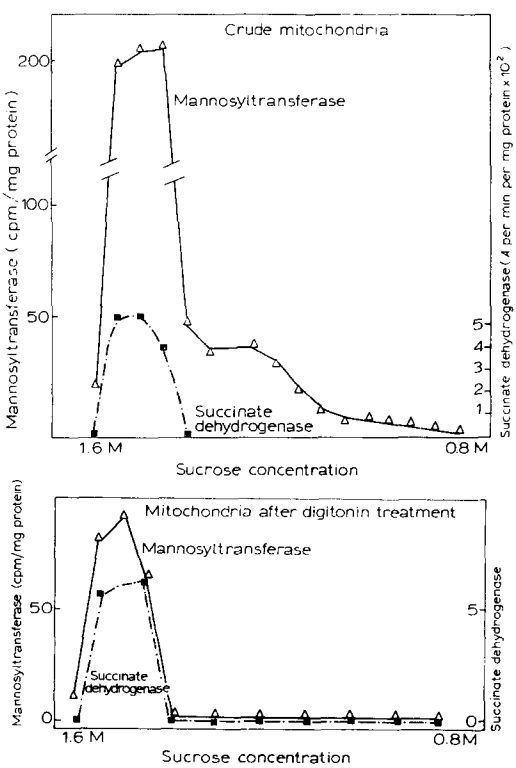

Fig. 1. Sedimentation in a continuous sucrose gradient (0.8–1.6 M) (2 h, 24 000 rev./min, Spinco L3-40, rotor SW 25/1) of the crude mitochondria fractions (a) and pretreated with digitonin (b).

sparing treatment by digitonin brings a certain loss of total mitochondrial mannosyltransferease activity, it eliminates all mannosyltransferase activity without correlation with succinate dehydrogenase.

Table I characterises the nature of the evicted contaminants: lysosomes, plasma membranes and microsomal membranes.

Influence of increasing concentrations of digitonin

These treatments have been accomplished with the aim of progressively eliminating the outer membrane of the mitochondria and to follow, in correlation, the destination of the mannosyltransferase. For each concentration of digitonin used, the enzymatic activities have been determined in the residual mitochondrial pellet and in the supernatant containing the components detached by the detergent. In Fig. 2 which reports the evolution of the activities of the enzymes remaining fixed in the mitochondrial pellet, we observe: the progressive elimination of the monoamine oxidase, characteristic of the outer membrane, between 50 and 100 μg of digitonin per milligram of protein; the absence of decrease of the succinate dehydrogenase, characteristic of the inner membrane, with the single exception of a slight decrease at a very strong concentration of detergent; the abrupt decrease of acid and basic 5'-nucleotidases, which liberates the customary contaminants (lysosomes and plasma membranes) at low concentrations of digitonin; the decrease of the mannosyltransferase in direct correlation with the disappearance of the contaminants; and the maintenance of a strong residual activity attached to the mitochondria and not liberated by the increasing concentrations of digitonin.

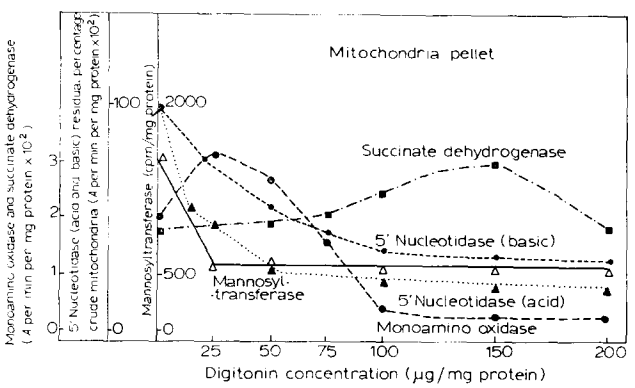

Fig. 2. Evolution of the enzymatic activities in the residual mitochondrial pellet, in terms of increasing amounts of digitonin (0–200 μg/mg of protein).

In Fig. 3, collecting the evolution of these same enzymes in the supernatant resulting from the digitonin treatment, we observe, in these supernatants, the increase in the rates of the enzymes progressively separated from the external surfaces of the mitochondria (monoamine oxidase) or of the contaminants (acid or basic 5'-nucleotidases and only one part of the mannosyltransferase).

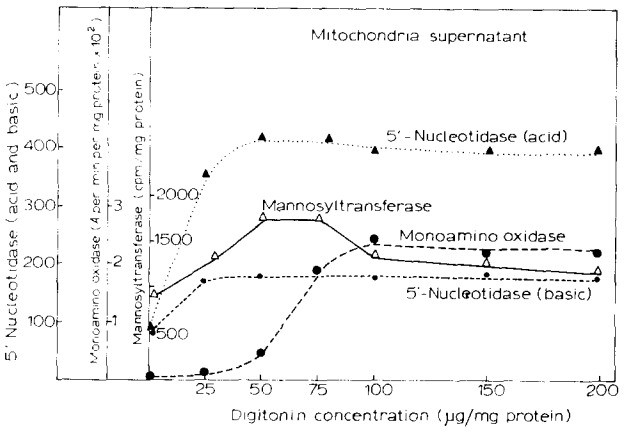

Fig. 3. Evolution of the enzymatic activities in the supernatant after treatment of the mitochondria with increasing amounts of digitonin (0–200 µg/mg of protein).

Study of the isolated outer membrane

The results obtained by the study of the progressive degradation of the outer membranes of the mitochondria and of the contaminants which were fixed onto them have been confirmed by the study of the isolated outer membranes. Figs 4 and 6 show the sedimentation of the outer mitochondrial membrane in sucrose continuous gradient. It shows the presence of mannosyltransferase in correlation with the succinate dehydrogenase (characteristic of inner mitochondrial membranes) and with the basic 5'-nucleotidase (characteristic of the contaminant plasma membranes).

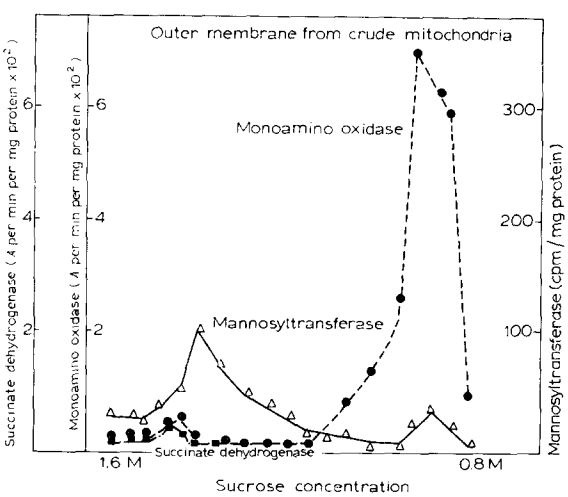

Fig. 4. Sedimentation in a continuous sucrose gradient (0.8–1.6 M) from a preparation of outer membranes from crude mitochondria (no previous passage over discontinuous gradient) (2 h, 24 000 rev./min, Spinco L3-40, rotor SW 25/1).

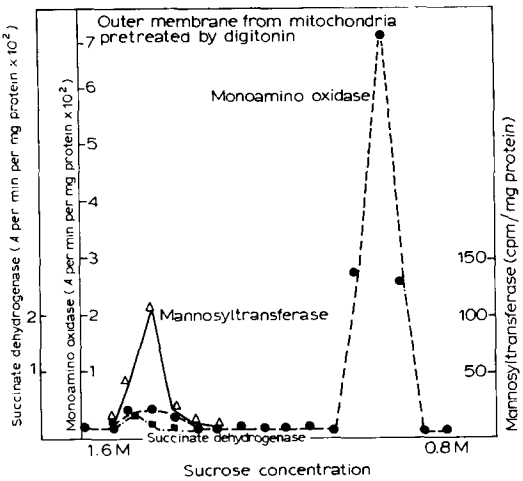

Fig. 5. Sedimentation on a continuous sucrose gradient (0.8–1.6 M) of outer membranes from mitochondria pretreated with digitonin (25 μg/mg of protein) but not purified by previous passage over discontinuous gradients (2 h, 24 000 rev./min, Spinco L3-40, rotor SW 25/1).

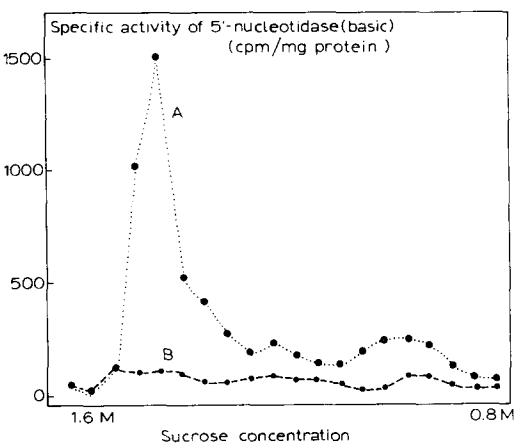

Fig. 6. Sedimentation on a continuous sucrose gradient (0.8–1.6 M) of outer membranes from crude mitochondria (A) and of mitochondria pretreated with digitonin (B): the graphs indicate the evolution of the basic 5'-nucleotidase profiles (2 h, 24 000 rev./min, Spinco L3-40, rotor SW 25/1).

Figs 5 and 6 correspond to the sedimentation, in sucrose gradient, of the outer membranes from mitochondria pretreated with digitonin (25 μg/mg of protein). We observe: at the botttom of the gradient, a slight mannosyltransferase in correlation with a little basic 5'-nucleotidase and succinate dehydrogenase activities; and, at the top of the gradient, the quasi-totality of the monoamine oxidase, with no trace of mannosyltransferase.

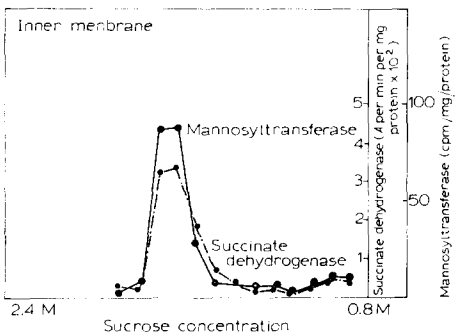

Fig. 7. Sedimentation on a continuous sucrose gradient (0.8–2.4 M) of the inner membranes from mitochondria (2 h, 24 000 rev./min, Spinco L3-40, rotor SW 25/1).

Study of the isolated inner membrane

The sedimentation in a continuous sucrose gradient (0.8–2.4 M) of mitochondrial inner membranes (Fig. 7) shows the perfect correlation between the mannosyltransferase and the succinate dehydrogenase.

Search for mannosyl transferase in the mitochondrial matrix

The separation of the matrix and the inner membrane and the study of their mannosyltransferase activities has led to the results set out in Table II. From the evidence, it seems that the main part of the mitochondrial mannosyltransferase is localized in the inner membrane, the specific activity of the matrix being so low as to be quite negligible.

TABLE II

DISTRIBUTION OF THE MANNOSYLTRANSFERASE ACTIVITY BETWEEN THE MATRIX AND THE INNER MEMBRANE OF THE MITOCHONDRIA

Fraction	Specific activity (cpm/mg)	Total activity (specific activity · volume)	Distribution (%)
Inner membrane	190	3762	98
Matrix	3	92	2

DISCUSSION

The methodology used in the purification of the mitochondrial and submitochondrial fractions has given very satisfactory results. The pretreatment with digitonin is useful in eliminating the cytoplasmic contaminants, and the treatment with digitonin at 100 μg/mg of protein then frees the outer mitochondrial membrane in a practically pure state.

The study of the inner membrane of mitochondria shows the direct correlation existing between the mannosyltransferase and the succinate dehydrogenase specific to that structure. The separation of the inner membrane from the matrix confirms

this point of view and now allows us to affirm that the mitochondrial mannosyltransferase is certainly localized in the inner membrane, accompanied by the requisite endogenous acceptors.

ACKNOWLEDGEMENTS

This work was supported by the "Centre National de la Recherche Scientifique", the "Fondation pour la Recherche Médicale Française" and the University of Lyon.

REFERENCES

1 Morelis, R. and Louisot, P. (1973) C. R. Acad. Sci. 276, 2219–2227
2 Morelis, R. and Louisot, P. (1973) Biochimie 55, 671–677
3 Weinbach, E. C. (1961) Anal. Biochem. 2, 335–343
4 Colbeau, A., Nachbaur, J. and Vignais, P. M. (1971) Biochim. Biophys. Acta 249, 462–492
5 Morgan, I. G., Wolfe, L. S., Mandel, P. and Gombos, G. (1971) Biochim. Biophys. Acta 241, 737–751
6 Schnaitman, C., Erwin, V. G. and Greemawalt, J. W. (1967) J. Cell Biol. 32, 719–735
7 Parsons, D. F., Williams, G. R., Thompson, W., Wilson, D. F. and Chance, B. (1967) in Mitochondrial Structure and Compartmentation (Quagliariello, E., Papa, S., Slater, E. C. and Tager, J. M., eds), p. 29, Adréatica Editrice, Bari
8 Devartanian, D. V. and Veeger, C. (1964) Biochim. Biophys. Acta 92, 233
9 Tabor, C. W., Tabor, M. and Rosenthal, S. M. (1955) in Methods in Enzymology (Colowick, S. P. and Kaplan, W. O., eds), Vol. 2, p. 390, Academic Press, New York
10 Nachbaur, J., Colbeau, A. and Vignais, P. M. (1972) Biochim. Biophys. Acta 274, 426–446
11 Schachter, H., Jabbal, I., Hudgin, R., Piwteric, L., McGuire, E. J. and Roseman, S. (1970) J. Biol. Chem. 245, 1090
12 Stecker, H. J. (1955) in Methods in Enzymology (Colowick, S. P. and Kaplan, W. O., eds), Vol. 2, p. 220, Academic Press, New York