GLYCOPROTEIN COMPONENTS, SIALIC ACID AND HEXOSAMINES, BOUND TO INNER AND OUTER MITOCHONDRIAL MEMBRANES

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1. Introduction

Much attention has been focused in the past on protein and phospholipid constituents of biological membranes. More recently, however, carbohydrate-containing materials have become subject of interest as important components of plasma membrane. These macromolecules have been recognized as responsible for a number of phenomena including contact-inhibition, transport and antigenic properties (for review see [1]). Intracellular membrane structures have also been shown to possess a glycoprotein complement [2–8] and an active synthesis of such components has been reported to occur *in vitro* in isolated mitochondria and microsomes [9–11]. The availability of a technique for the separation of inner and outer membranes [12] from isolated mitochondria makes it possible to investigate the intramitochondrial distribution of glycoproteins. In this paper we report the distribution between mitochondrial membranes of two typical constituents of glycoproteins such as sialic acid and hexosamines.

2. Methods

Mitochondria and microsomes as well as the outer mitochondrial membrane fractions have been obtained from rat liver according to the procedure already described [12]. Such a procedure provides a heavy fraction consisting of inner membrane + matrix. In order to get rid of the matrix content, the original technique has been modified as follows. Mitochondria adjusted to a protein concentration of 20 mg/ml were first swollen at 0° in 10 mM tris-phosphate buffer (pH 7.5) for 5 min. The suspension was then diluted 1:1 with 1.8 M sucrose and aliquots of 40 ml were subjected to sonic oscillation for 1 min at 2.5 A using a Branson "Sonifier" equipped with a "macro-tip". 19 ml of the treated suspension were layered on 8 ml of 1.11 M sucrose in centrifuge tubes of the SW-25 Spinco rotor. After centrifugation for 4 hr at 23,000 rpm, the sediment was resuspended in 10 mM tris-phosphate buffer (pH 7.5) at a protein concentration of 5–10 mg/ml. After resonication for 2 min at 2 A, the suspension was centrifuged in the no 40 Spinco rotor at top-speed for 1 hr. The sediment represented the purified inner membrane fraction.

Sucrose and other low molecular weight components were removed from all fractions by gel filtration on Sephadex G-25 (6 × 60 cm) pre-equilibrated with 10 mM phosphate buffer (pH 7.5). Lipids were removed from the lyophilized fractions by washing with the following sequence of solvents: chloroform–ethyl ether (2:1); chloroform–ethyl ether (1:1); pure chloroform; chloroform–methanol (1:1); methanol; acetone–H_2O (9:1); dry acetone; and finally, with *n*-heptane. After evaporation of the solvents the material was stored dry at −20°.

Rotenone-insensitive NADH–cytochrome *c* reductase, succinate–cytochrome *c* reductase, malic dehydrogenase and glucose-6-phosphatase activities were assayed as already described [12, 13]. These enzymes are markers for outer, inner membranes and matrix of mitochondria and microsomes, respectively.

N-Acetyl-neuraminic acid (NANA) was determined after hydrolysis in 5% trichloroacetic acid essentially according to Molnar et al. [14] by the reaction of

Warren [15]. Ion-exchange chromatography, included in the Molnar et al. procedure, allows deoxyribose interference to be excluded, as shown also by identity of spectrum of standard pure NANA and that of the eluate, after reaction with thiobarbituric acid.

Hexosamines were determined after hydrolysis in 6 N HCl at 100° for 6 hr by the Elson-Morgan reaction [16], as already described [17].

Submitochondrial fractions were assayed for protein content by the method of Waddell, as described by Romeo et al. [18]. Total nitrogen content of the lyophilized material was assayed by the Nessler reaction according to Minari and Zilversmit [19].

3. Results and discussion

Mitochondria were first assayed for glucose-6-phosphatase to assess the extent of microsomal contamination. By this parameter it was possible to conclude that the microsomal contamination of mitochondrial fraction was 6.2 ± 0.16% on the protein basis. The outer mitochondrial membrane fraction collected approximately 50% of the total rotenone-insensitive NADH–cytochrome *c* reductase activity with a 7-fold purification of the enzyme. Succinate–cytochrome *c* reductase still bound to this fraction accounted for only 6% of the original activity of the mitochondria. Such a degree of purity if perfectly comparable to that reported by one of us [12, 13, 20] in previous papers describing the detailed procedure. Both glucose-6-phosphatase activity and *P*-450 were virtually absent. The degree of purity of the inner mitochondrial membrane, as judged by the distribution of marker-enzymes in the subfractions, is shown in table 1. It may be seen that the first treatment (sonication and discontinuous density gradient centrifugation, see Methods) results in the release of approximately 90% of the outer membrane as indicated by the distribution of rotenone-insensitive NADH–

Table 1

Protein, rotenone-insensitive NADH–cytochrome *c* reductase, succinate–cytochrome *c* reductase and malic dehydrogenase of subfractions derived from rat liver mitochondria.

Fraction	Total protein (mg)	NADH–cyt. *c* reductase		Succ.–cyt. *c* reductase		Malic dehydrogenase (μmoles NADH ox. min^{-1})	
		(μmoles cyt. *c* red. min^{-1})					
		mg $prot^{-1}$	total	mg $prot^{-1}$	total	mg $prot^{-1}$	total
First treatment							
Swollen, shrunk and sonicated mitochondria	530	0.360	162.0	0.27	145	5.62	2980
Supernatant I	192	0.700	134.0	0.05	10	8.00	1540
Sediment I	295	0.050	15.2	0.43	129	3.94	1160
Second treatment							
Sonicated Sediment I	276	0.050	15.10	0.18	50.00	3.14	865
Supernatant II	145	0.006	0.88	0.01	1.75	4.64	720
Sediment II (Inner membrane)	132	0.080	10.60	0.30	39.40	0.42	56

Table 2
Hexosamines and *N*-acetyl-neuraminic acid content of rat liver microsomes, mitochondria, inner and outer mitochondrial membranes.

Fraction	Hexosamines	NANA	Hexosamines
	nmoles/mg N		NANA
Microsomes	316.00± 9.05 (7)	78.65±1.91 (5)	4.0
Mitochondria	168.86±10.78 (7)	19.54±0.57 (5)	8.6
Inner membrane	135.47± 7.08 (6)	11.08±0.78 (4)	12.3
Outer membrane	263.04± 6.74 (6)	40.82±2.91 (5)	6.4

Data + S.E.M. Number of experiments indicated in parenthesis.

cytochrome *c* reductase activity. About half of the matrix content is released by this procedure as indicated by the distribution of malic dehydrogenase. The second treatment results in an almost complete depletion of malic dehydrogenase from the sediment, indicative of a substantial removal of the matrix content from the inner membrane fraction. The contamination of the inner mitochondrial membrane fraction by outer membrane fragments and matrix protein can therefore be calculated as 6 and 2%, respectively.

Table 2 reports the content in hexosamines and sialic acid of microsomes, mitochondria and of the two mitochondrial membranes. In accordance with early findings by other authors [2–5], both microsomes and mitochondria contain hexosamines and sialic acid bound to their structure in high molecular weight complexes. On the basis of the technical procedure adopted to prepare materials, the best candidates for these macromolecules are glycoproteins. It is worth mentioning that our data, when recalculated in the same units, are in excellent agreement with the sialic acid content reported for rat liver microsomes and mitochondria by Patterson and Touster [2]. In contrast with that reported in literature for rabbit liver [4], the molar ratio of the two sugars is found to be twice as high in mitochondria than in microsomes. As compared with the intact mitochondrion, the subfractions derived therefrom have different contents in hexosamines and sialic acids on the protein basis. In particular the outer mitochondrial membrane shows a 1.5-fold purification of hexosamines and a two-fold purification of sialic acid. On the contrary, the inner membrane shows a lower content both of hexosamines and sialic acid than the intact mitochondrion. It is important to note, however, that the molar ratio hexosamines/sialic acid is different for each subfraction and that in neither case it corresponds to that of the original mitochondrion. The finding that the two mitochondrial membranes have different composition in hexosamines and sialic acid can be explained either by assuming that the two membranes have individual species or classes of glycoproteins complements or that similar glycoproteins are unevenly distributed between the two membranes. In either alternative these results provide further evidence of the chemical difference between the two mitochondrial membranes, on one hand, and outer mitochondrial membrane and microsomes, on the other, (for review see [21]).

On the basis of the percentage distribution of protein among outer, inner membranes and matrix + inter membrane space of liver mitochondria (14, 21 and 75% respectively [22]), it is possible to calculate that 80% of sialic acid and 77% of hexosamines are released in soluble form during the separation procedure. The surprising conclusion then is that about 80% of the total carbohydrate-containing material either does not represent an integral part of membrane structure or it is very loosely bound to it.

It follows that since virtually the same percentage of the two carbohydrates is solubilized, the average molar ratio of the released material must be the same as that of the intact mitochondrion. This conclusion allows to exclude that the amino-sugars found in the inner mitochondrial membrane fraction, may be accounted for by inclusion of matrix content in the inner membrane vescicles. In fact, if this were the case, the molar ratio hexosamines/sialic acid in this fraction would be the same as in the matrix.

The study of the glycoprotein complement of the two mitochondrial membranes is now under investigation in our laboratory.

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