A GLYCOPROTEIN LOCATED IN THE INTERMEMBRANE SPACE OF RAT LIVER MITOCHONDRIA

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1. Introduction

Recently much has been focused on the glycoprotein content of biological membranes and in particular of mitochondria [1–11]. In a previous paper on this subject [12], we have reported the distribution of glycoprotein components between inner and outer mitochondrial membranes. An interesting observation in that paper was that a large portion of sialic acid and hexosamine-containing material was released during the separation of the two membranes. No information was however available about the original intramitochondrial location of the released glycoprotein. We found therefore of interest to compare the supernatant obtained from mitochondria upon swelling and contraction with that obtained by sonication from pre-swollen and contracted mitochondria. In this paper we report an electrophoretic study of the proteins released in the soluble fraction by different procedures. A technique is also described for the isolation of the glycoprotein, released by swelling, in an electrophoretically homogenous form.

2. Materials and methods

Mitochondria were prepared in 0.2 M Mannitol from rat liver, essentially according to Johnson and Lardy [13]. Swelling of the mitochondria was performed by suspending the pellet in 10 mM Tris-phosphate buffer (pH 7.8). After standing in ice for 20 min an equal volume of 0.9 M sucrose, 2 mM ATP and 2 mM $MgSO_4$, was added. The suspension was immediately centrifuged at 105,000 *g* for 20 min and the supernatant (S_1) was saved. The pellet washed once with 0.45 M sucrose was taken up in 0.22 M sucrose containing 5 mM Tris-phosphate buffer (pH 7.8) at approximately 10 mg protein/ml and subjected to sonic ascillation. The sonication step was performed on 3.5 ml aliquots using a Branson sonifier, equipped with a microtip operated at 2.5 A for 15 sec.

The sonicated suspension was then subjected to centrifugation at 105,000 *g* for 1 hr. This supernatant (S_2) and S_1 were analyzed by polyacrylamide gels electrophoresis as follows. 4% acrylamide gels were prepared following the technique described by Davis [14]. The pH of the running and the spacer gels were 8.9 and 6.7, respectively. Samples were applied onto the gels after 12 hr dialysis against the spacer gel buffer containing 20% sucrose. Electrophoresis was run in a standard apparatus at a current of 5 mA per gel. For comparison purposes, the same procedure was applied to the supernatant obtained after separation of the two mitochondrial membranes by the swelling-shrinking-sonication technique, already reported [15]. After 30 min the fastest component had moved 6–7 cm from the origin. Gels were stained for proteins either with amido black according to Davis [14] or with coomassie blue according to Weber and Osborn [16]. In our hands amido black was found to be less sensitive but of faster use. Staining for glycoproteins was carried out by soaking the gels in 0.01% toluidine blue in 0.1 M acetate buffer (pH 4.5). After 8–10 hr the excess of the dye was removed by immersion in the same buffer till glycoproteins appeared as pink-violet bands contrasted on a virtually uncolored background. The glycoproteins were also stained after periodic acid oxidation with Schiff-reagent according to Evans [17] and by immersion of the gels in 0.01%

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water solution of rutenium red. Gels were scanned with white light by using the Eppendorf spectrophotometer equipped with a home-made automatic scanning device. Preparative electrophoresis has been performed using a simple home-made apparatus. The running gel was polymerized in a 22 mm inner diameter glass tube equipped with a cooling jacket (Sovirel-France), connected with an elution chamber allowing the displacement of the single bands by a stream of buffer with a flow-rate of approximately 20 ml/hr. Fractions were collected with an Ultrorac Fraction Collector (LKB) after continuous recording of absorbance at 294 nm by Uvicord Ultraviolet absorptiometer (LKB). Molecular weight estimation by electrophoretic gel permeation was carried out in SDS according to Weber and Osborn [16].

Malic dehydrogenase, a marker enzyme for matrix protein has been determined as previously described [18].

Protein content of mitochondria and submitochondrial fractions was determined by biuret reaction according to Gornall et al. [19].

The sialic acid, hexosamines and protein-nitrogen content of the purified glycoprotein was determined as previously reported [12].

Neutral sugar content was measured essentially according to Scott and Melcin [20].

3. Results and discussion

Trace a in fig. 1 shows the distribution of protein solubilized from mitochondria by swelling, shrinking and sonication and represents therefore the proteins set free in solution when the two membranes are separated [15]. Clearly a number of proteins are scattered through the gel. Interestingly, however, only

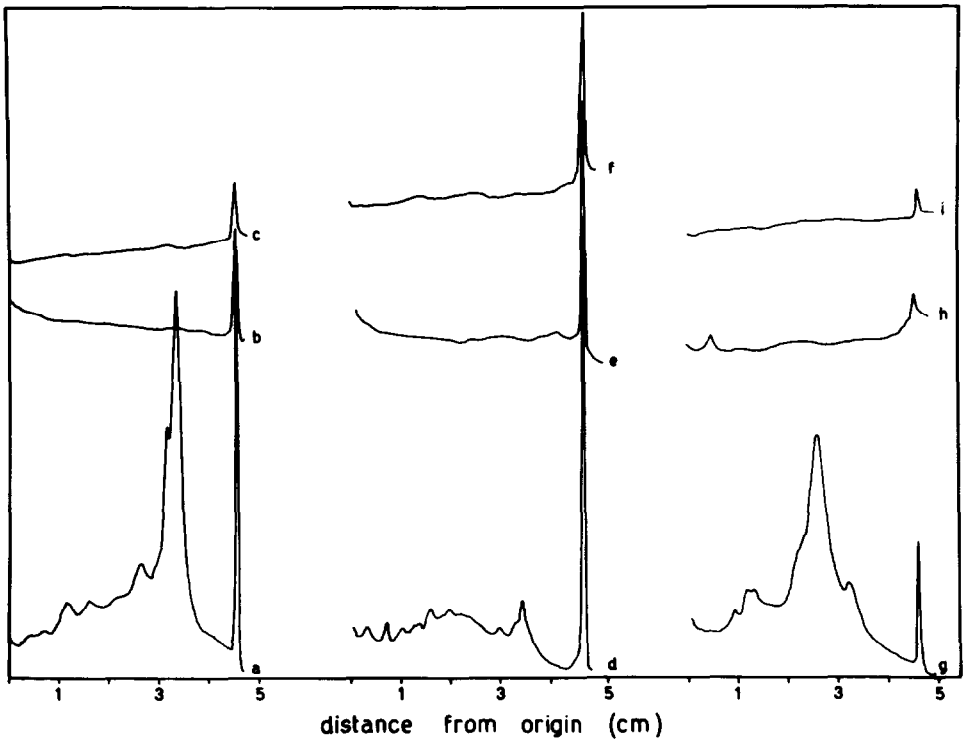

Fig. 1. Electrophoretic patterns of solubilized protein from rat liver mitochondria by different procedures. Left: swelling in Tris-phosphate buffer (pH 7.8) followed by shrinking and sonication; middle: swelling in Tris-phosphate buffer and shrinking; right: sonication of pre-swollen washed mitochondria (see Methods). *Experimental conditions*: 4% Polyacrylamide gels loaded with 0.70 mg protein of the fractions in traces a, d and g, stained with amido-black. The other traces refer to gels loaded with six times as much protein stained with toluidine blue traces b, e and h and with periodic acid-Schiff reagent traces c, f and i respectively.

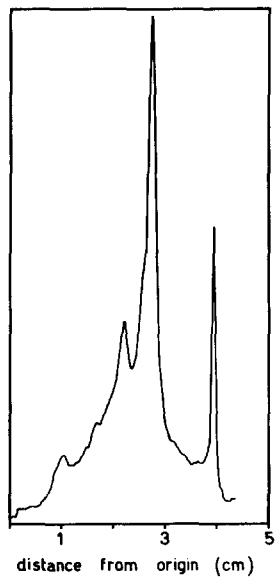

Fig. 2. Electrophoretic pattern of protein solubilized from rat liver mitochondria by hypotonic swelling in distilled water followed by shrinking in hypertonic sucrose. *Experimental conditions*: Mitochondria were treated as described under Methods for swelling and shrinking except that water was used instead of Tris-phosphate buffer. 0.230 mg protein of the fraction were loaded on 4% polyacrylamide gel. After electrophoresis staining of the protein was performed by amido-black.

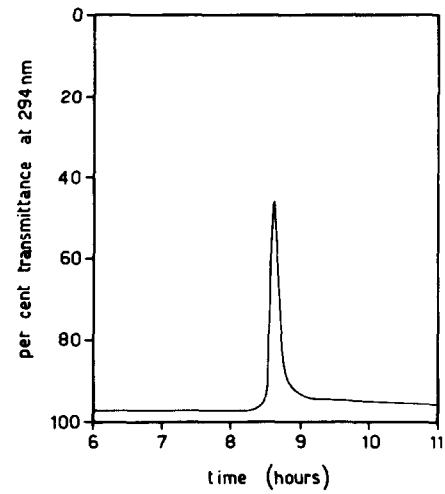

Fig. 3. Elution pattern from 4% polyacrylamide preparative gel electrophoresis of protein solubilized from rat liver mitochondria by swelling in Tris-phosphate and shrinking. *Experimental conditions*: 30 ml 4% polyacrylamide gel were polymerized in the apparatus and the material (80 mg protein) dialyzed against the spacer gel buffer was layered on top. The buffer in the two reservoirs and the elution buffer was Tris-glycine according to Davis [14] at pH 8.3. The flow-rate in the elution chamber was 20 ml/hr.

a major fast moving band is obtained which gives positive reaction with periodic acid-Schiff reagent (trace b) and shows metachromasy upon staining with toluidine blue (trace c). This finding suggests that a major glycoprotein component is released by the procedure and behaves as a single molecular species in gel electrophoresis under the conditions described. The glycoprotein nature of this component is further documented by the positive response to other specific dyes as alcian-blue [21] and rutenium red [22]. In the attempt to locate more precisely this interesting component, we have analyzed separately the fraction derived by swelling and that derived by sonication. The results of this experiment are reported in traces d–i of fig. 1. It may be noticed first of all that the two groups of densitometric traces are totally different from one another. The quantitatively most important component in the fraction derived by swelling is represented by the fast moving glycoprotei, whereas this band is present in a much smaller, though not insignificant, amount in the fraction derived by sonication.

These findings suggest that the two procedures are rather selective in extracting soluble proteins from mitochondria. Presumably the former treatment extracts proteins either included between the two membranes or loosely bound to them. The latter, on the contrary, releases the matrix content.

This conclusion is reinforced by distribution data of malic dehydrogenase and adenylate kinase. Upon swelling and contraction, in fact, less than 2% of the total malic dehydrogenase and more than 90% of adenylate kinase were set free in solution. On the contrary, pre-swollen and contracted mitochondria subjected to sonication for 15 sec released up to 50% of malic dehydrogenase activity, in good agreement with earlier data [18]. Surprisingly we found that the use of phosphate in the swelling medium is absolutely critical in maintaining the selectivity of extraction. In fact, if distilled water is used instead of 10 mM Tris-phosphate buffer, the electrophoretic pattern obtained with the supernatant after swelling is much

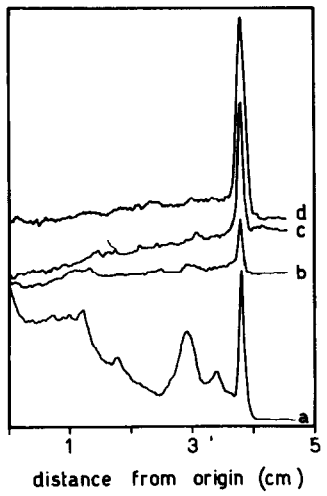

Fig. 4. Electrophoretic pattern on 10% polyacrylamide gel of purified glycoprotein fraction obtained by 4% polyacrylamide preparative electrophoresis. *Experimental conditions*: 0.150 mg protein of the fraction derived from swollen, shrunk mitochondria by preparative electrophoresis on 4% polyacrylamide gel dialyzed against spacer gel buffer loaded on a 10% polyacrylamide gel. After electrophoresis proteins were stained by amido-black (trace a). Staining of parallel gels loaded with twice as much protein was performed with periodic acid-Schiff reagent (trace b), rutenium red (trace c) and toluidine blue (trace d).

closer to that obtained by the combined swelling-sonication procedure as shown by data in fig. 2. Whether this result reflects a specific action of phosphate cannot be stated at the moment for sure.

The amount of protein released by swelling in phosphate accounts for 30.40 ± 1.37% of the total mitochondrial protein (15 experiments). On the basis of the integral of the curve obtained by densitometric recording it has been found that consistently the major band accounts for 20% of the total protein of this fraction. This calculation suffers from the limitation that amido-black binding the proteins may be uneven. On the basis of these data, it should be concluded that this glycoprotein represents some 5–6% of the whole mitochondrion. In order to further investigate the properties of the mitochondrial glycoprotein released by swelling we have subjected the material solubilized from mitochondria by this treatment to preparative gel-electrophoresis as described under Methods and in the legends to the figures. Part of the elution pattern from the column is presented in fig. 3. It may be noticed that the protein is eluted as a single and a very sharp peak. This peak could include more than one component and we have subjected therefore this fraction to gel electrophoresis

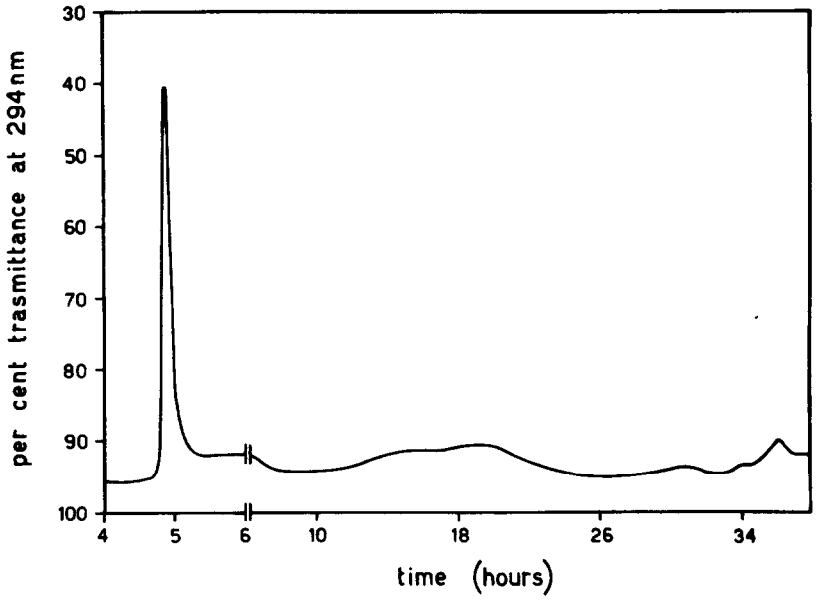

Fig. 5. Elution pattern from a combined 3% and 10% polyacrylamide gel electrophoresis. *Experimental conditions*: As in fig. 3 except that the apparatus was set up by polimerizing in sequence 10 ml 10% polyacrylamide and 10 ml 3% polyacrylamide.

in 10% polyacrylamide gel. Under these conditions the severe sieving effect did resolve components with identical electrical mobility but different sizes. As illustrated in fig. 4, clearly a number of different proteins are separated by disc electrophoresis under these conditions. Specific reactions for carbohydrates revealed however, that the only component positive to metachromatic dyes, rutenium red and periodic acid-Schiff reagent is still the fastest moving band. On the basis of the data presented showing that electrophoresis on 4% polyacrylamide gels does not resolve completely the glycoprotein from other components with high electrophoretic mobility, we have undertaken the purification of the glycoprotein by means of preparative electrophoresis using as combined system 3% and 10% polyacrylamide gels. The elution pattern from such a column is reported in fig. 5. Also in this case it may be noticed that the protein is eluted as a single sharp peak. In agreement with the data obtained by analytical electrophoresis (see fig. 1 trace d) a number of protein peaks are retarded on the column and each of them constitutes but a minor portion of the total protein. In addition, none of them give positive reactions with specific stains for glycoprotein. The electrophoretic homogeneity of the glycoprotein isolated by this procedure was assessed by 7% polyacrylamide gel electrophoresis in SDS. The results of this experiment is presented in fig. 6. Clearly only one component may be stained by coomassie blue in the gel. By comparison with appropriate standard proteins the molecular weight of about 51,000 daltons has been calculated.

Preliminary chemical analysis of purified preparations of the fast moving glycoprotein obtained by preparative disc electrophoresis on 10% gels have shown that the protein contains hexosamines and sialic acid in the ratio of 8:1, as expected from our previous data [12]. On nitrogen basis the content of sialic acid and hexosamines was 3–4 times higher than in the whole mitochondrion. The glycoprotein nature of the compound isolated is further documented by the presence of neutral sugars determined by the anthrone method.

Data presented strongly support the view that the major glycoprotein is located between the two membranes. It may be quoted in this connection that recently Hall and Crane [23] have reported electron microscopic evidence of protein in the intracristael space, a compartment continuous with that delimited by the two membranes. The finding that ionic irradiation of pre-swollen-washed mitochondria results in a further release of the same glycoprotein may be explained by one of the two following alternatives: (a) the same glycoprotein is present both in the intermembrane and in the matrix space; (b) there exists an equilibrium between the soluble intermembrane glyco-

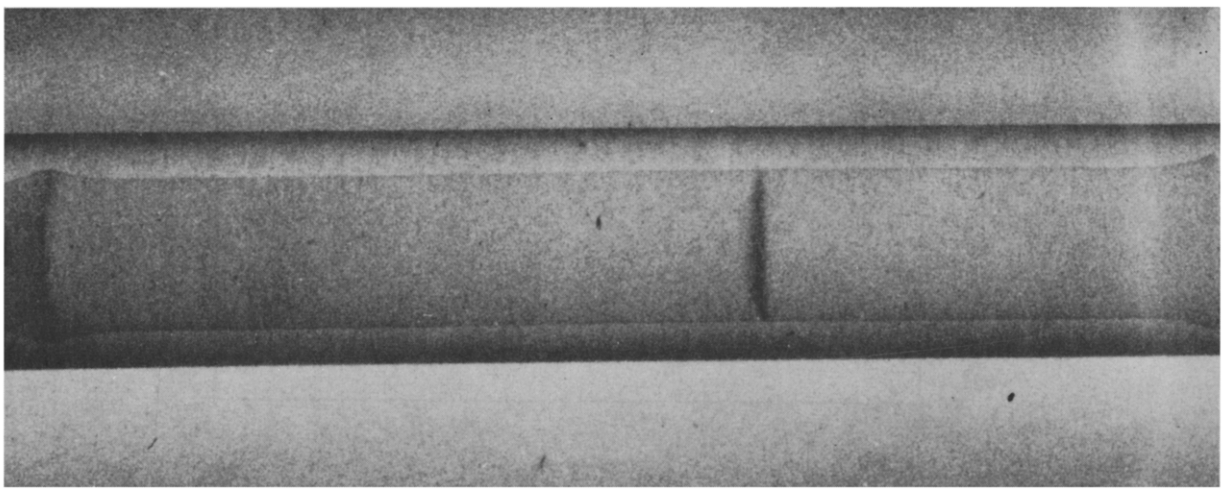

Fig. 6. Electrophoresis on polyacrylamide gel of the purified glycoprotein, in the presence of SDS. *Experimental conditions*: Electrophoresis at pH 8.9 in 0.1% SDS according to Weber and Osborn [16]. 0.200 mg protein/gel.

protein and that bound to the outer surface of the inner membrane. At the moment we are not in the position to decide for either possibility.

As for the physiological role of the glycoproteins an interesting observation is that the band on analytical electrophoresis stains yellow when soaked in 0.70 mM murexide solution, indicative of a firm binding of calcium to it. The relation of the glycoprotein to the calcium carrier [24] is now under investigation in cooperation with E. Carafoli, P. Gazzotti and F. Vasington (Modena, Italy).

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