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THE ABILITY OF THE MITOCHONDRIAL Ca^{2+}-BINDING GLYCOPROTEIN TO RESTORE Ca^{2+} TRANSPORT IN GLYCOPROTEIN-DEPLETED RAT LIVER MITOCHONDRIA

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Summary

Rat liver mitochondria may be subfractionated in sediment and supernatant fractions by swelling in the presence of EDTA and oxaloacetate. The sediment is largely depleted of the Ca^{2+}-binding glycoprotein and its Ca^{2+}-transporting activity may be as low as 10—20% of the starting value. Both the rate of Ca^{2+} uptake and the capacity to maintain a high Ca^{2+} concentration gradient across the membrane are depressed. Addition of an osmotic supernatant to the assay mixture may partially restore the original Ca^{2+}-transporting ability. The active component in the supernatant is the Ca^{2+}-binding glycoprotein. This is shown by the following facts: (a) the effect is enhanced by the addition of the purified glycoprotein to the supernatant; (b) precipitation of the glycoprotein from the supernatant by affinity chromatography-purified antibodies abolishes the stimulatory effect, and (c) in the presence of 130 μM Mg^{2+}, the glycoprotein alone may restore fully the Ca^{2+}-transporting ability of the particles. The maximal velocity is already reached at 0.1 μg glycoprotein/mg mitochondrial protein.

Introduction

A glycoprotein with high-affinity Ca^{2+}-binding capacity was isolated in our laboratory in 1971 [1]. Its involvement in Ca^{2+} transport has been suggested on the basis of large, although indirect, evidence (see review Ref. 2). Much more direct evidence came from immunological studies. Antiglycoprotein, purified by affinity chromatography, blocks, at very low concentrations, both the active Ca^{2+} uptake [3] and Ca^{2+} release induced by uncouplers [4]. Perhaps even more interesting, the glycoprotein is the site of action of metabolic regulation

of mitochondrial Ca^{2+} fluxes. It has been recently shown, in fact, that oxaloacetate-induced Ca^{2+} release, first described by Lehninger et al. [5—7], is inhibited by antibodies directed to the glycoprotein. It has also been found, in addition, that NAD^+ may modulate the Ca^{2+}-binding ability of the isolated glycoprotein by binding to the molecule at four sites. This binding shows, as expected, a positive cooperativity (Panfili, E., Sandri, G., Liut, G. and Sottocasa, G., unpublished results).

A natural development of these studies was the attempt to reconstitute in vitro Ca^{2+} transport. Experiments aimed at this goal were so far without success, both in our and other laboratories (see for discussion Ref. 8). The reason of the failure depends on the experimental design. Experiments carried out with liposomes in which a proton or potassium gradient was expected to drive Ca^{2+} transport in the presence of the glycoprotein indicated clearly that this mitochondrial component cannot act as a simple ionophore. This conclusion has been arrived at also by Carafoli et al. [8] on the basis of experiments carried out with black phospholipid films [9]. Another approach we investigated was the incorporation of the glycoprotein into yeast mitochondria where Ca^{2+} transport is genetically absent. Also in this case no reproducible positive results were obtained. The most feasible experiment, namely to use as starting material liver mitochondria devoid of glycoprotein and incapable of Ca^{2+} transport, was very difficult to perform in that the compound is present in mitochondria in large excess and the removal of the last traces of it is virtually impossible.

Taking advantage, however, of the recent knowledge that the addition of oxaloacetate to mitochondria promotes Ca^{2+} efflux [5—7], a phenomenon accompanied by a conformational change of the Ca^{2+}-binding glycoprotein (Panfili, E., unpublished results), we have set up a system by which glycoprotein-depleted mitochondria may be prepared in the presence of oxaloacetate. Data are presented in this paper showing that using these particles trace amounts of purified Ca^{2+}-binding glycoprotein can fully restore active Ca^{2+} uptake.

Materials and Methods

Rat liver mitochondria were prepared from albino rats according to Schnaitman and Greenawalt [10]. Ca^{2+} uptake was measured using Arsenazo III as a free Ca^{2+} indicator [11]. The compound was purified before use according to Dipolo et al. [12]. Protein content of mitochondrial suspensions and submitochondrial fractions was estimated according to Gornall et al. [13]. Affinity chromatography-purified antibodies to the glycoprotein were prepared as already described [3]. The protein content of the antibody solution was estimated according to Waddell [14] as modified by Romeo et al. [15].

Subfractionation of mitochondria

Step 1: swelling of mitochondria. A mitochondrial pellet was suspended in a swelling medium consisting of 10 mM Tris-KH_2PO_4 buffer (pH 7.8), 1 mM EDTA, 2 mM Tris-oxaloacetate (pH 7.8). The final concentration of protein was adjusted to 15 mg/ml. A good homogenization was obtained by blowing the suspension up and down a 10-ml pipette. After standing on ice for 20 min,

the suspension was centrifuged at 100 000 × *g* for 1 h in a n.40 Spinco rotor. The supernatant was very carefully decanted. The surface of the pellet was rinsed with the medium described in step 2.

Step 2: washing. The pellet from step 1 was homogenized by a hand-operated Potter homogenizer in the same volume of a solution consisting of 10 mM Tris P_i buffer (pH 7.8), 1 mM EDTA, 15 μM rotenone, 10 mM sodium succinate and 0.5 mg/ml bovine serum albumin. The suspension was centrifuged as in step 1. Occasionally, when the Ca^{2+}-transporting activity of this second sediment was too high, step 2 was repeated exactly as described. The sediment, after rinsing the surface with 0.2 M mannitol containing 0.5 mg/ml albumin, was resuspended at approx. 50 mg/ml in the same medium.

Reconstitution experiments

Reconstitution experiments were carried out either with an osmotic supernatant or with pure glycoprotein. Since the first supernatant obtained from step 1 still contained oxaloacetate or its reduction product (malate), we used routinely a different osmotic supernatant obtained from mitochondria swollen as described in step 1, but in the absence of oxaloacetate. When the pure glycoprotein was used, this, prepared as described [16] and lyophilized, was dissolved in distilled water. This glycoprotein preparation was desalted by gel filtration on Bio-Gel P2 before lyophilization and did not contain salts in the final solution.

Results

Standard preparations of mitochondria could take up calcium aerobically, with succinate as substrate, at an initial rate of 678.4 ± 59.6 ng ion · min^{-1} · mg^{-1} protein. The first step of the subfractionation procedure resulted in a loss of soluble protein and the initial rate of calcium uptake was 723.0 ± 60.8 ng ion · min^{-1} · mg^{-1} protein. The increase in specific calcium transport capacity obviously is a reflection of protein loss. The particles obtained after the second step, on the contrary, show a drastic decrease in calcium transport capacity: the initial rate was 97.7 ± 15.9 ng ion · min^{-1} · mg^{-1} protein. It is important to recall here that the second step consists in a simple washing with a medium containing albumin, succinate, rotenone and EDTA. The composition of the medium has been designed so as to protect succinate oxidase acitivity which is, as expected, unaltered by the procedure. The decline in calcium-uptake ability may derive therefore either from uncoupling (unlikely to occur simply by washing) or by removal of the last traces of the calcium-binding glycoprotein. The following experiments rule out the former possibility. Trace a in Fig. 1 clearly indicates that the second mitochondrial sediment not only takes up Ca^{2+} at low rate, but it is also incapable of maintaining a high concentration gradient of Ca^{2+} across the membrane. On the other hand, trace b in the same figure clearly shows that addition of osmotic supernatant considerably enhances the rate of Ca^{2+} uptake and improves the capacity of the organelle to maintain a high concentration gradient of Ca^{2+} across the membrane. Even better results can be obtained by adding a combination of the supernatant and low amounts of the purified glycoprotein (trace c). Fig. 2 shows the concentra-

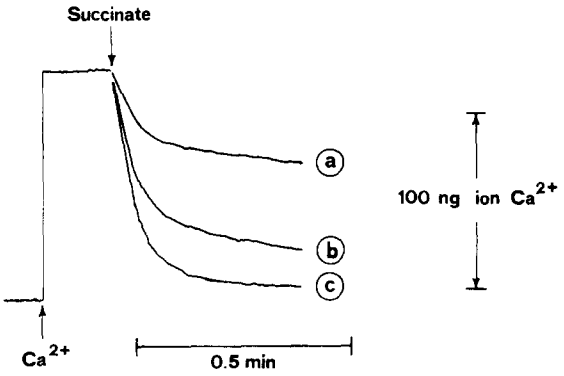

Fig. 1. Photometric traces of Ca^{2+} uptake obtained with glycoprotein-depleted particles. Experimental conditions: Spectrophotometric measurements (Phoenix Dual Wavelength Recording Spectrophotometer) using Arsenazo III as free calcium indicator. The reaction mixture contained in 3 ml 30 μM Arsenazo III, 0.3 mM Tris-phosphate (pH 7.8), 0.2 M mannitol, 2 mM acetate, 0.5 mg/ml albumin, 5 mM succinate, 15 μM rotenone. Calcium was added in aliquots of 100 ng ions. The experiments were carried out at room temperature. Adjustements at 40 μM calcium final concentration were necessary, when trace amounts of EDTA were present due to the addition of supernatant. Trace a: 2.3 mg particle protein; trace b: 2.3 mg particle plus 180 μg osmotic supernatant protein; trace c: 2.3 mg particle protein plus 180 μg osmotic supernatant protein plus 0.2 μg purified glycoprotein.

tion dependence of the stimulatory effect brought about by the osmotic supernatant on the rate of Ca^{2+} uptake. Half maximal stimulatory effect is obtained already at about 30 μg supernatant protein per sample (10 μg supernatant protein/mg mitochondrial protein). Obviously the effect could be due to the addition of an unknown factor present in the supernatant and different from the Ca^{2+}-binding glycoprotein. To rule out this possibility we have precipitated specifically the glycoprotein from the supernatant by preincubation with an

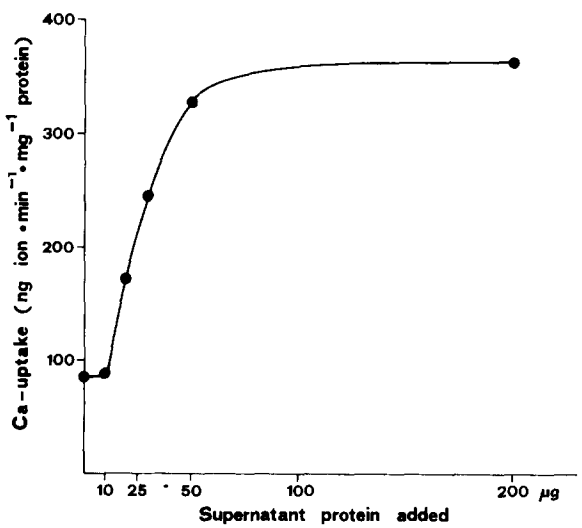

Fig. 2. Concentration dependence of the stimulatory effect brought about by the osmotic supernatant. Experimental conditions: as in Fig. 1; 3 mg particles per sample.

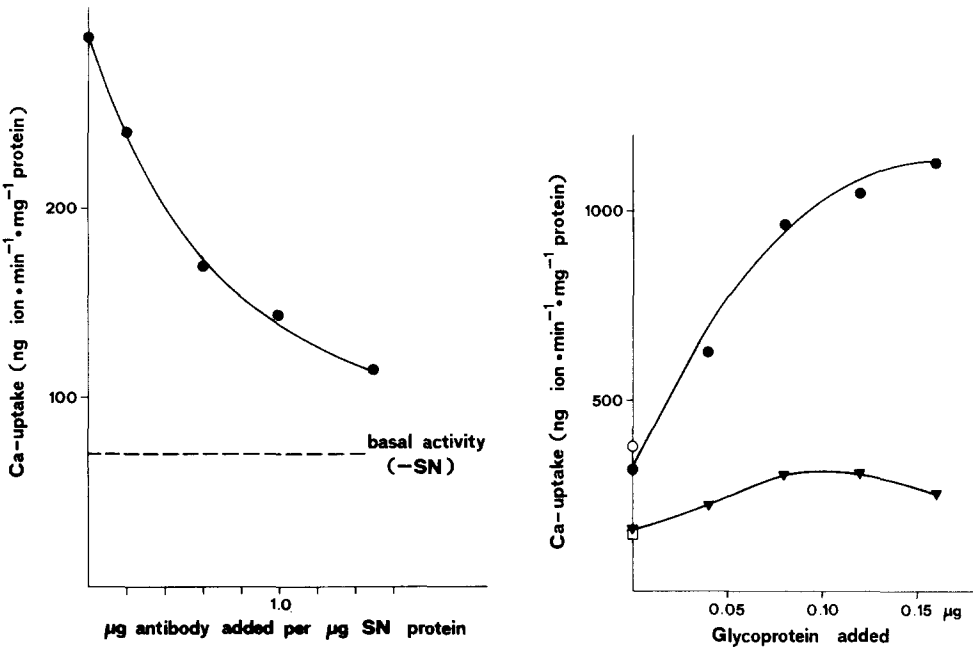

Fig. 3. Effect of specific antibodies on the reconstitutive capacity of the osmotic supernatant. Experimental conditions: samples of the osmotic supernatant were preincubated for 10 min at 30°C with the affinity chromatography-purified antibodies in the ratios indicated in the figure. The precipitate was removed by centrifugation in an Eppendorf Model 3200 centrifuge. Aliquots of 180 μg osmotic supernatant (SN) were included in the assay mixture described in Fig. 1 together with 3.4 mg glycoprotein-depleted particles.

Fig. 4. Reconstitution of Ca^{2+} transport in glycoprotein-depleted submitochondrial particles by the Ca^{2+}-binding glycoprotein. Experimental conditions: as in Fig. 1. 1.65 mg of particles per sample. □, basal activity without further addition; ○, basal activity in the presence of 130 μM Mg^{2+}; ▲, activity in the presence of 20 μg osmotic supernatant per mg mitochondrial protein plus the glycoprotein; ●, activity in the presence of 130 μM Mg^{2+} plus the calcium-binding glycoprotein.

affinity chromatography-purified antiglycoprotein. After centrifugation the supernatant has been added to the particles and the Ca^{2+} uptake measured in the usual way. The results of this experiment are reported in Fig. 3. Data clearly show that the effect is due to the presence in the supernatant of the glycoprotein and can be therefore prevented by very low amounts of specific antibody. Even more convincing evidence can be obtained by experiments aimed at reconstituting the system using particles plus pure glycoprotein. Fig. 4 shows data of an experiment in which this approach has been attempted. The activity of the original preparation was very low (open square). A slight stimulation can be obtained by addition of suboptimal amounts of osmotic supernatant (in this case 20 μg/mg) (black triangle at the origin). This effect can be further enhanced by addition of increasing concentrations of the purified glycoprotein (lower curve). It is important to known that the glycoprotein in the absence of traces of the supernatant shows very soon a strong inhibitory effect, an effect only partially counteracted by the supernatant. We suspected that the phenomenon involved the presence in the supernatant of the

endogenous Mg^{2+} lost during the preparation (EDTA is present in the swelling medium). We have therefore tested the influence of this cation on the reconstitution. The optimal concentration was found to be 130 μM. At this concentration the residual Ca^{2+}-uptake activity of the mitochondrial preparation is enhanced (open circle at the origin). Under these conditions, however, the glycoprotein alone may restore the full capacity for Ca^{2+} transport (upper curve). Under these conditions the specific rate of Ca^{2+} uptake approaches the theoretical value which can be calculated on the basis of a loss of approx. 30% of the mitochondrial protein during fractionation.

Discussion

The data reported in this paper show that rat liver mitochondria can be depleted of their Ca^{2+} transport capacity by removal of the Ca^{2+}-binding glycoprotein. The procedure described to achieve this includes a crucial step consisting in swelling of mitochondria after addition of oxaloacetate in the presence of EDTA (a reagent known to promote release of the Ca^{2+}-binding glycoprotein in soluble form [17,18]). All previous experiments in which oxaloacetate was absent invariably resulted in preparations still fully capable of Ca^{2+} transport. This result implies that the glycoprotein is present in large excess in mitochondria and by no means is the rate-limiting factor in Ca^{2+} uptake. This consideration explains also the finding that mitoplasts and other submitochondrial particles devoid of outer membrane have been reported to display active Ca^{2+} transport [8,19,20—23]. In addition, these data suggest that NAD^+-bound Ca^{2+}-binding glycoprotein is much less firmly bound to the inner membrane so that it can be removed almost quantitatively. Incidentally, the inclusion of succinate and rotenone as well as albumin in the washing medium was found to be the optimal condition to maintain the full succinate oxidase activity of the subfraction. Our reconstitution experiments, on the other hand, clearly show that the only necessary ingredient to restore the Ca^{2+} transport in glycoprotein-depleted mitochondria is the isolated Ca^{2+}-binding glycoprotein itself, provided an optimal concentration of Mg^{2+} is re-added. In our opinion it is at least doubtful that an additional component with ionophoretic activity exists in the inner mitochondrial membrane. If such a compound, in fact, exists under the conditions of our experiments, it should not be able to operate unless the glycoprotein is added. The principle of the least number of assumptions makes us think that an ionophore does not exist provided the experimental data can be accounted for without it. Experimental data so far collected in model systems suggest that the glycoprotein does not behave as an ionophore [8]. This finding, far from excluding an active participation of the glycoprotein in Ca^{2+} transport, may simply suggest that during Ca^{2+} translocation the glycoprotein undergoes an energy-consuming conformational transition which may take place only in the inner mitochondrial membrane under appropriate conditions of membrane potential and/or conformational state of surrounding components. These conditions may not be easily reproduced in model systems but it cannot be excluded that, in the future, reconstitution in a simplified model membrane can be achieved.

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