INTERACTIONS OF A MITOCHONDRIAL Ca^{2+}-BINDING GLYCOPROTEIN WITH LIPID BILAYER MEMBRANES

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1. Introduction

Attempts to resolve the molecular system for the transport of Ca^{2+} across the mitochondrial membrane have led to the isolation of a glycoprotein with high affinity for Ca^{2+} [1–3]. The glycoprotein appears to be associated with the mitochondrial membranes with various degrees of tightness. Part of it is easily solubilized into aqueous media by mild hypotonic shocks applied to mitochondria, part of it requires treatment with the glycoprotein-specific chaotropic agent lithium-di-jodosalycilate (LIS) [4]. The chemical and functional properties of the osmotic and LIS-protein are very similar, but one possible explanation for their different dissociability from the membrane environment is their different phospholipid content.

The functional properties of the isolated glycoprotein (e.g., affinity for Ca^{2+}, sensitivity to inhibitors, absence from Ca-negative mitochondria, etc.) are closely related to those of the high-affinity Ca^{2+} binding sites, and of the energy-linked transport of Ca^{2+}, of intact mitochondria. The participation of the glycoprotein in the process of Ca^{2+} translocation in intact mitochondria seems thus a likely possibility. Whether the glycoprotein acts as a mobile carrier which translocates Ca^{2+} across the apolar region of the membrane, or whether it forms a fixed, Ca^{2+}-specific tunnel across the entire width of the membrane, is open to question. The highly polar character of the glycoprotein is not per se a sufficient reason to exclude the mobile carrier mechanism: the solubilization of highly polar proteins like cytochrome *c*, or rhodopsin, into apolar solvents has recently been achieved [5,6] and has been shown to depend on the formation of ion pairs with Ca^{2+} and acidic phospholipids. The alternative possibility should also be considered that the glycoprotein is not directly involved in the transmembrane translocation of Ca^{2+}, but acts as a Ca^{2+}-specific, membrane recognition site which operates in series with other sites directly involved in the translocation.

To test these possibilities, experiments have been carried out on lipid bilayers. It has been found that the conductance of lecithin bilayers, formed in the presence of Ca^{2+}, increases considerably upon addition of the glycoprotein to the aqueous medium.

2. Materials and methods

The glycoprotein was dissociated from rat liver mitochondria using osmotic shocks under the conditions described in [1] and [3]. The treatment with lithium di-jodosalycilate, used to extract additional glycoprotein, was applied to mitochondria from which the 'osmotic' glycoprotein had already been separated, and is described in [7]. The glycoprotein was purified from the 'osmotic' or 'LIS' extracts by preparative polyacrylamide gel electrophoresis, as described in [1,3] and in [2] for the case of beef liver mitochondria.

Lipid bilayers were obtained from a 50 mg/ml solution in *n*-decane of purified egg lecithin, a generous gift of Dr. G. Rialdi of Vevy (Genoa, Italy). The black films, 1.75 mm in diameter, were formed on a lucite septum separating two identical solutions of various monovalent or divalent chloride salts. In most experiments the salt concentration in solution

ranged between 1 mM and 4 mM. Measurements of membrane resistance were made by applying known potential steps across a precision resistor (10^{10}, 10^9 or 10^8 ohms) in series with the membrane, and measuring the resulting potential drop across the membrane. Ag–AgCl electrodes were used both as current and as potential electrodes, since no significant effect of electrodes polarization could be detected with the currents normally employed. The electric potential across the membrane was measured with a high impedance electrometer (Keytley 610 BR); in all measurements it was kept between ± 20 mV. A switch allowed to insert in the measuring system the resistor in series which had the resistance value closer to the membrane resistance. Previous to the addition of the glycoprotein the resistance of the black films varied usually between 2×10^8 and 4×10^8 ohms/cm^2. Lipid bilayers with lower initial resistances were usually discarded. The effect of the glycoprotein or of other substances was studied by adding small equal amounts of concentrated solutions on both sides of the membranes, and stirring with two small, Teflon-coated, magnets at the bottom of the two compartments separated by the membrane.

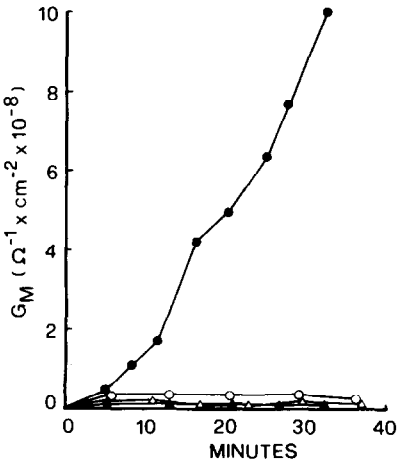

Fig. 1. Change in conductance of a lipid bilayer, upon addition to the aqueous media of the lis-Ca^{2+}-binding glycoprotein (–•–). The glycoprotein was added at equal concentrations (1.48×10^{-7} M) on both sides of the bilayer at time zero. The bathing solutions contained 4 mM $CaCl_2$ and 1 M Tris–Cl, pH 8. For comparison the effects of the addition of 5×10^{-7} M horse radish peroxidase (–○–), 1.25×10^{-7} M immunoglobulin G (–▲–), 4.22×10^{-7} M ovalbumin (–△–) to the same type of lipid bilayer preparation are shown.

3. Results

When added to lecithin bilayers in the presence of 4 mM Ca^{2+} and no other cations, the glycoprotein induces an evident increase in the electrical conductance of the artificial membrane. The magnitude of the response was somewhat variable, perhaps in relation to the age and functional properties of the glycoprotein preparation, but in general two different forms of behaviour could be identified.

Either there was a relatively small and rapid increase in the conductance (half-time of the order of 2–4 min) followed by a slower continuous rise which in some cases lasted up to 2 hr, or the conductance rapidly rose to very high values after the addition of the glycoprotein, until the bilayer broke in 30–40 min. It was also discovered that, as a rule, the LIS glycoprotein showed a tendency to induce effects of the second type and was quantitatively more effective than the osmotic glycoprotein. Given the variable response of the glycoprotein, in the experiments to be reported the effects induced by the different agents were compared on preparations which reacted homogeneously. Fig. 1 shows an experiment in which the LIS-glycoprotein has been used in the presence of 4 mM Ca^{2+}. The electrical conductance of the bilayer before the addition of 10^{-8} M glycoprotein was 5×10^{-9} Ω^{-1} cm^{-2}; after the addition of the glycoprotein it rose in 30 min. to about 10^{-7} Ω^{-1} cm^{-2} The figure also shows that three more commercially available glycoproteins, added to the aqueous medium in the presence of Ca^{2+}, had no effect on the conductance of the bilayer. Also without effect were other proteins like hemoglobin, cytochrome *c*, bovine serum albumin.

In the experiment shown in fig. 2, the osmotic glycoprotein has been used, and the effect seen with Ca^{2+} has been compared with that seen in the presence of monovalent cations. In the presence of all monovalent cations tested, the glycoprotein induced an increase of the electrical conductance. The increase was, however, small, when compared with that observed in the presence of the same concentration of Ca^{2+}, and was limited to the first few minutes

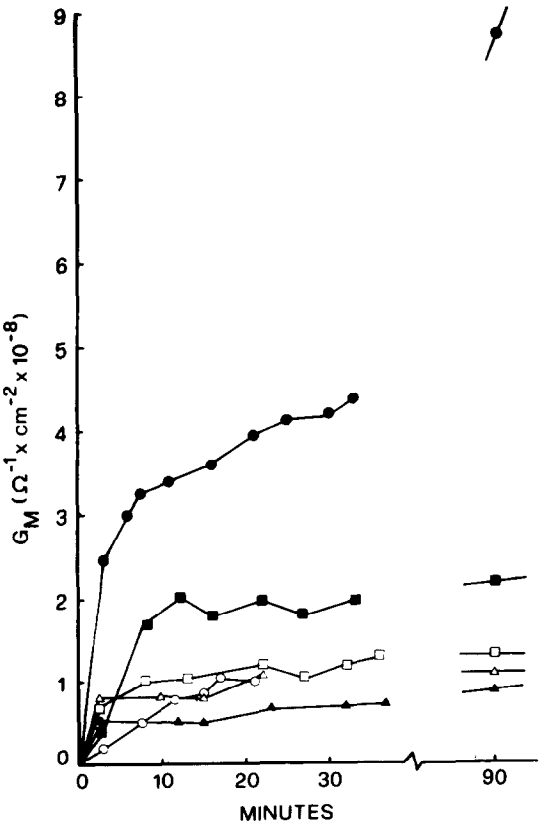

Fig. 2. Comparison of the changes in membrane conductance, produced by the addition at time zero of 1.92×10^{-7} M Ca^{2+}-binding glycoprotein, to bathing solutions containing identical concentrations (4 mM) of monovalent cations or Ca^{2+}. All the curves reported in the figure were obtained with the same batch of osmotic glycoprotein and within 3 days of time between each other (–●–), Ca^{2+}; (○), K^+; (–▲–), Na^+; (–△–), Li^+; (–■–), Rb^+; (–□–) Cs^+.

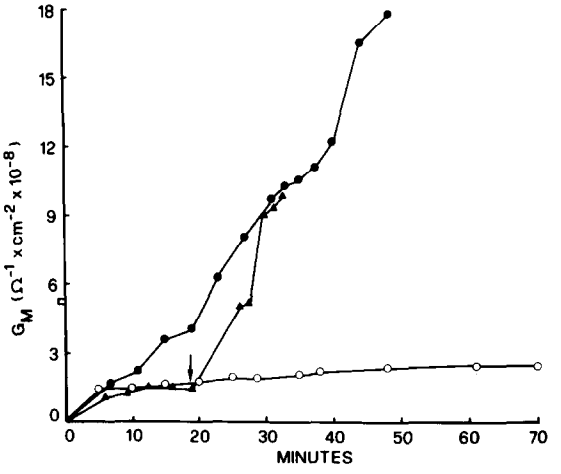

Fig. 3. Superimposition of the effects induced by addition of 1.48×10^{-7} M LIS protein, in the presence of KCl and $CaCl_2$. (–●–), Membrane conductance change in the presence of 4 mM $CaCl_2$; (–○–), membrane conductance change in the presence of 4 mM KCl; (–▲–), effect of the addition of 4 mM $CaCl_2$ to a membrane exposed to the glycoprotein in the presence of 4 mM KCl.

after the addition of the glycoprotein. By contrast, the figure shows clearly that the conductance in the presence of Ca^{2+} rose progressively to values which after 90 min were 4 to 10 times higher than in the presence of monovalent cations. As a further extension of the experiment shown in fig. 2, fig. 3 shows that Ca^{2+}, added to the solution after the glycoprotein had induced the usual small increase in conductance in the presence of 4 mM K^+, rapidly shifted the conductance curve to the values observed in the presence of Ca^{2+} alone. In preliminary experiments it has also been found that other divalent cations, particularly Sr^{2+}, also induced increases of the electrical conductance in the presence of the glycoprotein. The responses observed in the presence of Mg^{2+} were however very variable: the reasons for this variability are currently under investigation.

Fig. 4 shows that Ruthenium Red, which specifically inhibits the reaction of the isolated glycoprotein with Ca^{2+} [1–3], abolishes the effect of the glycoprotein on the conductance of the artificial membrane. The other inhibitor of the binding of Ca^{2+} to the isolated glycoprotein, La^{3+}, was also tested. The results obtained were however inconclusive, since La^{3+} had a direct effect on the bilayer itself, inducing an evident thickening of the artificial membrane. It was also found that the effects of the glycoprotein are optimally seen at concentrations of Ca^{2+} in the mM range. Upon increasing the Ca^{2+} concentrations to values of 40 to 50 mM, the effects on the conductance are gradually reversed, most likely due to the precipitation of the glycoprotein at high Ca^{2+} concentrations. Precipitation of the glycoprotein from solutions containing elevated concentrations of Ca^{2+} has already been observed in our laboratories, and has even been used in attempts to simplify the purification procedure.

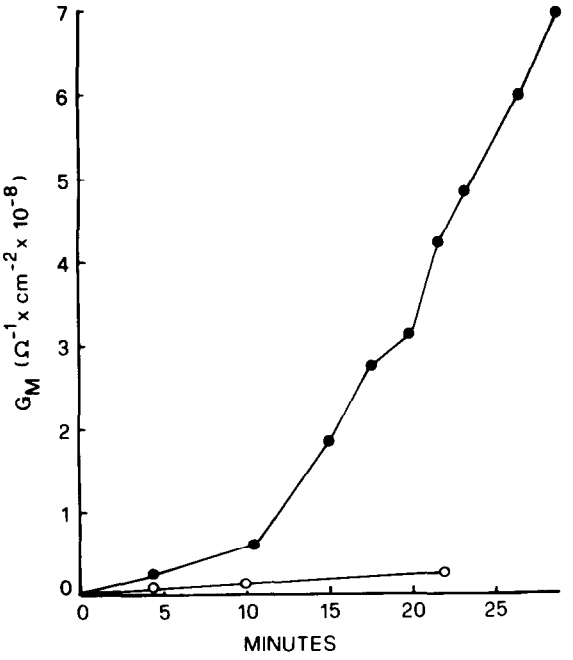

Fig. 4. The inhibitory effect of Ruthenium Red. The two curves show the change in membrane conductance following the addition of the same glycoprotein to a membrane preparation containing 4 mM $CaCl_2$ (–•–), or to a similar membrane preparation to which 10^{-6} M Ruthenium Red was added 10 min before the glycoprotein –o–.

4. Discussion

The importance of reconstituting a transport activity using isolated membrane proteins and artificical membranes cannot be overemphasized. It is perhaps the most direct and valid test for the involvement of the isolated protein in the transport system of the intact membrane. Following the original observation of Storelli et al. [8] on the reconstitution of a sucrase-mediated sugar transport in lipid bilayer membranes, other reports of successful reconstitution of transport (or binding) activities have recently appeared [9–12]. The reconstitution of the mitochondrial transport activities appears particularly challenging, in view of the peculiar complexity of the mitochondrial membrane, and of the fact that, at least in the case of Ca^{2+}, the transport system has probably more than one essential component [7,13]. The experiments described here have shown that the Ca^{2+}-binding glycoprotein of the mitochondrial membrane increases the electrical conductance of the lipid bilayers in a reaction which is sensitive to Ruthenium Red, and thus repeats at least one of the important characteristics of the binding and transport process in intact mitochondria. Several possibilities could at this point be considered. The experiments reported could be taken to indicate that the glycoprotein acts as a mobile or immobile carrier, which specifically transfers Ca^{2+} across the lipid bilayer. In view of the recently obtained solubilization of other polar proteins in apolar solvents [5–6] there can be no theoretical objection against this possibility. Indeed, preliminary experiments in our laboratories indicate that the LIS-glycoprotein can be solubilized in apolar solvents. However, at the present stage the conclusion that the glycoprotein actually transfers Ca^{2+} from one side of the artificial membrane to the other, would be premature, since neither fluxes of Ca^{2+} across vesicular artificial membranes, nor membrane potentials due to Ca^{2+} gradients across lipid bilayers, have yet been measured.

One could also consider the possibility that the glycoprotein does *not* easily become associated with the bilayer unless Ca^{2+} is present, much as is the case for cytochrome *c* in the experiments by Gitler and Montal [5] or of rhodopsin in those of Montal and Korenbrot [6]. The function of Ca^{2+} would in this case be that of permitting the interaction bilayer–glycoprotein; once associated with the bilayer, the protein could discharge Ca^{2+} to the opposite side, and then accept more Ca^{2+} to avoid rediffusing back out of the bilayer. It is actually unnecessary to postulate that Ca^{2+} itself is transferred across the artificial membrane. However, even if the current across the bilayer membrane were not carried by Ca^{2+} ions, Ca^{2+} would in this case still have the essential role of permitting the association of the glycoprotein with the bilayer membrane.

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