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Original Article

Solubilization and Reconstitution of the Mitochondrial Peptide-Sensitive Channel

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In addition to the voltage-dependent anion channel (VDAC), mitochondrial outer membranes contain a cationic channel of large conductance, which is blocked by a mitochondrial addressing peptide (peptide-sensitive channel, PSC). Bovine adrenal cortex mitochondria were solubilized in 1.5% octyl β-glucoside, and membrane vesicles were reconstituted by slow dilution with a low ionic strength buffer. The reconstituted vesicles contained a functional channel possessing the electrical characteristics of the cationic channel, including its sensitivity to the mitochondrial addressing peptide. Important features of the described protocol are the nature of the detergent, its concentration, and the addition of glycerol during the whole procedure. No solubilization could be observed in the presence of cholate.

KEY WORDS: Ionic channel; mitochondria; addressing peptide; membrane solubilization.

INTRODUCTION

In recent years, methodological progress has resulted in an increase of the number of ionic channels described in mitochondria (Schein *et al.*, 1976; Sorgato *et al.*, 1987; Tedeschi *et al.*, 1987; Thieffry *et al.*, 1988; Dihanich *et al.*, 1989; Petronilli *et al.*, 1989; Moran *et al.*, 1990; Inoue *et al.*, 1991). Using the "tip-dip" technique, we have characterized a cationic channel of large conductance (Thieffry *et al.*, 1988). This channel is localized on the outer membrane (Chich *et al.*, 1991), but it is different from the voltage-dependent anion channel (VDAC). This conclusion is supported by the observation that the cationic channel of a yeast porin-deficient mutant is not different from that of the wild type (Fèvre *et al.*, 1990). An interesting property of the cationic channel is that it is blocked by a 13-residue peptide possessing the sequence of the N-terminal extremity of cytochrome *c* oxidase subunit IV precursor (Henry *et al.*, 1989). This peptide-sensitive channel (PSC) might thus be a candidate for the translocation of mitochondrial protein precursors synthesized in the cytoplasm (Verner and Schatz, 1988; Neupert *et al.*, 1990).

The characterization of a membrane protein implies its solubilization and its purification in the solubilized state. This approach has been followed successfully to identify the VDAC as the major mitochondrial porin (Colombini, 1983). As a preliminary step toward this goal, we present in this communication the solubilization of the PSC by octyl β-glucoside in a fully active form, which can be reconstituted in lipid bilayers.

MATERIALS AND METHODS

Materials

Peptide M (MLSLRQSIRFFKY, pCOX IV (1–12)Y) was synthesized by Neosystem (Strasbourg, France). Octyl β-glucoside (*n*-octyl β-D-glucopyranoside) was purchased from Sigma (St. Louis, Missouri). Bovine adrenal cortex mitochondria were prepared as described previously (Chich *et al.*, 1991).

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Solubilization and Reconstitution Procedure

Frozen mitochondria were resuspended at a concentration of 8 mg of protein/ml in 2 ml of buffer containing 50 mM NaCl, 1 mM EDTA, 10% glycerol (v/v), 1.25 μg/ml leupeptin, 2 μg/ml pepstatin, and 20 mM Hepes, pH 7.3 (buffer A). A 10% octyl β-glucoside solution was added under vigorous stirring at room temperature to give the indicated final concentration. Phenylmethylsulfonyl fluoride (PMSF) was then added to a final concentration of 1 mM. The mixture was kept at 0°C for 30 min and then centrifuged for 1 h at 130 000 $\times$ g. The pellet was resuspended in 1.5 ml of 0.15 M NaCl and 20 mM Hepes, pH 7.3. To reconstitute the solubilized material, the supernatant (1.8 ml) was diluted 40 times by slow addition of buffer A over a 100-min period of time using a peristaltic pump and the particulate material was collected by centrifugation at 130 000 $\times$ g for 3 h. The pellet thus obtained was resuspended in 0.6 ml of 0.15 M NaCl and 20 mM Hepes, pH 7.3.

To test the effect of cholate, mitochondria (3 mg of protein/ml) were resuspended in 100 mM KCl and 20 mM Hepes, pH 7.3, containing 1 mM EDTA, 1.25 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 mM PMSF (2 ml final volume). A concentrated cholate solution (20% in the same buffer) was added dropwise to 1.5% final concentration. After 30 min at 0°C, the mixture was centrifuged as described above. To remove the detergent from the solubilized material, the supernatant was dialyzed against 3 litres of the same buffer for 48 h, with several buffer changes.

Assay of the Fractions

The electrical activity was assayed by the "tip-dip" method after formation of a bilayer at the tip of a microelectrode (Thieffry *et al.*, 1988; Chich *et al.*, 1991). The pipette and the bath contained 150 mM NaCl, 1 mM $MgCl_2$, and 20 mM Hepes adjusted to pH 7.3 using NaOH. For selectivity measurement, 90% of the bath NaCl was replaced by an isoosmotic equivalent of mannitol. The sample to be tested was first fused with phosphatidylserine:phosphatidylethanolamine liposomes (3:7), as described. Comparisons of various fractions were performed at constant protein concentration in the proteoliposomes, generally 13 or 26 μg of protein/mg phospholipid. Protein was estimated by the technique of Lowry *et al.* (1951).

RESULTS

Solubilization of Mitochondrial PSC

The electrical activity of adrenal cortex PSC is shown in Fig. 1, left. The relative abundance of this channel can be quantified by the frequency of observation of the characteristic electrical activity of a given fraction, defined as the total number of channels found divided by the total number of trials (Chich *et al.*, 1991).

Mitochondrial membranes were progressively solubilized by addition of octyl β-glucoside in low-salt buffer (Table I). At a final concentration of 1.5% ($\approx$ 50 mM), about 65% of the initial mitochondrial proteins were found in the supernatant. Since 15% of the initial proteins were found in the supernatant in the absence of detergent, this corresponds to a solubilization yield of 50%. This figure was not significantly increased at 2% detergent concentration. Protein solubilization was accompanied by a loss of channel activity in the pellet (Table I).

After slow dilution of the solubilized material by a detergent-free buffer and centrifugation, the pellet, which contained 14% of the solubilized material, was examined by electron microscopy following negative staining. It appeared composed of spherical particulate material, approximately 100 nm in diameter (data not shown). When this material was incorporated in proteoliposomes, the electrical activity characterizing the PSC was observed (Fig. 1, right and Table I), thus indicating that octyl β-glucoside solubilized the channel in an active form.

A different result was observed when cholate was used in place of octyl β-glucoside. Proteins were solubilized with a similar yield; however, no electrical activity could be detected after removal of the detergent by dialysis. Such a failure might have originated from either a lack of solubilization or an inactivation of the solubilized material. The former hypothesis is more likely since the solubilization pellets were found to be active.

Functional Integrity of the Reconstituted PSC

The electrical activity observed by the adopted procedure using 1.5% octyl β-glucoside had the characteristics previously reported for the PSC (Thieffry *et al.*, 1988). The conductance (two jumps of 220 pS suggesting a dimeric structure), the kinetics (Fig. 1), and the potential dependence (Fig. 2) of the native and of the reconstituted channel were similar.

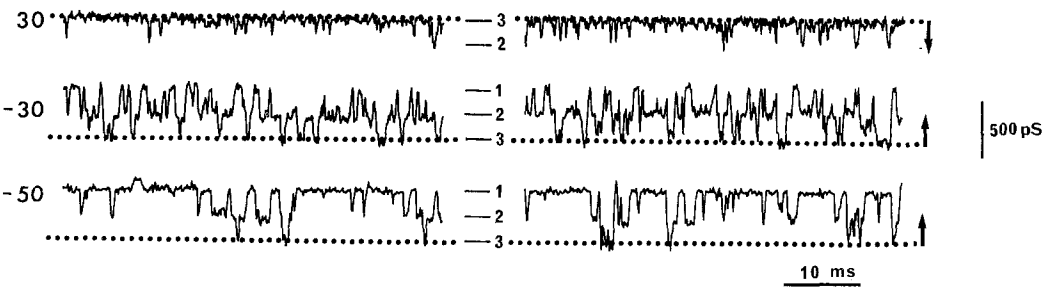

Fig. 1. Electrical activity of a native channel (left) and of a reconstituted channel (right). Samples of current fluctuations recorded at 30, −30, and −50 mV from a bilayer formed at the tip of a microelectrode from a surface monolayer. For the native channel, the bilayer was derived from a monolayer enriched in crude mitochondrial membranes. For the reconstituted channel, it was derived from a monolayer containing the membrane components solubilized using 1.5% octyl β-glucoside as described in the text. The three main levels of increasing conductance of the channel are indicated at each potential by numbers 1, 2, and 3. For this figure and the following ones, the channel maximum conductance is indicated in each current trace by a dotted line. At the end of the trace, an arrow starting from this line points to the direction of channel closures. Signal filtered at 2.5 kHz and sampled at 10 kHz.

The reconstituted channel was, like the native channel (Thieffry *et al.*, 1988), sensitive to trypsin which eliminated the voltage-dependent gating mechanism of the channel (data not shown). Finally, the reconstituted channel was sensitive to peptide M irrespective of the orientation of the channel in the bilayer. The transfer of such a channel in a bath containing 50 μM peptide increased the number of rapid closures (Fig. 3), as previously described (Henry *et al.*, 1989).

When the concentration of octyl β-glucoside was increased to 2%, two modifications were noted. First, the percentage of active patches was reduced from 70% to 35%. Second, among the channels which were then observed, only 25% had the characteristics of PSC. Samples of electrical activities recorded in other cases are shown in Figs. 4 and 5. The activity most often observed is shown in Fig. 4. Following a voltage jump from 0 to positive potentials, the channel remains open with only brief (< 1 ms) closures, then closes at a level of about 200 pS below the maximum conductance. Generally, it does not reopen spontaneously, but recovers immediately its full conductance following reversal of the transmembrane potential. The long-lasting closures develop faster at potentials of larger magnitude. At negative potentials, bursts of brief closures are observed and long-lasting closures of 200 pS occur only rarely. The channel may

Table I. Protein Content and Channel Activity as a Function of Octyl β-Glucoside Concentration

Octyl β-Glucoside (%)	0	1	1.5	2
Protein in the pellet[a]	85	64	36	35
Activity in the pellet[c]	69	75	9	3
Protein in the supernatant[a]	15	36	64	65
Protein in the reconstituted pellet[b]	—	1.2	14	14
Activity in the reconstituted pellet[c]	—	0	70	35
PSC frequency[d]			100	25

[a] Percentage relative to the material before octyl β-glucoside addition.
[b] Percentage relative to the solubilized material.
[c] 100 × number of channels/number of trials.
[d] 100 × number of PSC channels/number of channels.

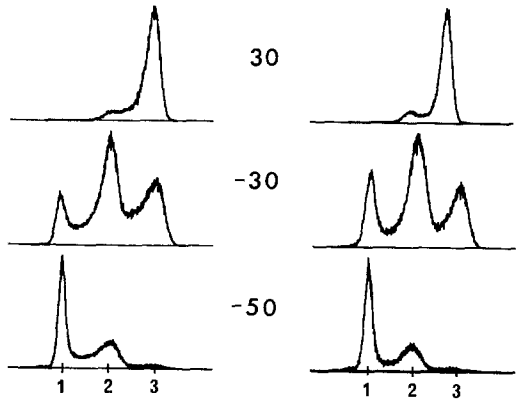

Fig. 2. Voltage dependence of the native channel (left) and of the reconstituted channel (right) shown in Fig. 1. Conductance histograms were derived at each potential from current histograms computed over periods of at least 9 secs. Numbers 1–3 in the abcissa refer to the three main conductance levels.

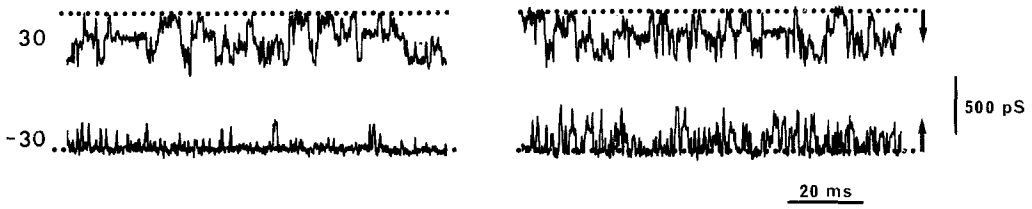

Fig. 3. Blockade of a reconstituted channel by a mitochondrial addressing peptide. Left: activity recorded at 30 and −30 mV from a bilayer in which a reconstituted channel was inserted upside down when compared to the channel shown in Fig. 1. Right: activity recorded at the same potentials after transfer of the tip to a bath containing 50 μM peptide M. The blockade, characterized by more frequent brief transitions to the lowest conductance levels, is stronger at negative potential. Filtering and sampling rate as in Fig. 1.

be observed with the reverse orientation in the bilayer. In the presence of a tenfold NaCl gradient, the reversal potential was shifted by about −30 mV, indicating some selectivity for cations ($p_{Na}/p_{Cl} \approx 4.5$). No effect could be detected when the channel was exposed to 50 μM peptide M.

In other patches, we identified a 100-pS voltage-dependent channel with fast kinetics (Fig. 5). Slower conductance changes of about 25 pS were also found. They were not studied in detail and we do not know whether they reflect the existence of a distinct channel or are substates of the 100-pS channel.

An important feature of the solubilization procedure is the addition of 10% glycerol to the solubilization buffer. In the absence of glycerol, the reconstituted pellet obtained either by rapid dilution or by dialysis of the detergent had an atypical activity, not reproducible from record to record and often changing with time within the same record. Jumps ranging from 30 to 560 pS in 0.15 M NaCl were detected. In some patches, activities characterized by jumps of about 100 and 200 pS were observed, but they never exhibited the typical voltage dependence of PSC.

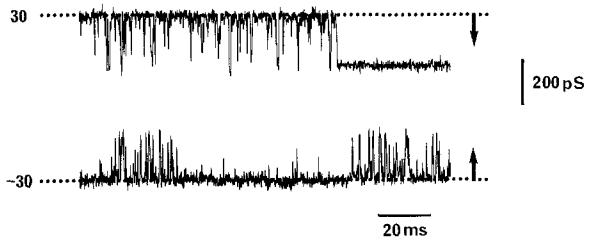

Fig. 4. Electrical activity of a channel, different from PSC, reconstituted following solubilization by 2% octyl β-glucoside and exhibiting 200 pS conductance jumps. This form is the most often found when octyl β-glucoside is used at this concentration. Signal recorded at 30 and −30 mV, filtered at 2.5 kHz, and sampled at 10 kHz.

DISCUSSION

Among the various procedures tested, the most efficient one was derived from that previously described for the solubilization of functional rough endoplasmic reticulum (Yu *et al.*, 1989). The important features of this protocol are the nature of the detergent, its concentration, and the addition of glycerol during the whole procedure.

From a quantitative point of view, this process might not be very efficient since the electrical activities of the reconstituted pellet (in the best case, i.e., using 1.5% detergent) and of the initial material, measured on the same amount of protein, are comparable, whereas only 14% of the solubilized proteins are found in the reconstituted pellet. Such figures have to be compared with some caution because assay of the electrical activity is difficult. They are likely to reflect the "fragility" of the electrical activity.

In fact, the fragility of the channel is indicated by two types of experiments. First, in the absence of glycerol, some activity was observed, but its characteristics were so variable and so different from that of the initial PSC that it was difficult to conclude as to their origin. Glycerol might thus protect the protein against the deleterious effects resulting from the change of environment induced by the detergent (Yu *et al.*, 1989). Second, even in the presence of glycerol, the highest detergent concentration we used induced both a decrease in the number of active patches and modifications of the observed electrical activities. Most of the channels differing from typical PSC which were then recorded had conductance jumps of the same amplitude as PSC, and their selectivity was about the same. However, they differed from the native channels by both their voltage dependence and their sensitivity to peptide M. It is thus likely that this activity is carried

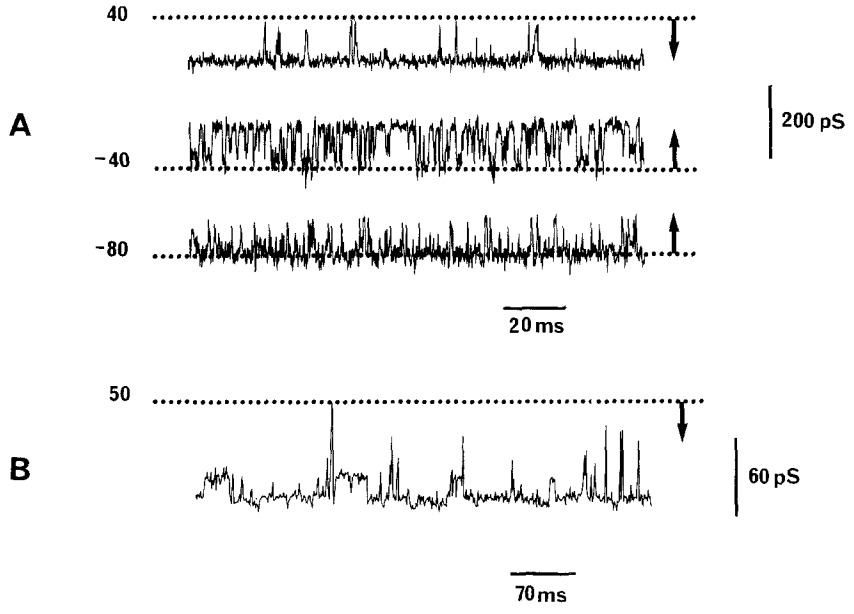

Fig. 5. Other activities recorded following reconstitution after solubilization using 2% octyl β-glucoside. A: Voltage dependence of a 100-pS channel. A 25-pS channel is also present in the bilayer. Current traces recorded at 40, −40, and −80 mV. Signal filtered at 2.5 kHz and sampled at 10 kHz. B: Current trace recorded from the same bilayer at 50 mV. The signal was filtered at 500 Hz and sampled at 1 kHz in order to show the 25-pS activity.

by the protein(s) carrying the PSC activity, but that the native structure was lost and could not be reconstituted.

Two types of channels that we recorded in fractions reconstituted after solubilization by 2% octyl β-glucoside (the 100-pS voltage-dependent channel and the 25-pS channel) remind us of channels previously described by Moran and colleagues using patch clamp of giant liposomes enriched in different fractions from mitochondrial membranes (Moran *et al.*, 1990). The reason why we never recorded these channels in fractions from either native of 1.5% solubilized membranes remains obscure. However, it is noteworthy that the observation of inner membrane channels (including a 100-pS channel) in patch-clamped mitoplasts has also been shown to depend on experimental protocols (Kinnally *et al.*, 1991). It is thus likely that the poor consensus existing at the present time about the repertoire of mitochondrial channels results from difficulties in maintaining the integrity of complex structures, even when recordings are done from native membranes.

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