Methodology for assaying iodide conductance in proteoliposomes: specific induction by thyroid membrane protein

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A sensitive assay is developed to assess the existence of an iodide channel in a fraction of solubilized membrane proteins. This step is critical when considering various procedures for purification of this channel. Sodium cholate is used as a detergent as it does not denature the iodide channel. A simple and rapid method involving gel-filtration chromatography is used simultaneously to remove the detergent and to adjust the buffer composition, before protein insertion into liposomes. The presence of an iodide channel is investigated by measuring the iodide conductance of these proteoliposomes at 4 °C. An outward iodide gradient is set up across the proteoliposomal membrane by anion-exchange chromatography, allowing uptake of radiolabelled iodide. This uptake is conductive as it is abolished by valinomycin in the presence of potassium. It is specifically mediated by a thyroid plasma-membrane protein inserted into liposomes, as its

denaturation before insertion totally abolished uptake. It was observed only within a well-defined fraction of thyroid membrane proteins collected by size-exclusion chromatography (molecular mass between 100 and 200 kDa). Furthermore, it was not observed with other membrane proteins such as ileal brush-border-membrane proteins or bacteriorhodopsin. Like many anion channels, this conductance was also inhibited by *N*-phenylanthranilic acid. Optimization of the assay is described, validating the measurement of conductive iodide uptake at 30 s by proteoliposomes reconstituted in a ratio of 10 µg of protein to 90 µg of lipid, with an outward iodide gradient (KI 15 mM inside and 1 µM outside). This assay provides a test of the biological activity of the iodide channel at each step of the purification; it can be applied to any anionic channel.

INTRODUCTION

Channels are integral membrane proteins which in their open state transport about one million ions per second [1]. This high transport capacity probably explains their usual low abundance in terms of percentage of total membrane protein. Obviously this complicates the channel purification which generally requires several sequential steps. After each of them, the function of the channel under investigation should be appropriately tested, and, to this end, reconstitution of the transport protein into proteoliposomes is proposed.

With the ultimate goal of isolating an iodide-selective channel presumably located within the apical membrane of thyrocytes [2], we have solubilized plasma-membrane proteins from bovine thyroid and devised a simple and rapid procedure for their reconstitution into liposomes. The optimal conditions for testing the function of the iodide channel were determined.

MATERIALS AND METHODS

Materials

Fresh bovine thyroid and ileum were obtained from the local slaughterhouse. Na¹²⁵I (carrier-free) was purchased from New England Nuclear (Wilmington, DE, U.S.A.). Dowex 1 (× 10; 50–100 mesh; Cl[−] form) was obtained from Fluka Chemie (Buchs, Switzerland). Prepacked columns PD-10 (Sephadex G-25M), Sephadex G-50M and Superdex 200 HR 10/30 column were supplied by Pharmacia (Uppsala, Sweden). Egg phosphatidylcholine (PC) was generously given by Lipoid KG (Ludwigshafen, Germany) and *N*-phenylanthranilic acid (DPC)

by Professor R. Greger, University of Freiburg, Freiburg, Germany. CHAPS, *n*-octyl glucoside and BSA were purchased from Sigma (St. Louis, MO, U.S.A.); sodium cholate was from Aldrich Chemie (Bornem, Belgium). All other chemicals were of the highest purity available either from Sigma or from Merck (Darmstadt, Germany).

The bicinchoninic acid (BCA) protein assay reagent was used to quantify protein (Pierce Europe, Oud Beijerland, The Netherlands) [3].

Preparation of the protein fraction inserted into liposomes

Preparation of thyroid plasma membrane

Plasma-membrane vesicle proteins from bovine thyroid were obtained by differential centrifugation, as previously described [2] and were suspended in about 1–1.5 ml of 5 mM Mops/KOH buffer, pH 7.2, containing 200 mM KCl and 0.5 mM EGTA or 25 mM Hepes/KOH buffer, pH 7.2, containing 100 mM KCl and 0.5 mM EDTA.

Solubilization

Thyroid membrane proteins were mixed with an equal volume of the Mops/KOH buffer containing sodium cholate or with an equal volume of the Hepes/KOH buffer containing CHAPS or *n*-octyl glucoside.

They were then incubated at 4 °C for 10 min and centrifuged at 4 °C for 30 min at 100 000 *g* (Beckman centrifuge; rotor SW50). The volume of the supernatant corresponding to the solubilized material represented about 2–2.5 ml.

Detergent removal and buffer exchange

About 35 mg of solubilized protein in a volume of 2.5 ml was eluted with 10 mM imidazole/gluconic acid buffer, pH 7.5, containing 250 mM sucrose, through a prepacked Sephadex G-25M column (Sephadex PD-10 column), pre-equilibrated with 25 ml of the same buffer. The first 2.5 ml was discarded (void volume) and the protein material in the next 2.5 ml of eluate, from 2.75 to 5.25 ml, was collected. This operation was repeated and yielded $76.1 \pm 2\%$ ($n = 8$) of the solubilized protein. This procedure aimed to eliminate almost all detergent and ions present in the solubilization medium. The eluted material was further centrifuged on Centricon 100 (Amicon) (4 °C; 1 h; 1000 *g*; Sorvall RC2B centrifuge; rotor SS34) to concentrate and eliminate proteins of molecular mass less than 100 kDa. This final material was stored at -80 °C until used for proteoliposome reconstitution.

Preparation and solubilization of ileal brush-border membrane

Brush-border-membrane vesicles from bovine ileum were prepared by the Mg/EDTA precipitation method [4]. The vesicles were suspended in about 1–1.5 ml of the Mops/KOH buffer and solubilized in an equal volume of this buffer containing sodium cholate, at a detergent/protein ratio of 0.5:1 (i.e. cholate concentration of 1%). The same procedure as for solubilized thyroid protein was then applied for detergent removal and buffer exchange.

Proteoliposome reconstitution

Phospholipid films (PC/cholesterol, 4:3; 10 mg/ml) were suspended (unless otherwise specified) in 110 mM potassium gluconate/15 mM KI/10 mM imidazole buffered to pH 7.5 with gluconic acid. This suspension (10 mg/ml) yielded unilamellar liposomes after sonication at 20 kHz, 20 W on ice for two periods of 5 min, under nitrogen (Soniprep 150; titanium probe; 9.5 mm diameter). When iodide concentration were varied (1.5 and 50 mM), potassium gluconate was adjusted to keep the osmolarity constant. The unilamellar vesicles were incubated with solubilized proteins at a 9:1 (w/w) ratio, in a water bath at 25 °C for 20 min and vortexed vigorously every 5 min. The proteoliposomes were stored on ice.

Uptake of radiolabelled iodide driven by an outward iodide gradient

Each experiment was carried out at 4 °C with freshly reconstituted proteoliposomes. The method developed by Garty et al. [5] for Na⁺ transport was adapted for measuring the conductive uptake of iodide. An outwardly directed iodide gradient (KI 15 mM inside, 1 μ M outside) was generated by eluting 250 μ l of proteoliposome suspension (about 1400 μ g of PC/cholesterol and 155 μ g of protein) with 1 ml of imidazole/gluconic acid buffer on a chilled 4.5 cm Dowex 1 ($\times$ 10) column, prepared in gluconic acid [6], and equilibrated with 2 ml of the same buffer. The eluate was mixed with an equal volume of incubation medium which contained 125 mM potassium gluconate/10 mM imidazole adjusted to pH 7.5 with gluconic acid and 2 μ M ¹²⁵I (about 6 μ Ci/ml). After an appropriate incubation time, 130 μ l of the mixture was pipetted on to an ice-cold Dowex 1 ($\times$ 10) column and eluted with 1.5 ml of the ice-cold imidazole/gluconic acid buffer. The eluted ¹²⁵I corresponded to isotope trapped in the proteoliposomes. Radioactivity was counted in a γ counter (LKB Pharmacia). Under these experimental conditions, the columns retained virtually all the free

isotope (less than 0.01 % of the radioactivity was collected when 130 μ l containing about 0.5 μ Ci of ¹²⁵I was eluted in the absence of proteoliposomes). This was checked particularly when the highest outwardly directed iodide gradient (KI 50 mM) was used.

Elution through the Dowex column, as compared with the rapid filtration technique, considerably reduced background radioactivity. Furthermore, it obviated the need to consider the size of the liposomes.

To study the selectivity of the anionic conductance, intra-liposomal iodide was replaced with different anions (Cl⁻, NO₃⁻ or SO₄²⁻); osmolarity was kept constant by adjusting potassium gluconate.

Valinomycin (Sigma) dissolved in ethanol was added to the proteoliposomes 2 min before the uptake was started, at a final concentration of 9 μ M.

The difference in uptakes without and with valinomycin, which abolished the (proteo)liposomal membrane potential, represents potential or conductive iodide uptake. Furthermore, this difference at 30 s, an early time in the linear phase of the uptake, is defined as the initial conductive iodide uptake.

DPC, an anion channel inhibitor [7], was dissolved in DMSO and added at 1:100 dilution (final concentration of 100 μ M) to the proteoliposomes and incubated for 5 min before the uptake was started.

Proteoliposomes were in the presence of a maximum of 0.4 % ethanol or 1 % DMSO; control preparations received the same volume of solvent with no significant effect on the initial iodide uptake.

Size-exclusion chromatography

Solubilized plasma-membrane proteins (about 2.5 mg) suspended in Mops/KOH buffer containing 1 % sodium cholate were loaded on to a Superdex 200 HR column (1 cm $\times$ 30 cm) equilibrated with the same buffer. Elution was performed at a flow rate of 0.25 ml/min and ten fractions of 1 ml each were collected every 4 min, from 28 to 68 min. The protein content was determined and the activity of the iodide channel was evaluated in each fraction.

Presentation of results

In each Figure, the values are given as means $\pm$ S.E.M. (S.E.M.s are not shown when their values fall within the symbol).

RESULTS AND DISCUSSION

Solubilization of plasma-membrane vesicle proteins

The choice of detergent is the first step towards designing a purification scheme for membrane protein, which should ideally be solubilized without losing biological activity. Furthermore, as removal of the detergent may also be critical, only detergents presenting a high critical micellar concentration were considered [8].

Three detergents belonging to three different families were tested: the anionic detergent, sodium cholate, the zwitterionic detergent, CHAPS, and the non-ionic detergent, *n*-octyl glucoside. The solubilized proteins are presented as a function of detergent/protein ratio in Figure 1. Cholate achieved the highest solubilization for the lowest amount of detergent and was therefore selected. Of interest, it is also noticeably cheaper than the others.

Proteoliposomes were reconstituted with 10 μ g of protein solubilized with three different cholate concentrations of 0.5–2 %, corresponding respectively to detergent/protein ratios of 0.25:1,

Figure 1 Solubilization of thyroid plasma-membrane proteins

Thyroid plasma-membrane proteins were solubilized with sodium cholate (□), CHAPS (●) ($n = 3-4$) or *n*-octyl glucoside (○) ($n = 3-4$). The cholate/protein ratio of 0.25:1, 0.5:1 and 1:1 solubilized respectively 45.9 ± 2.8 ($n = 4$), 65.3 ± 5.4 ($n = 17$) and 73.9 ± 5.2 ($n = 17$)% of plasma-membrane proteins.

Figure 2 Dependence of iodide uptake on the cholate/protein ratio

Initial conductive iodide uptake was measured on proteoliposomes reconstituted with $90 \mu\text{g}$ of lipid and $10 \mu\text{g}$ of protein solubilized by three different cholate/protein ratios ($n = 4$).

0.5:1 and 1:1. The initial conductive iodide uptake is given in Figure 2. The 0.5:1 cholate/protein ratio yielded the highest initial conductive iodide uptake [84.08 ± 3.1 pmol/mg of protein] ($n = 6$) although it solubilized less plasma-membrane protein than higher cholate/protein ratios, suggesting more selective solubilization and/or a smaller loss of biological activity. Therefore a cholate/protein ratio of 0.5:1 was used throughout the study.

Preparation of membrane protein for reconstitution

Proteoliposome reconstitution is usually achieved by mixing phospholipids in high-ionic-strength medium containing the solubilized proteins and detergent. Detergent is then removed by using Bio-Beads and dialysis against the desired buffer [9,10]. However, this technique does not allow perfect control of the intraliposomal medium. Membrane proteins are usually most effectively solubilized in a medium containing high KCl concentration (100–200 mM), in which case the intraliposomal space will obviously be contaminated by chloride. As measurement of iodide conductance requires a chloride-free medium, we elected to prepare the membrane proteins in a buffer devoid of chloride and also removed the detergent by gel filtration before proteoliposomal reconstitution. To this end, proteins were eluted with imidazole/gluconic acid buffer on a Sephadex G-25M column and collected from 2.75 to 5.25 ml. This step was repeated to ensure maximal removal of unbound detergent and chloride.

Liposomes were separately prepared by sonication in the desired intraliposomal buffer and mixed with the protein material eluted from the Sephadex column. This rapid procedure allowed complete control of the intra- and extra-liposomal medium as well as simultaneous removal of cholate.

Conductive iodide uptake by proteoliposomes

Iodide uptake driven by membrane potential

A channel can be operationally defined as mediating a conductive ionic flux, i.e. it is critically dependent on the membrane potential, and importantly can still function at 4°C . In contrast, exchange diffusion (as mediated for instance by the band 3 protein) is insensitive to membrane potential but is more sensitive to the physical state of the lipid bilayer which can undergo a phase transition from the liquid to the solid state, usually below 15°C .

Na^+/I^- co-transport, which mediates iodide uptake at the basolateral blood-facing membrane, is not operational under the conditions selected here [2] since all experiments were performed at 4°C in the absence of Na^+ .

To demonstrate the presence of an iodide channel reconstituted into liposomes, an outward iodide gradient was generated by ion-exchange chromatography [Dowex 1 ($\times 10$) column]. At this concentration gradient (15 mM inside and $1 \mu\text{M}$ outside), iodide leaves the proteoliposome having an iodide conductance and induces a membrane potential (positive inside) which drives the uptake of radiolabelled iodide added to the extraliposomal medium. This uptake, measured at 4°C , is abolished by valinomycin, as this ionophore short-circuits the membrane potential in the presence of potassium (Figure 3); this clearly demonstrates the conductive nature of the iodide uptake at 4°C and hence the presence of an iodide channel, and rules out exchange diffusion.

Anion selectivity

Intraliposomal iodide was replaced with Cl^- , NO_3^- or SO_4^{2-} (15 mM). The outward anionic gradient was generated on an

Figure 3 Time course of iodide uptake

Iodide uptake was measured in proteoliposomes (10 μ g of protein and 90 μ g of lipid) in the absence (●) or presence (○) of 9 μ M valinomycin or in the presence of 100 μ M DPC (▲) ($n = 3$).

Table 1 Initial iodide uptake with different anions inside the proteoliposomes

The anions (potassium salts) were present at a concentration of 15 mM inside the proteoliposomes. Results are means $\pm$ S.E.M. for the number of determinations given in parentheses.

Anion inside the proteoliposomes	Iodide uptake (pmol/mg per 30 s)		
	Control	Valinomycin	Conductive
I ⁻	167.17 $\pm$ 18.65 (18)	2.61 $\pm$ 0.47 (18)	164.55
Cl ⁻	1.04 $\pm$ 0.04 (4)	1.04 $\pm$ 0.06 (4)	0
NO ₃ ⁻	1.21 $\pm$ 0.12 (4)	1.17 $\pm$ 0.08 (4)	0.04
SO ₄ ²⁻	1.09 $\pm$ 0.11 (4)	1.05 $\pm$ 0.1 (4)	0.04

anionic-exchange resin, as for iodide. Table 1 shows iodide uptake measured in proteoliposomes (10 μ g of protein and 90 μ g of lipid) in the absence and presence of valinomycin. Conductive iodide uptake was only observed in the presence of iodide. This indicates that none of these anions was able to generate a membrane potential. The conductance exhibited by thyroid membrane proteins is therefore highly selective for iodide.

Dependence of iodide uptake on the amount of lipid (PC/cholesterol)

Proteoliposomes were reconstituted with a low constant amount of protein (10 μ g) and variable amounts of lipid (30–120 μ g). Initial conductive iodide uptake was then measured and compared with that in liposomes prepared with the same variable

Figure 4 Dependence of iodide uptake on the amount of lipid

Initial conductive iodide was measured in proteoliposomes reconstituted with a constant amount of protein (10 μ g) but with variable amounts of lipid (30–120 μ g) (●) and compared with the initial conductive iodide uptake measured in liposomes prepared with the same variable amounts of lipid (○) ($n = 4$).

amounts of lipid (30–120 μ g) (Figure 4). Iodide uptake by these liposomes showed a slow steady increase directly proportional to the amount of lipid, indicating a larger distribution space for entrapped radiolabelled iodide. However, with proteoliposomes, the uptake increased more dramatically between 30 and 90 μ g of lipid and more slowly near 120 μ g; this demonstrates the insertion of a membrane protein conducting iodide into the liposomes. For the same amount of lipid (60–120 μ g), the non-specific iodide uptake by liposomes or iodide bound to the lipids represented about 23 % of the initial conductive iodide uptake of proteoliposomes. At 30 μ g of lipid, however, the uptakes were quite similar, perhaps as the amount of lipid becomes rate-limiting for protein insertion. Throughout the present study, 90 μ g of lipid was selected.

Dependence of iodide uptake on protein

Dependence on the amount of protein

Using a constant amount of liposomes (90 μ g of lipid), the amount of protein was varied between 5 and 60 μ g. The initial conductive iodide uptake increased linearly from 5 to 20 μ g and appeared to be saturated above 40 μ g of protein (Figure 5). As the signal is proportional to the amount of protein between 5 and 20 μ g, the uptake computed in pmol/mg is identical in this range of proteins.

However, non-specific iodide uptake by liposomes (90 μ g of lipid) represented about 40, 20 and 11 % of the initial conductive iodide uptake of proteoliposomes reconstituted respectively with 5, 10 and 20 μ g of protein. Therefore 10 μ g was selected, as the amount of protein will become rate-limiting as purification

Figure 5 Dependence of iodide uptake on the amount of protein

Initial conductive iodide uptake was measured in proteoliposomes reconstituted with a constant amount of lipid (90 μg) with variable amounts of protein (5–50 μg) ($n = 4$).

progresses (see below). The initial conductive iodide uptake was 167.17 ± 18.6 ($n = 18$) pmol/mg of protein for proteoliposomes reconstituted with 90 μg of lipid and 10 μg of protein. The values for iodide uptake were reliable, as the coefficient of variation derived from duplicate measurements in eight independent experiments was $100 \pm 3\%$.

Dependence on the nature of the protein

Conductive iodide uptake was measured in proteoliposomes reconstituted with 90 μg of lipid and 10 μg of protein either native or denatured by heat treatment for 5 min at 95 °C. The latter treatment abolished the uptake indicating that denaturation destroys the iodide conductance induced by thyroid membrane proteins (Figure 6). Furthermore, as shown below, only a defined fraction of thyroid membrane protein exhibited this iodide conductance (see Figure 8).

Proteoliposomes reconstituted with bacteriorhodopsin (60 μg), a membrane protein easily inserted into liposomes [11] that does not exhibit any iodide conductance, exhibited an iodide uptake that was the same as in proteoliposomes reconstituted with denatured protein. Proteoliposomes reconstituted with ileal brush-border-membrane proteins also failed to exhibit iodide conductance (Figure 6), further confirming that iodide uptake is mediated by a specific thyroid membrane protein component.

It should be stressed that the conductive uptake by liposomes (90 μg of lipid) was higher than with proteoliposomes reconstituted with denatured proteins (10 μg of protein and 90 μg of lipid) or bacteriorhodopsin (60 μg of protein and 90 μg of lipid). This indicates that insertion of non-conductive protein into liposomes may decrease their 'natural' or 'ground' permeability to ions.

Figure 6 Dependence of iodide uptake on the quality of the protein

Conductive iodide uptake was measured in proteoliposomes reconstituted with 90 μg of lipid and 10 μg of native (●) or denatured (○) thyroid proteins, 10 μg of ileal brush-border membrane proteins (□), 60 μg of bacteriorhodopsin (▲) or liposomes alone (90 μg of lipid) (▲) ($n = 3$).

Dependence of iodide uptake on the magnitude of the imposed outward iodide gradient

Conductive iodide uptake is dependent on the magnitude of the outward iodide gradient (usually 15 mM inside and 1 μM outside) which sets up a membrane potential (positive inside) across the proteoliposomal membrane.

Proteoliposomes (10 μg of protein and 90 μg of lipid) and liposomes (90 μg of lipid) were loaded with buffers containing various iodide concentration (1.5, 15 and 50 mM KI), and the initial conductive iodide uptake was measured. As expected, it increased as a function of the magnitude of the iodide gradient (Figure 7). The outward gradient generated by 15 mM intra-liposomal iodide appeared to be a good compromise between a sizeable signal and a moderate non-specific iodide leakage.

Time course of iodide uptake

Iodide uptake has been presented as conductive uptake, i.e. the difference in iodide uptake in the absence and presence of valinomycin. The relative contributions of each component are presented separately in Figure 3 for proteoliposomes reconstituted with 10 μg of protein and 90 μg of lipid. The uptake in the presence of valinomycin is extremely low, less than 5% of that in the absence of valinomycin, and probably represents non-specific iodide binding to the external side of the liposomal membrane. This drastic reduction in iodide uptake caused by valinomycin treatment demonstrates its conductive nature, as the ionophore short-circuits the liposomal membrane potential in the presence of potassium. In the absence of valinomycin, the uptake is linear up to 1 min, reaching a plateau

Figure 7 Magnitude of the imposed outward iodide gradient

The initial conductive iodide uptake was measured in the presence of different outward iodide gradients (1.5, 15 or 50 mM inside and 1 μ M outside) in proteoliposomes reconstituted with 10 μ g of protein and 90 μ g of lipid (□) and compared with the initial conductive iodide uptake measured in liposomes loaded with the same KI-containing buffers (■) ($n = 4$).

Figure 8 Size-exclusion chromatography of solubilized plasma-membrane proteins

Solubilized plasma-membrane proteins were loaded on to a Superdex 200 HR 10/30 column and eluted with Mops/KOH buffer containing 1% sodium cholate. The flow rate was 0.25 ml/min, and 1 ml fractions were collected every 4 min. The protein content of each fraction was measured by the BCA method (●). After removal of chloride and detergent, proteins in each fraction were reconstituted into proteoliposomes (10 μ g of protein and 90 μ g of lipid) and iodide channel activity was measured as the initial conductive iodide uptake (▽) ($n = 4$).

only after 60 min (results not shown). This confirms that its value at 30 s is in the linear phase of the uptake, validating its use to compute the initial iodide influx. In the absence of valinomycin,

100 μ M DPC, an anion channel inhibitor, considerably reduced iodide uptake (by about 70% at 30 s), suggesting that the iodide channel may share some general properties of anion channels.

Size-exclusion chromatography of the solubilized plasma-membrane proteins

Figure 8 shows the elution profile of solubilized plasma-membrane proteins and the activity profile of the iodide channel in the fractions collected. Activity was evaluated by measuring the initial conductive iodide uptake in proteoliposomes reconstituted with 10 μ g of protein and 90 μ g of lipid. It was maximal in fractions 5 and 6, corresponding to a molecular mass of between 100 and 200 kDa and representing 247.27 ± 7.65 pmol/mg ($n = 4$) which corresponds to about 10% of the protein. No further activity appeared in fractions 8 and 9, demonstrating that iodide conductance is specifically mediated by a thyroid membrane protein.

Conclusions

The iodide uptake is specifically mediated by a thyroid protein because: (1) it can be correlated with the amount of solubilized protein inserted into the liposomes (Figure 5); (2) it is mediated by a native protein component, and denaturation before reconstitution abolishes it (Figure 6); (3) it is mediated by a thyroid membrane protein as insertion of other proteins devoid of anionic conductance properties, such as bacteriorhodopsin and ileal membrane proteins, failed to induce uptake (Figure 6); (4) it is only observed with a well-defined fraction of thyroid membrane proteins (molecular mass between 100 and 200 kDa) (Figure 6); (5) it is considerably reduced by the anionic channel inhibitor DPC, suggesting that the iodide channel has some general properties of anion channels (Figure 3).

In conclusion, we have developed an optimal assay to assess the presence of iodide conductance in liposomes reconstituted with thyroid membrane proteins. This procedure can be used for screening any anionic conductance in solubilized or purified protein fractions.

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