

Chapter 3

Use of Liposomes to Study Cellular Osmosensors

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Abstract

When cells are exposed to changes in the osmotic pressure of the external medium, they respond with mechanisms of osmoregulation. An increase of the extracellular osmolality leads to the accumulation of internal solutes by biosynthesis or uptake. Particular bacterial transporters act as osmosensors and respond to increased osmotic pressure by catalyzing uptake of compatible solutes. The functions of osmosensing, osmoregulation, and solute transport of these transporters can be analyzed in molecular detail after solubilization, isolation, and reconstitution into phospholipid vesicles. Using this approach, intrinsic functions of osmosensing transporters are studied in a defined hydrophilic (access to both sides of the membrane) and hydrophobic surrounding (phospholipid membrane), and free of putative interacting cofactors and regulatory proteins.

Key words: Osmotic stress, Osmosensing, Transport, Reconstitution, Liposome, Proteoliposome, Membrane protein, Phospholipid, Signal transduction

1. Introduction

Under steady state conditions, cells maintain a certain ratio of internal versus external osmotic pressure which results in a particular cell turgor. A change in the external osmolality leads to a change in the turgor, and cells have developed sophisticated mechanisms of osmoregulation to respond to this challenge. Changing osmolality is one of the most frequent types of environmental stress for many cells, in particular microbial cells. In order to properly respond to this stress, cells harbor sensory mechanisms which perceive stimuli related to osmotic stress, transduce these stimuli into appropriate intracellular signals, or directly respond to these stimuli by appropriate actions.

In the case of hypo-osmotic stress, the major elements of stimulus response are mechanosensitive channels, which are dealt with in another chapter of this book. For the response to hyperosmotic stress, a major reaction is the activation of transport systems leading to the accumulation of compatible solutes. On the other hand, membrane bound sensory systems for hyperosmotic stress must be available, leading to appropriate responses in the cell on the level of transcription.

In vitro systems for the study of membrane bound osmosensing transporters for compatible solutes require the presence of topologically closed vesicles and thus the use of liposomes. Functional reconstitution of transporter proteins in proteoliposomes allows the study of these systems in the absence of any other interfering mechanisms, cofactors or proteins, facilitating experimental access to both sides of the membrane, as well as to the composition of the phospholipid membrane in which the proteins are embedded. On the other hand, proteoliposomes have a number of drawbacks for the study of osmosensing in comparison to intact cells, mainly due to the inherent lack of a cell wall. As a consequence, proteoliposomes are in general more fragile than cells, and they lack turgor pressure. Consequently, osmosensory events related to turgor cannot be studied using proteoliposomes.

As a further consequence, the fact that the morphological response of liposomes to hyperosmotic stress is different from cells has to be taken into consideration. Since liposomes behave as osmometers, i.e., they change their volume by water efflux according to the changing external osmotic pressure, and membranes are rigid in terms of their surface dimension; on the other, liposomes do not shrink in size but just invaginate under conditions of hyperosmotic stress to adapt the internal volume.

The physical nature of stimuli, perceived by cellular osmosensors, is difficult to define, and in most cases not known (1). There are, however, a number of transport systems (1–3) in which these stimuli have been defined to a significant extent, and one of these model systems, the betaine transporter BetP from the gram-positive soil bacterium *Corynebacterium glutamicum*, is used as an example here.

In order to study osmosensing by a transport system, solute transport has to be measured. This necessarily requires some knowledge of transport kinetics and energetics, which, at least at a basic level, can be treated exactly as enzyme kinetics and energetics, and will not be further discussed here. Another basic requirement for the issue dealt with here is the proper handling of membrane proteins, their solubilization, isolation, purification, and, in particular, reconstitution. In this article, the relevant procedures of functional reconstitution of BetP from *C. glutamicum* will be described; however, no detailed reference to appropriate

procedures for obtaining solubilized membrane proteins in a functional active state will be made.

2. Materials

2.1. Preparation of Preformed Liposomes for Reconstitution

1. *E. coli* polar lipid extract (Avanti, Alabaster, USA).
2. Liposome buffer: 100 mM phosphate buffer, pH 7.5.

2.2. Reconstitution of BetP Into Preformed Liposomes

1. Reconstitution buffer: 100 mM phosphate buffer, pH 7.5.
2. Solubilized and purified BetP protein (minimal protein concentration should be around 0.3 mg/ml, detergent concentration about 0.1% dodecyl maltoside (DDM solgrade, Anatrace, Maumee, USA). The protein concentration has to be measured with an appropriate method, e.g., using amido black (4).
3. 20% v/v Triton X-100 solution in water.
4. Spectrophotometer for monitoring liposome titration with detergent.
5. Biobeads as absorbent for detergents (Biobeads SM2, Biorad, Munich, Germany; washing of the beads in methanol/water should be carried out as described by the manufacturer). The beads are stored in water. Directly before the use for absorbing detergent, the “wet beads” are prepared by placing biobeads suspended in water on filter paper, which removes the surplus water. These “wet beads” are used for weighing.
6. Minivial ultracentrifuge for harvesting liposomes.
7. Avanti Mini Extruder System (Avanti, Alabaster, USA).
8. Polycarbonate filters, pore size 400 nm (Nucleopore, Schleicher & Schuell, Dassel, Germany).

2.3. Betaine Transport as a Response of BetP to an Osmotic Shift

1. Rapid filtration unit (FH225V, Hofer, Holliston, USA).
2. Membrane filters (Nitrocellulose, 0.45 μm pore size; Millipore, Schwalbach, Germany).
3. Radioactively labeled ^{14}C -betaine (synthesized from ^{14}C -choline by the use of choline oxidase)(5).
4. Transport buffer: 20 mM phosphate buffer, pH 7.5, 25 mM NaCl (if necessary, an increased osmolality is adjusted by addition of ionic solutes (e.g., NaCl), or neutral solutes, e.g., proline, if the ionic strength is not be changed significantly), 15 μM ^{14}C -betaine, and 0.5 μM valinomycin.
5. Washing buffer: 0.1 M LiCl.
6. Szintillation fluid and szintillation counter.

3. Methods

In the following, functional reconstitution of BetP, an osmosensing membrane protein, and the quantification of its response to osmotic stress is described. BetP is a secondary active glycine betaine transporter from the grampositive soil bacterium *C. glutamicum* (3). This carrier is energetically coupled to the co-transport of two sodium ions and thus driven by the electrochemical sodium potential. The transport activity of BetP is regulated by the actual osmotic stress, and it has been shown that the carrier protein is able to sense the extent of osmotic stress without any additional factors or components (3, 6).

Isolation and purification of membrane proteins in a functionally active state requiring optimization of the kind and concentration of detergent used, of the solubilization conditions (ionic strength, type of buffer, pH, temperature, etc.), on the one hand, as well as optimization of the purification procedure (type of tag used, variation of isolation conditions, etc.), on the other. The protocol described here starts with membrane protein(s) in solubilized and functionally active form. The stability of solubilized membrane proteins in terms of functionality is in general a serious problem and needs attention (see Note 1).

In order to obtain an appropriate in vitro test system, the solubilized osmosensing membrane protein is reconstituted into liposomes, often prepared from *E. coli* lipids. There are a large number of different methods for membrane protein reconstitution; the method of choice used in most of the successful procedures recently published is the integration into preformed liposomes developed by Rigaud and colleagues (7, 8). A number of different aspects have to be tested and optimized when trying to obtain an appropriate in vitro system for testing functional properties of a reconstituted membrane protein. The preformed liposomes may differ in the type of lipids used depending on the requirement of the particular membrane protein to be reconstituted (see Note 2). Furthermore, lipid quality is an important issue, which means purity and lipid stability. The latter critically depends on how they are handled and stored (see Note 3).

3.1. Preparation of Preformed Liposomes for Reconstitution

1. Purified lipids dissolved in organic solvent (chloroform/methanol) are mixed. The mixture is evaporated to dryness in a rotary evaporator (30°C; temperature should be above phase transition temperature of the lipids).
2. Traces of solvent are removed overnight by freeze-drying (lyophilization).
3. Liposome buffer (plus 2 mM β -mercaptoethanol) is added at a concentration of 20 mg lipid/ml and lipids are suspended by stirring at room temperature (RT) for about 2 h.

4. The lipid suspension is flushed with N₂ gas, frozen in liquid N₂, and stored at -80°C until use.
5. For reconstitution an aliquot of lipids is thawed gently at RT. Thereafter, liposomes are prepared by extrusion through polycarbonate filters (pore size 400 nm, multiple extrusions 17 times).

3.2. Reconstitution of BetP Into Preformed Liposomes

1. The preformed liposomes are diluted fourfold into liposome buffer. 20% v/v Triton X-100 is added stepwise (in 2–4 μl portions, thorough mixing by the use of a pipette) until detergent saturation of liposomes is achieved. Reaching the correct physical state of the liposomes for reconstitution is monitored by measuring optical density at 540 nm (7, 8). Optimal conditions for incorporation of the solubilized protein are obtained right after reaching the maximum value of light scatter at 540 nm.
2. The solubilized protein is then added drop by drop with thorough mixing. The amount of detergent added to the mixture together with the solubilized protein should be small in comparison to the amount of Triton X-100 added before. This mixture is shaken gently for 30 min at RT.
3. For removal of detergent according to the batch-procedure (7, 8), polystyrol beads (biobeads are added according to their absorption capacity for particular detergents (9), e.g., 5 mg and 10 mg of wet beads per mg of triton or DDM, respectively) are added. After shaking for 1 h at RT the same amount of beads is added and the mixture is shaken again for 1 h. The sample is then shaken overnight at 4°C with an additional twofold amount of beads. Subsequently, wet beads (same amount as for the first addition) are again added and shaken for 45 min at 4°C (see Note 4).
4. Finally, the proteoliposomes are separated from the beads with a pipette and washed twice with ice cold liposome buffer by centrifugation (Beckman Optima TLX tabletop ultracentrifuge, 350.000 g, 20 min, 4°C) before being resuspended in liposome buffer at a concentration of about 60 mg/ml lipid. The proteoliposomes are frozen in liquid N₂ and stored at -80°C.
5. In order to assess the efficiency of reconstitution, the protein content of each batch of reconstituted protein has to be thoroughly measured by protein determination, e.g., using the amido black assay (4) (see Note 5).

3.3. Betaine Transport as a Response of BetP to an Osmotic Shift

1. For transport measurements, an aliquot of the proteoliposomes is thawed gently at RT, and extruded (polycarbonate filter, pore-size 400 nm) 17 times in a total volume of up to 1 ml, before being concentrated to the original volume by centrifugation (ultracentrifuge for mini vials, 350.000 g, 20 min, 20°C) and resuspended in liposome buffer.
2. In the transport assay the proteoliposomes, supplemented with internal K⁺ during the preparation in liposome buffer, are rapidly

diluted into transport buffer containing valinomycin and the labeled substrate, betaine, and thoroughly mixed. In a standard experiment, the reaction is started by pipetting 5 μl of concentrated proteoliposomes into 1000 μl of transport buffer. According to the dilution ratio, this will lead to a K^+ -diffusion potential of about 140 mV. For measuring the activity of a particular transport protein, an appropriate driving force has to be provided. The driving force for BetP is the electrochemical Na^+ potential, consequently, a sufficient amount of Na^+ has to be present in the medium, in addition to the membrane potential, supplied here as K^+ -diffusion potential.

3. For each time-point in the following kinetic assay, 200 μl samples are used. These samples are withdrawn from the transport assay and instantly filtered through membrane filters in a filtration unit. The time intervals chosen depend on the velocity of the reaction under study; experienced experimenters are able to handle time points down to 5 s intervals. The filters are washed immediately twice with about 2 ml of washing buffer each (see Note 6).
4. After finishing the kinetic experiment, the filters are removed with tweezers and transferred into a scintillation vial each, which is filled with scintillation fluid, and counted.
5. The amount of betaine taken up into the proteoliposomes by the reconstituted carrier protein at the chosen time-points is calculated on the basis of the specific radioactivity applied in the transport buffer and the amount of protein present in the 200 μl sample of proteoliposomes. Protein determination should be carried out for each experiment, since variable losses during the extrusion procedure may give rise to variation.
6. Substrate uptake activity is interpreted in terms of transport kinetics, and specific transport rates are calculated (see Note 7). Beside the activity of the reconstituted protein, the result of uptake measurements may depend on a variety of other factors (see Note 8).
7. Testing a transport protein for its osmosensing function will need variation of the medium on both sides of the membrane, with respect to concentration and quality of solutes, e.g., ionic and nonionic solutes, or type of cations and anions, respectively. The solute composition in the interior of the proteoliposomes can be varied during liposome preparation (Step 1 of Subheading 3.3.). The composition of the internal compartment can also be changed after proteoliposome preparation by applying additional freezing and thawing cycles in a medium of changed composition followed by liposome generation using the extrusion procedure. The lipid composition can also be varied during the preparation of the preformed liposomes.

Additional lipids can be integrated by repeated cycles of freezing and thawing mixtures of (proteo) liposomes with different lipid compositions.

8. It should be noted, that there are two different types of experiments testing the dependence of a membrane inserted protein on variation of osmotic properties. On the one hand, the external osmolality (or solute composition) can be changed at the start of the transport experiment, leading to an osmotic gradient and a subsequent volume change of the proteoliposomes. A variation of solute concentration or composition on both sides of the membrane at the same time, i.e., during proteoliposome preparation, on the other hand, changes the osmotic pressure without introducing an osmotic gradient and without concomitant volume changes (6).

4. Notes

1. The stability of solubilized membrane (carrier) proteins is a serious issue for the success of this type of experiment. The conditions of solubilization, isolation, and storage, if necessary, have to be optimized carefully. Most transport proteins are notoriously unstable in the solubilized state, but, in general, rather stable once properly inserted into a phospholipid membrane. Consequently, they should be purified quickly and reconstituted into proteoliposomes right after purification.
2. Often, selected lipids have to be used for the reconstitution of a particular membrane protein, in order to reach optimum reconstitution efficiency or functionality.. Different membrane proteins have different preference for lipids, with respect to the phospholipid headgroup, the headgroup charge, and the fatty acid composition. Typical lipids to start with are lipids from *E. coli* membranes, asolectin (soy bean lipids), or egg yolk lipids. Once, a basic activity of the reconstituted protein is observed, variation of lipids will normally improve the results. An obvious strategy to overcome some of these problems is the use of synthetic phospholipids; however, for this, a balanced composition of unsaturated (essential for most membrane proteins) and saturated fatty acids is required. For unknown reasons, effective reconstitution into synthetic lipid mixtures seems to be more difficult compared with natural lipids, e.g., from *E. coli*. In any case, the fact that proteins in general prefer mixtures of phospholipids, both with respect to the headgroup and the fatty acid composition over the membranes prepared from single compounds has to be taken into account.

3. The same as stated for handling of the protein is also true for handling of lipids. Always the best lipids available should be used. In general, Avanti is a good choice as a supplier for lipids, however, be careful for variation of lipid quality from batch to batch. After having established an optimized protocol for reconstitution, try to examine different batches of the lipids of choice and then buy a stock of lipids of the optimal batch. In general, if the proteoliposome test system, after having been successfully established, fails to work for unknown reasons, the quality of lipids is a good candidate to think about. For this reason, it is recommended always that part of a lipid batch which was proven to function is stored away, to serve as a control for later experiments. Other reasons for failure, of course, are the functionality of the solubilized protein, and, rather often, the efficiency of detergent removal by adsorbance to biobeads, which directly affects the efficiency of reconstitution and/or the permeability properties of the proteoliposomes.
4. The stability of the lipids used is a serious issue, too. The manufacturer supplies lipids in general sealed under nitrogen gas; it is recommended that the same condition is kept when storing lipids in the lab, preferably at -80°C . Useful recommendations for lipid handling and storage are found in (10–12).
5. Detergent removal is an important step during reconstitution and is prone to a number of difficulties. Complete removal of detergent from the proteoliposomal sample can simply be tested by the absence of foam after shaking. It has to be taken into account that the beads not only absorb detergents, but all amphiphilic (and even hydrophilic) compounds, in general in the order detergent > lipid > protein. It is thus important to limit the amount of biobeads used for absorption of the detergent in order not to lose too much protein. An alternative strategy partially avoiding this problem is to presaturate the biobeads with lipids (7, 8).
6. The efficiency of reconstitution is a complex problem. First, it refers to the amount of protein successfully integrated. This value may vary with the kind of detergent and lipid used, as well as with particular aspects of the reconstitution procedure. It has to be quantified by protein determination in the proteoliposomes, e.g., by the amido black method (4). In general, the efficiency of protein insertion decreases with increasing protein/lipid ratio during reconstitution. Second, reconstitution efficiency also means the fraction of functionally active protein in comparison to the total amount of integrated protein. Only the latter is measured by protein determination. This ratio is rather difficult to determine and in general needs additional methods, e.g., quantification of substrate binding. Third, protein orientation may be an issue. In most cases, the

orientation of the reconstituted membrane protein is not known. This, however, may be of crucial significance for interpreting its function in the in proteoliposomal in vitro system, in particular for transport proteins being dependent on oriented driving forces. There are a number of methods available for defining the orientation of a membrane-inserted protein, e.g., the use of antibodies, proteases, and impermeable labeling reagents for specific amino acids.

7. Although seeming apparently simple, fast, and efficient liposome filtration is a crucial step in the whole procedure. It should be taken into account that filtration, as used here, is to a large extent adsorption to the filter material, in view of the pore size of the filters used. Before application to the filtration unit, the membrane filters have to be presoaked in washing buffer, in order to avoid labeled substrate to be soaked into the external ring of the filter which is fixed in the filtration unit. Before running the kinetic experiment, the filters should be briefly soaked by application of vacuum. When applied to the filter during transport kinetics, the proteoliposome sample should quickly be distributed over the entire filter surface. The same holds true for the washing solution, which preferentially should be applied along the walls of the filtration chamber.
8. Transport kinetics has been elaborated in relevant text books. In general, when establishing transport kinetics of a particular carrier protein, a time course including several experimental points (five at least) has to be used in order to find out in which time window the uptake kinetics is reasonably linear with time. Once established, experiments are frequently carried out using two time points only within the linear kinetic phase, because of the requirement of rapid assays and/or frequently because of a limited range of linearity (mainly due to the small size of the proteoliposomes used). In order to provide sufficient experimental reliability, these two point-kinetics have to be carried out in multiple sets, e.g., fivefold. When changing experimental conditions, multiple time point kinetics has to be applied again to verify linearity. It should furthermore be mentioned that the linearity of a transport assay also depends on the amount of substrate taken up during the time course monitored in the kinetic experiment. For this reason, the amount of labeled substrate taken up at the end of the time course should not exceed one third of the added label. This amount, however, may sometimes be much lower, if higher substrate concentrations have to be used because of a relatively low substrate affinity of the transport protein under study.
9. It should be pointed out that the result of the kinetic analysis of a reconstituted transport protein not only depends on the functionality of the protein, and the experimental conditions

applied (e.g., kind and extent of driving force, pH, ionic strength, kind of lipid provided, etc.), but to a large extent also on the quality of liposomes used. This refers, on the one hand, to the integrity and stability of liposomes, which in the presence of residual detergent, or high amounts of protein integrated, can be an issue. The integrity of the proteoliposomes used can be tested by the incorporation of calceine (13). Another reason for unexpected failure of transport experiments is partial leakiness of the proteoliposomes to small ions, e.g., protons, leading to a quick collapse of the driving force. This will in general not lead to leakiness of the larger solutes, like substrates (or calceine), and is frequently caused by residual amounts of detergent present. Furthermore, the size of the liposomes used is of critical significance. An early deviation of transport kinetics from linearity may indicate the presence of liposomes too small in size for the particular transport experiment. The impact of the liposome size on the shape of the transport kinetics observed is also a major reason for using liposomes sized by the extrusion procedure through polycarbonate filters. All other methods of proteoliposome formation lead to mixtures of vesicles with a broad size distribution. This fact will have a complex influence on the resulting transport kinetics.

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