Functional Analysis of Detergent-Solubilized and Membrane-Reconstituted ATP-Binding Cassette Transporters

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[25] Functional Analysis of Detergent-Solubilized and Membrane-Reconstituted ATP-Binding Cassette Transporters

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Abstract

ATP-binding cassette (ABC) transporters are vital to any living system and are involved in the translocation of a wide variety of substances, from ions and nutrients to high molecular weight proteins. This chapter describes methods used to purify and membrane reconstitute ABC transporters in a fully functional state. The procedures are largely based on our experience with substrate-binding protein-dependent ABC uptake systems from bacteria, but the approaches should be applicable to multisubunit membrane complexes in general. Also, we present simple methods, based on substrate binding or translocation, to follow the activity of the protein complexes in detergent-solubilized and/or membrane-reconstituted state(s).

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Introduction

ATP-binding cassette (ABC) proteins comprise one of the largest superfamilies of proteins known to date. The majority of ABC proteins are involved in the translocation of solutes across the membrane, for example, nutrient uptake, drug and antibiotic excretion, cell volume regulation, lipid trafficking, and biogenesis. Additionally, a subset of ABC proteins is involved in DNA maintenance and protein synthesis, for example, recombination, DNA repair, chromosome condensation and segregation, and translation elongation. These latter proteins exert their functions in the cytoplasm and/or nucleus and are not considered here; for a comprehensive overview on the different types of ABC proteins and their functions, we refer to Holland *et al.* (2003). ABC proteins facilitating solute translocation, referred to as ABC transporters, reside in the cytoplasmic membrane of bacteria, archaea, and eukaryotes and can also be found in the organellar membranes of the higher organisms, that is, the inner mitochondrial membrane, endoplasmic reticulum, and peroxisomal and vacuolar membranes.

ABC transporters use the hydrolysis of ATP to translocate solutes across cellular membranes. The translocator component of the ABC transporters is composed of two transmembrane and two intracellular ATP-binding subunits (Fig. 1A), with the individual subunits expressed as separate polypeptides or fused to each other in any possible combination (Holland *et al.*, 2003). In addition to these ubiquitous components, prokaryotic ABC transporters involved in solute uptake employ a specific extracellular ligand-binding protein to capture the substrate (Fig. 1B–D). These substrate-binding proteins (SBPs), which are the main determinants

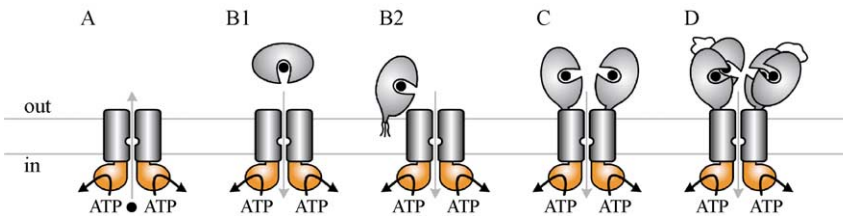

FIG. 1. Schematic representation of the domain organization of ABC transporters. (A) Efflux system. (B) Conventional SBP-dependent uptake system with periplasmic (B1) or lipid-anchored SBP (B2). The chimeric substrate-binding/translocator systems with two and four substrate-binding sites per functional complex are shown in C and D, respectively. "'Out'' and ''in'' indicate the extra- and intracellular side of the membrane, respectively; the translocator and NBD subunits are in gray and orange, respectively.

of the specificity of SBP-dependent ABC transporters, were first identified in gram-negative bacteria, where they reside in the periplasmic space (Neu *et al*., 1965) (Fig. 1B1). In gram-positive bacteria and Archaea, which lack a periplasm, SBPs are anchored to the outer surface of the cell membrane via a N-terminal lipid moiety (Sutcliffe *et al*., 1995) (Fig. 1B2), a N-terminal transmembrane segment (observed for Archaea only) (Albers *et al*., 1999), or fused to either N or C terminus of the translocator protein (van der Heide *et al*., 2002). With systems that have one or two substrate-binding domains fused to the translocator protein, there may be two (Fig. 1C) or even four (Fig. 1D) substrate-binding sites functioning in the translocation process (Biemans-Oldehinkel *et al*., 2003).

Crystal structures of ABC transporters are available for the lipid A exporter MsbA from *Escherichia coli* (Chang *et al*., 2001) and *Vibrio cholera* (Chang, 2003) and the vitamin B_{12} uptake system BtuCD from

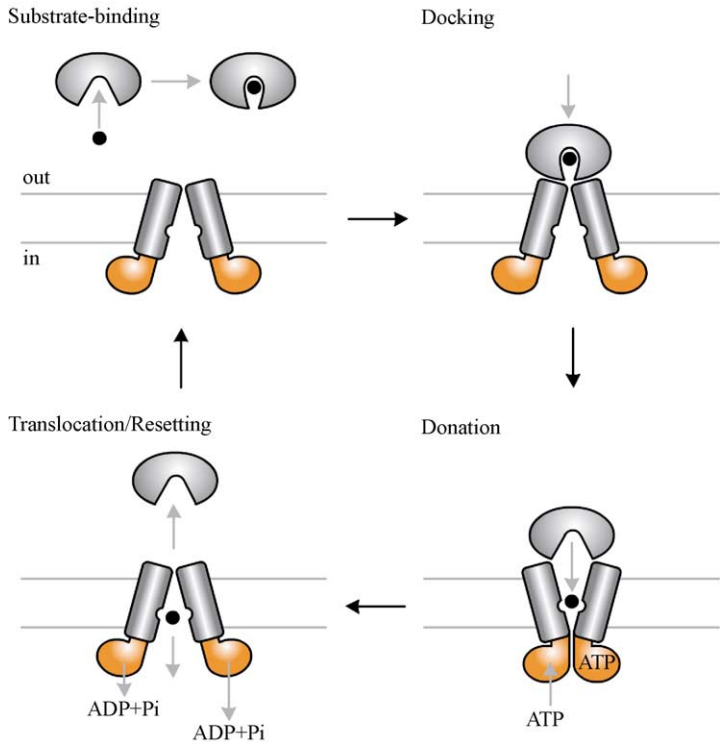

FIG. 2. Model for the transport cycle of a SBP-dependent ABC transporter. For explanation, see text.

E. coli (Locher *et al*., 2002). In addition to these structures of complete complexes, crystal structures are available for many of the SBPs and ATP-binding cassette subunits (Holland *et al*., 2003). From these structures and mutational analyses and kinetic studies on efflux and uptake systems, an understanding of the translocation mechanism of ABC transporters is beginning to emerge (indicated schematically for a SBP-dependent uptake system in Fig. 2). The model is based on data obtained from both SBP-dependent uptake and SBP-independent efflux systems (Chen *et al*., 2001; Davidson, 2002; Higgins *et al*., 2004; Liu *et al*., 1999; Locher *et al*., 2002). Although details of the model may not be the same for all ABC transporters, the main mechanistic steps seem to be well conserved. Translocation via SBP-dependent ABC transporters starts with binding of the substrate to the SBP. Upon docking of the liganded SBP onto the transmembrane domains (TMDs), a signal is transmitted to the nucleotide-binding domains (NBDs). This enhances the cooperative binding of two ATP molecules, which in turn facilitates closed dimer formation. The closing of the NBD dimer is coupled mechanistically to critical rearrangements in the TMDs, and the affinity for substrate is reduced by opening of the SBP, facilitating the donation of the substrate to a binding site in the TMDs or to the cytoplasm directly. After the substrate has crossed the membrane, the SBP dissociates from the translocator. Hydrolysis of two ATPs initiates resetting of the system for another translocation cycle, that is, after the sequential release of inorganic phosphate and ADP. In an extension of this model (van der Does *et al*., 2004), it has been postulated that in some ABC transporters, the dimer may be stabilized by one ATP and subsequent hydrolysis of a single ATP may be sufficient for translocation. The issue of the number of ATP molecules hydrolysed per substrate translocated has not been completely settled, and an experimental strategy for ATP/substrate stoichiometry determination is outlined later. Finally, in the model presented in Fig. 2, a single SBP is involved in substrate delivery to the translocator; in case of ABC transporters with multiple substrate-binding domains (SBDs), cooperative interactions between SBDs and the translocator domain may occur (Biemans-Oldehinkel *et al*., 2003).

This chapter deals with the analysis of purified ABC transporters in detergent-solubilized and membrane-reconstituted states. The focus is on the strategies to incorporate ABC transport systems into lipid vesicles, so-called large unilamellar vesicles (LUVs; diameter of 100–300 nm) and giant unilamellar vesicles (GUVs; diameter of 5–100 μm). In addition, we provide protocols based on radiolabel distribution and fluorescence measurements, yielding kinetic information on substrate binding and substrate translocation by ABC transporters.

ABC Transporters in the Detergent-Solubilized State

Protein Purification and Stability of Oligomeric Complexes

Most membrane proteins, including ABC transporters, are purified at present by affinity chromatography, taking advantage of, in most cases, an amino- or carboxyl-terminal 6- or 10-his tag, typically fused to one of the subunits of the transporter. The affinity tag is often flanked by a specific proteolytic cleavage site to remove the tag after purification. Protocols used to purify proteins by affinity chromatography can be found in numerous papers and are not detailed here. However, because many translocator complexes of ABC transporters are composed of multiple subunits and tend to dissociate upon solubilization, giving rise to specific complications and opportunities, we provide pointers to obtain complexes of stoichiometric amounts of polypeptides after purification.

Chromatography. To facilitate purification, the expression of ABC genes is often increased by using a strong promoter, which can lead to nonstoichiometric production of the individual subunits. In such cases, it is desirable to have the his tag fused to the subunit that is produced in the lowest amount, which allows removal of an excess nontagged subunit(s) by washing of the affinity resin. Alternatively, size exclusion

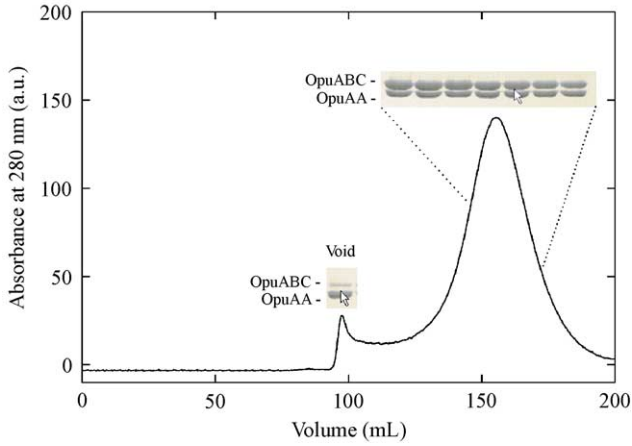

FIG. 3. Size-exclusion chromatography of OpuA from *L. lactis* (unpublished experiment). OpuA was purified by Ni-NTA chromatography, essentially as described (van der Heide *et al.*, 2000), concentrated to 20 mg/ml on Amicon Ultra (100,000 MWCO,) and 40 mg of protein was loaded onto Sephadex S300 in 10 m*M* Na-HEPES, pH 8.0, 100 m*M* NaCl, 15% (v/v) glycerol, and 0.15 % (w/v) CyMal5. (Inset) A Coomassie brilliant blue-stained SDS–polyacrylamide gel of the individual protein fractions.

chromatography (SEC) can be used to remove the free, noncomplexed subunit, which has the additional advantage that aggregated protein can also be separated from the native complex. An example of purification of the osmoregulatory transporter OpuA from *Lactococcus lactis* (Fig. 1C) is presented in Fig. 3. The overexpression of *opuA* genes yielded a small but significant excess of the ATPase subunit (OpuAA), which in SEC experiments appeared as aggregated material in the void volume of the column.

Cosolvents. To obtain stoichiometric amounts of polypeptides after purification, composition of the chromatography media is critical. Relevant parameters are the choice of detergent, the presence of cosolvents, ionic strength, and the presence or absence of specific additives. In the case of OpuA from *L. lactis* (Fig. 1C), minimally 15% (v/v) glycerol is needed as a cosolvent to prevent the complex from dissociating during the chromatography steps (metal affinity chromatography followed by SEC) (Biemans-Oldehinkel *et al.*, 2003). Because dissociation of the OpuA complex (two substrate-binding/translocator subunits, OpuABC, plus two ATPase subunits, OpuAA) proved to be reversible, we took advantage of this property to form unique heterodimeric complexes (e.g., one wild type and one mutated ''nonfunctional'' OpuABC subunit plus two OpuAA subunits). This allowed us to dissect the roles of the individual subunits in the oligomeric complex (Biemans-Oldehinkel *et al.*, 2003).

PROCEDURE 1. Purified wild-type and mutant OpuA complexes in buffer A [50 m*M* KPi, pH 8.0, 200 m*M* KCl, 20% (v/v) glycerol, 0.05% (w/v) dodecyl-maltoside (DDM)] are mixed at different ratios (final protein concentration of 0.4 mg/ml) and, subsequently, dissociated by decreasing the glycerol concentration to 5% (v/v). For this purpose, the protein mixture is diluted fourfold with buffer A without glycerol. After 30 min of incubation at 4°, the glycerol concentration is increased again to 20% by addition of buffer A containing 60% (v/v) glycerol. Reassembly of the complexes is allowed to continue for 30 min at 4°, after which the proteins are incorporated into liposomes (see later).

Imidazole. The oligopeptide transporter Opp from *L. lactis* is composed of a lipid-anchored substrate-binding protein (OppA), two integral membrane proteins (OppB and C), and two ATP-binding cassettes (OppD and F) (Fig. 1B2). Although metal affinity-based purification protocols generally employ low concentrations (5–30 m*M*) of imidazole in the protein binding to the resin and washing steps, imidazole severely compromises the stability of the OppBCDF translocator (Doeven *et al.*, 2004). Initial conditions for maintaining an intact OppBCDF complex during purification were screened for ionic strength (0–500 m*M* KCl) and pH (6.0–8.0) using 20 m*M* imidazole, 0.05% (w/v) DDM, and 20% (v/v)

glycerol as basal medium. This did not lead to purification of the complete translocator, but rather resulted in purification of the His_6-tagged component OppC only. However, when imidazole was omitted from the buffer during solubilization and binding of the complex to the metal affinity resin, the OppBCDF proteins could be obtained in an approximate 1:1:1:1 ratio. In contrast to OpuA, varying the glycerol concentration from 0 to 40% (v/v) did not have any effect on the polypeptide stoichiometry of Opp obtained after purification.

Detergents. ABC transporters OpuA (Fig. 1C) and GlnPQ (Fig. 1D) have been solubilized and purified successfully in alkyl-maltosides [decyl to tridecyl; critical micelle concentration (CMC) values ranging from 1.8 to 0.03 m*M*], cyclohexyl-alkyl-maltosides [CyMal-5, -6, and -7; CMC values ranging from 2.4 to 0.19 m*M*], alkyl-phosphocholines (FOS-choline-10, -12, and -14; CMC values ranging from 11 to 0.14 m*M*), and Triton X-100 (CMC of 0.23 mM); detergents are Anagrade and obtained from Anatrace Inc. (Maumee, USA). In general, the longer the alkyl chains (lower CMC), the more stable the protein complexes, but in the case of OpuA, this parameter was less critical than the glycerol concentration. The membrane complexes $MalFGK_2$ and $HisQMP_2$ of the well-characterized maltose transport system from *Escherichia coli* and the histidine transport system from *Salmonella typhimurium*, respectively, were initially purified in octyl β-D-glucopyranoside (OG) (Ames *et al.*, 2001; Davidson *et al.*, 1991). Although OG with its high CMC (~30 m*M*) has the advantage that it is removed readily by detergent dilution or dialysis in membrane reconstitution experiments, membrane protein complexes are generally not very stable in this detergent (Knol *et al.*, 1996). Indeed, $MalFGK_2$ was inactivated by OG when used in purification; activity was observed when the protein complex was isolated and purified in DDM and reconstitution was mediated by OG (Davidson *et al.*, 1991).

Binding of Substrates to SBPs and Translocator Subunits

Here, we discriminate between SBP-dependent uptake systems that employ a specific extracellular receptor to deliver the substrate to the translocator (as in Fig. 1B–D) and SBP-independent efflux systems where substrate binding exclusively takes place in the transmembrane domains (Fig. 1A). The general mechanism of substrate association to SBPs is described by the Venus flytrap mechanism (Quiocho *et al.*, 1996), that is, the ligand binds in the cleft between two globular domains and, upon binding of the ligand, the protein closes. The binding of substrate to SBPs is often of relatively high affinity with dissociation constants (K_D) in the submicromolar to low micromolar range and can be monitored by equilib-

rium dialysis (Silhavy *et al.*, 1975), rapid filtration of protein trapped with ligand (Detmers *et al.*, 2000; Richarme *et al.*, 1983), or spectroscopic (Miller *et al.*, 1983) methods.

For glycine betaine binding to OpuA, we have successfully employed the ammonium sulfate precipitation method described by Richarme *et al.* (1983).

PROCEDURE 2. Detergent-solubilized protein (0.5–2 μM) and [^{3}H] glycine betaine (0.1–20 μM) are mixed in a total volume of 0.1 ml and binding is allowed to proceed for 2 min at 30° (Biemans-Oldehinkel *et al.*, 2003). The reaction is stopped by adding 2 ml ice-cold 70% (v/v) saturated ammonium sulfate and rapid filtration of the mixture through 0.45-μm pore-size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassel, Germany). The filters are washed twice with 2 ml ice-cold 70% (v/v) saturated ammonium sulfate and radioactivity is counted by liquid scintillation spectrometry.

This method is very simple but is not suitable for every protein; the ammonium sulfate-precipitated SBP may not sufficiently trap the substrate and dissociation may occur during the washing steps or the ammonium sulfate may also precipitate free ligand [oligopeptides such as bradykinin start precipitating above 50% (v/v) saturated ammonium sulfate]. Therefore, for the peptide-binding protein OppA from *L. lactis*, an alternative strategy was devised (Detmers *et al.*, 2000). Because OppA is synthesized with an N-terminal lipid moiety, the protein was tethered to the surface of liposomes (for procedure, see later), which increased the retention of the protein on the filters.

PROCEDURE 3. Peptide binding to OppA is measured by making use of the high-affinity ligand [3,4(n)-^{3}H]bradykinin, which is a cationic peptide with the sequence RPPGFSPFR. Bradykinin (0.02–20 μM) is incubated with liposome-tethered OppA at 25° for 4 min in assay buffer [final volume of 0.1 ml; final OppA concentration is 20 μg/ml (~0.3 μM)], followed by a 1-min incubation with antibodies raised against OppA using a titer of 1:10. Subsequently, the assay mix is diluted with 2 ml ice-cold 8% (w/v) PEG 6000 and filtered over 0.2-μm pore-size cellulose acetate (OE66) filters (Schleicher and Schuell GmbH, Dassel, Germany), after which the filters are washed again with 2 ml ice-cold 8% (w/v) PEG 6000 and radioactivity is counted.

Some aspects of this assay require explanation. First, cellulose acetate instead of cellulose nitrate filters were used to minimize nonspecific binding of bradykinin to the filters; cationic compounds such as bradykinin bind strongly to nitrocellulose filters. Second, antibodies raised against OppA, together with PEG 6000, were used to collect the proteoliposomes on the filters more effectively. Without these treatments, more than 60% of

the material passed through the filters. Although similar K_D values were observed in the absence of the antibodies, the amount of peptide binding, reflecting the maximal number of binding sites (B_{max}), was lower due to the loss of liposomes with OppA. The smaller the proteoliposomes, the less well retained they are by the cellulose acetate filters. Third, with neutral or anionic substrates, cellulose nitrate filters can be used, and SBP-specific antibodies plus PEG 6000 are not needed to quantitatively recover the proteoliposomes because these filters trap (proteo)liposomes more efficiently than those made of cellulose acetate due to charge interactions between the lipids and the filter.

For most SBPs that we and others have analyzed, it appears that substrate binding to SBPs elicits conformational changes that can be probed by fluorescence spectroscopy (Lanfermeijer *et al*., 1999, 2000; Miller *et al*., 1983). With this method, presteady-state kinetics of binding and dissociation can also be monitored accurately. The spectroscopic method is often preferred over equilibrium dialysis (slow and time-consuming) and rapid filtration (possible loss of ligand) methods because measurements can be performed on-line and estimates of binding constants can be obtained for both high- and low-affinity ligands (Lanfermeijer *et al*., 1999). One needs to be aware of bleaching of the protein fluorophores by the excitation light, however, and perform the appropriate mock controls. Moreover, with some SBPs, the change in conformation upon substrate binding does not elicit a significant change in protein fluorescence.

PROCEDURE 4. Fluorescence spectra of OppA are obtained with 1 ml of protein solution (0.5–2 μM) in filtered and thoroughly degassed buffers in a quartz cuvette (stirred continuously and kept at 15° with a circulating water bath). Effects of peptides on fluorescence are measured by exciting at 280 $\pm$ 2 nm and measuring the emission at 315 $\pm$ 8 nm. The effect of a saturating concentration of peptide on the intrinsic protein fluorescence of OppA is shown in Fig. 4A; the concentration dependence of the fluorescence increase induced by peptide is shown in Fig. 4B. For a full analysis of steady-state and presteady-state data of ligand binding to SBP, refer to Miller *et al.* (1983) and Lanfermeijer *et al*. (1999).

With respect to substrate binding and transport in ABC transporters, it is worth emphasizing that for the initiation of translocation the kinetically relevant species is the liganded substrate-binding protein rather than the free substrate. In case of the histidine transporter from *S. typhimurium* and maltose transporter from *E. coli* (Dean *et al*., 1992; Prossnitz *et al*., 1989), it has been shown that unliganded SBP competes with liganded SBP for binding to the translocator and that too high a SBP concentration may thus inhibit transport. This finding could not be confirmed for the Opp oligopeptide transporter from *L. lactis* (Doeven *et al*., 2004), where the

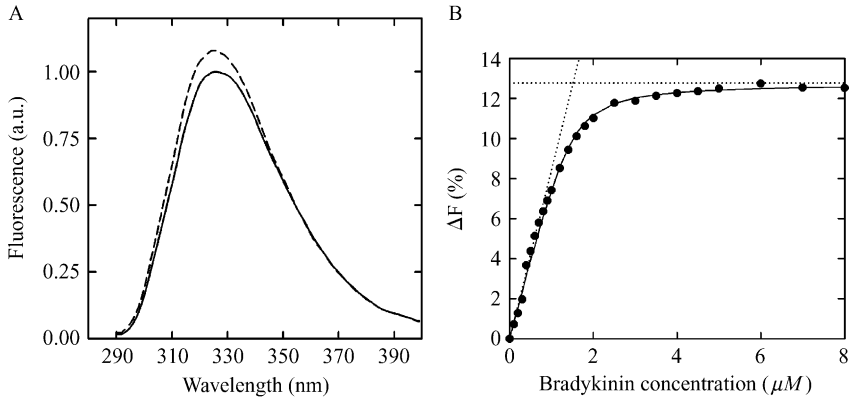

FIG. 4. Peptide binding to OppA. (A) Effect of saturating concentrations of peptide on the intrinsic protein fluorescence of OppA. Emission spectra were recorded in the absence (solid line) and presence (dashed line) of 30 μM of SLSQSKVLP. (B) Concentration dependence of the fluorescence increase (ΔF) induced by bradykinin. The intercept of the two dotted lines is at a peptide concentration of 1.53 μM. Because the concentration of OppA in this experiment was 1.49 μM, approximately 1 mol of bradykinin is bound per mole of OppA. The solid line through the data points represents the best fit; data were taken from Lanfermeijer *et al.* (1999).

affinity of the OppBCDF translocator for liganded OppA appears to be much higher than for unliganded OppA. The affinity of the OppBCDF translocator for liganded OppA was determined by measuring in proteoliposomes (see later) uptake rates at increasing OppA concentrations and saturating amounts of substrate (essentially all the OppA molecules had a peptide bound). The affinity for unliganded OppA was estimated at limiting concentration of substrate, thereby keeping the concentration of liganded OppA essentially constant and low compared to the concentration of unliganded OppA. Although these transport-based assays do not yield direct estimates of K_D values for SBP binding (transport instead of binding activity is measured), the experiments can provide important information on the initial steps in translocation by ABC transporters.

In an elegant series of experiments, Davidson and co-workers (Chen *et al.*, 2001; Davidson, 2002) characterized the binding of maltose to maltose-binding protein (MalE) in the presence of DDM-solubilized $MalFGK_2$ and under conditions where Mg-ATP (or Mg-ADP) was present, either with or without added *ortho*-vanadate. Vanadate inhibits ABC transporters and, in case of the maltose system, trapped ADP in one of the two nucleotide-binding sites immediately after ATP hydrolysis. [γ-^{32}P] ATP-Mg and [α-^{32}P]ATP-Mg were used to monitor binding of ATP and

ADP, respectively, to establish the nucleotide-bound state(s) of the transporter. In these experiments, glycerol was used as a cosolvent to stabilize the oligomeric complex. MalE, $MalFGK_2$, and radiolabeled substrates were separated by ion-exchange chromatography, and the radioactivity associated with each of the protein fractions was determined. These experiments demonstrated that in the vanadate-trapped state, MalE was bound tightly to $MalFGK_2$, whereas both molecules eluted separately in the absence of vanadate. Moreover, they showed that [^{14}C]maltose, tightly bound to free MalE, did not coelute with MalE-$MalFGK_2$. Apparently, upon docking of liganded MalE onto $MalFGK_2$, a ''vanadate-stabilized'' transition state is formed, provided ATP was present. ATP binding to NBDs opens MalE and allows maltose to enter the translocator and subsequently be released to the cytoplasm. In Fig. 2, these steps are indicated schematically as ''docking'' and ''donation.'' Thus, more complex ligand–protein and protein–protein-binding assays are possible with ABC transporter complexes in the detergent-solubilized state, and these studies have been instrumental in dissecting the individual steps of the translocation process.

For SBP-independent efflux systems, the substrate binds only in the transmembrane domains and one needs the detergent-solubilized or membrane-reconstituted translocator complex for binding studies. The best studied example is P-glycoprotein[1] (Lugo *et al*., 2005), but data on drug binding to the multidrug transporter LmrA (Alqawi *et al*., 2003) from *L. lactis* have also been reported. These systems bind and export relatively large and hydrophobic substrates, and a wide variety of fluorescent and photoaffinity probes are available as reporters of substrate binding and/or transport. Other substrates, such as daunomycin, verapamil, steroid and bile acid conjugates, glucuronide, and glutathione conjugates, are available in radiolabeled form (see Holland *et al*., 2003). Despite complications in data analysis, there is compelling evidence that P-glycoprotein and members of the MRP family have multiple, partially overlapping, substrate-binding sites in the hydrophobic domain. The broad specificity of these systems for hydrophobic substrates offers an advantage in the choice of ligands, but it also has the disadvantage that these molecules readily partition into detergent micelles and lipid bilayers so that binding and transport data are often complex. Moreover, certain detergents and/or lipids can represent substrates of the multiple drug resistance (MDR)-type

[1] There is an enormous amount of data on the binding and transport of substrates to P-glycoprotein and other MDR types of ABC transporters for which Holland *et al.* (2003) is a good starting point; the majority of these studies involve crude membrane preparations with amplified levels of transporter rather than purified and reconstituted proteins.

transporters, which complicates the analysis even further (Borst *et al*., 2003; Putman *et al*., 1999). It is beyond the scope of this chapter to discuss these issues further.

Binding of Nucleotides and Fluorescent Nucleotide Analogues to NBDs

ATP, ADP, nonhydrolyzable ATP analogues such as AMP-PNP and ATP-γS, and azido derivatives of ATP and ADP, either with or without vanadate-induced trapping of nucleotides, have been used in numerous studies to delineate the mechanism by which ATP binding and hydrolysis are coupled to substrate translocation. It is beyond the scope of this chapter to evaluate each of these methods. This section describes a simple protocol to evaluate the functional integrity of ABC transporters in the detergent-solubilized state by monitoring binding of the fluorescent nucleotide derivative 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) or the ADP derivative TNP-ADP. These compounds bind to ATP-binding cassettes with much higher affinity than ATP or ADP and are hydrolyzed slowly (Qu *et al*., 2003). TNP-labeled nucleotides are weakly fluorescent in aqueous solution, but their quantum yield is enhanced greatly upon transfer to a hydrophobic environment, such as the nucleotide-binding site of a protein. The high affinity of TNP-ATP, compared to ATP, is most likely caused by hydrophobic interactions of the TNP moiety with the protein, whereas the specificity of binding is fully conserved by the ATP moiety.

In ABC transporters such as P-glycoprotein, ATP hydrolysis and drug transport can be poorly coupled and, in the absence of substrate, significant ATPase activity can be observed even in the detergent-solubilized state.[2] In transporters such as OpuA, ATP hydrolysis and substrate uptake are tightly coupled and, irrespective of the presence of substrate, ATPase activity is negligible in the detergent-solubilized state. However, upon membrane reconstitution, the OpuA system is fully functional and hydrolyzes ATP in the presence of substrate. Instead of reconstituting individual protein fractions, it is much more convenient to obtain first an indication of the functional and structural integrity of a system by measuring TNP-ATP binding, for example, for crystallization trials and detergent or cosolvent screens. TNP-ATP binding to a number of ABC transporters has been reported, including P-glycoprotein (Qu *et al*., 2003), the nucleotide-binding domains of human CFTR (Kidd *et al*., 2004) and OpuA from *B. subtilis* (Horn *et al*., 2003), the MDR transporter BmrA from *B. subitilis* (Steinfels *et al*., 2004),

[2] In some cases, it is not clear if the lipid or detergent served as substrate and thereby enhanced ATPase activity.

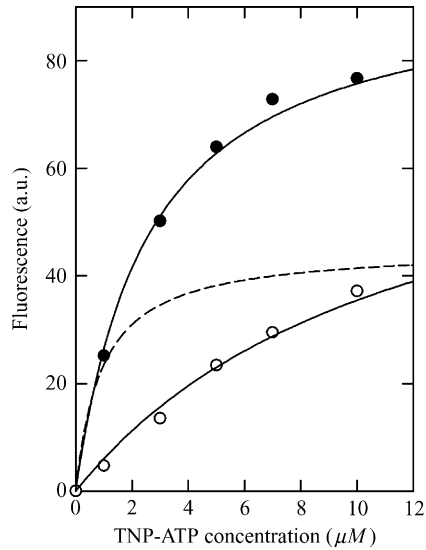

FIG. 5. Binding of TNP-ATP to OpuA (unpublished experiment). TNP-ATP was added stepwise to 50 m*M* potassium phosphate, pH 7.0, 200 m*M* KCl, 20% (v/v) glycerol, and 0.05% (w/v) DDM in the absence (○; A) and presence of 0.5 μM OpuA (●; B). The dashed line represents B–A, from which a K_D for TNP-ATP binding to OpuA of 0.9 μM was estimated. Intrinsic protein fluorescence was measured at excitation wavelength settings of 408 ± 2 nm, and fluorescence emission was collected from 520 to 560 nm; measurements were carried out at 20° in a total volume of 1.0 ml.

and OpuA from *L. lactis* (unpublished), but the method is not yet widely used.

PROCEDURE 5. Binding of TNP-ATP (or TNP-ADP) is conveniently observed at protein concentrations of 0.5 μM and fluorescence excitation at 408 ± 2 nm and emission settings at 520–560 nm. To obtain the K_D of TNP nucleotide binding, titration data in the absence of protein need to be subtracted from data in the presence of protein; a typical example of TNP-ATP binding to DDM-solubilized OpuA is shown in Fig. 5. Moreover, by titrating protein-bound TNP-ATP with ATP or ADP, one can obtain an estimate of the affinity constant for binding of these genuine nucleotides. An analysis of the stoichiometry of TNP nucleotide binding to P-glycoprotein is presented in Qu *et al.* (2003). In case of OpuA, negligible hydrolysis of ATP or TNP-ATP is observed at 20–30°. For systems that hydrolyze TNP-nucleotides, measurements may have to be carried out at 4° and/or in the absence of Mg^{2+} to minimize hydrolysis.

Membrane Reconstitution

Strategy and Procedure

The most commonly employed methods for the reconstitution of membrane proteins are based on mixing detergent-solubilized protein together with lipid, either dispersed in detergent or in the form of detergent-destabilized preformed liposomes, followed by subsequent removal of detergent by dilution, dialysis, gel filtration, or adsorption to polystyrene beads (Rigaud *et al*., 2003). Although each of these methods has been used in combination with a variety of detergents, the dilution, dialysis, and gel filtration techniques are not very efficient in removing low CMC detergents. Because proteins are generally more stable in low CMC detergents such as DDM (and equivalents of the Fos-choline or cyclohexyl-alkyl series) and Triton X-100, these detergents are often preferred for membrane reconstitution and are removed most efficiently by adsorption to polystyrene beads (BioBeads SM2, Bio-Rad Inc.). These beads have an adsorption capacity for detergents of 0.2–0.45 mmol/g of wet beads, whereas the binding of lipid is low, provided the bead-to-detergent ratio is low in the initial stages of reconstitution. In the following experiments, the ABC transporters are generally purified in 0.05% (w/v) DDM or 0.05% Triton X-100, corresponding to ~1 m*M* of detergent. Due to binding to the proteins, however, the actual concentration of detergent in a protein solution will be significantly higher (see later).

To find optimal conditions for membrane reconstitution, the strategy based on the stepwise solubilization of preformed liposomes and protein incorporation at the different stages of liposome solubilization is used (Knol *et al*., 1998; Lichtenberg, 1985; Rigaud *et al*., 1988). The physical state of the liposomes during the titration with detergent is followed by measuring the optical density at 540 nm. The equilibration of detergent and lipid is temperature dependent, and the mixture is generally kept at 20° when the liposomes are titrated with detergent. The solubilization of preformed liposomes can be divided into three stages (Lichtenberg, 1985). During stage I, detergent molecules partition between the aqueous buffer and the bilayer. Stage II starts when liposomes are saturated with detergent, which defines the onset of solubilization ($=R_{sat}$) and continues upon a further increase of the detergent concentration, thereby inducing liposome solubilization and the formation of micelles. At stage III, when the optical density has reached its minimal value (R_{sol}), the mixture consists of micelles at varying detergent/lipid ratios. The parameters R_{sat} and R_{sol} for various detergents describing the solubilization of liposomes can be found in Rigaud *et al*. (2003).

A good starting point is to mix purified detergent-solubilized protein with preformed liposomes that have been treated with an amount of deter-

gent corresponding to R_{sat} plus 10–20%. For liposomes at a lipid concentration of 4 mg/ml and suspended in 100 m*M* potassium phosphate, pH 7.0, this corresponds to 4–5 m*M* of DDM or 1.6–2 m*M* of Triton X-100. Little or no insertion of protein occurs at detergent concentrations below R_{sat}. The efficiency of membrane reconstitution beyond R_{sat} depends heavily on the detergents used (Knol *et al.*, 1998; Rigaud *et al.*, 1988). Triton X-100 mediates an efficient reconstitution in the range of R_{sat} to R_{sol}, whereas other detergents such as DDM are most effective at R_{sat}. These variations reflect in part the types of structures formed when preformed liposomes are treated with different detergents (Knol *et al.*, 1998). In addition, the optimal detergent concentration to use also depends on the protein. In our hands, Triton X-100-mediated incorporation consistently leads to a better reconstitution efficiency in terms of ''translocation activity'' than when DDM or other detergents are used. However, we prefer DDM (and other detergents) over Triton X-100 for solubilization and purification because of the high UV absorbance associated with Triton X-100, which precludes accurate protein determination and turbidity measurements by UV spectroscopy. Therefore, many of our reconstitutions involve two detergents: DDM (or other amphiphile) to purify the protein and Triton X-100 to destabilize the preformed liposomes and facilitate reconstitution.

The equilibration of liposomes with detergent requires seconds to minutes for Triton X-100, whereas DDM equilibrates very slowly when the detergent is added stepwise to the liposomes. The low equilibration rate is probably caused by a slow flip-flop of detergent molecules in the bilayer and accompanying rearrangement of lipid and detergent molecules. It is therefore important to follow the titration carefully by measuring the A_{540} to ensure that equilibrium is reached prior to adding the protein. The reconstitution efficiency may be low if the system has not come to equilibrium. Following equilibration of the liposomes with the appropriate concentration of detergent, which has to be determined empirically, the mixture is equilibrated with purified protein in detergent. We routinely use protein stock solutions of ~1 mg/ml for these preparations. Following addition of protein, the detergent is subsequently removed by adsorption onto polystyrene beads (BioBeads SM2).[3] Detergent-solubilized ABC transporter is mixed with the detergent-destabilized liposomes to yield a

[3] Routinely, the purified protein and titrated liposomes are mixed and incubated for 30 min at room temperature under gentle agitation before BioBeads are added at a wet weight of 40 mg per milliliter of sample. After 15 min of incubation, fresh BioBeads (40 mg/ml) are added. The sample is incubated at 4° and subsequent additions of BioBeads (40 mg/ml) take place after intervals of 15 and 30 min, overnight, and 2 h before the BioBeads are removed by filtration. Before use, the BioBeads are washed extensively with methanol and water and stored in water at 4°.

final lipid-to-protein of 5000–50,000 to 1 (mol/mol); for a transporter complex with a molecular mass of 200 kDa (OpuA), this corresponds to 17.5–175 to 1 (g/g). At these lipid-to-protein ratios, the amount of detergent brought into the system via the protein is small compared to that needed for destabilization of the preformed liposomes (R_{sat} plus 10–20%). One can minimize the amount carried over by concentrating the protein, for example, by ultrafiltration, which may be needed when reconstitution experiments are carried out at lipid-to-protein ratios below 5000 to 1 (mol/mol). It is our experience that the membrane cutoff of Amicon Ultra (100,000 MWCO) allows passage of detergent micelles (generally <50,000 Da), while concentrating the protein–detergent complex (generally >100,000 Da). In general, we keep the volume of purified protein solution below 10% of the final volume of the liposome mixture. To calculate the contribution of detergent brought into the system via the protein, not only does the ''free'' detergent concentration need to be considered but also the amount bound to the protein. The amount of bound detergent is evaluated most conveniently by equilibrating the protein with isotopically labeled detergent, with subsequent determination of the protein concentration and radioactivity in fractions following elution from affinity or ion-exchange resins (Friesen *et al*., 2000; LeMaire *et al*., 2000; Moller *et al*., 1993).

In case of the major facilitator superfamily transporters LacS and XylP (12 predicted transmembrane segments per subunit), a binding stoichiometry of ~200 mol of DDM/mol of polypeptide was determined (Friesen *et al*., 2000; Heuberger *et al*., 2002); for a protein solution at 1 mg/ml (~14 μM LacS) purified in 1 mM DDM, this corresponds to a total detergent concentration of $1 + 2.8 = 3.8$ mM. For the ABC transporter BmrA, the number of DDM molecules bound per dimeric BmrA (12 transmembrane α helices) was found to be 380 (Ravaud *et al*., 2005), which is significantly higher than observed for LacS and XylP but consistent with data for DDM-purified OpuA. By SEC, OpuA in DDM runs at an apparent molecular mass of 460 kDa (unpublished). With a protein mass of ~200 kDa, this corresponds to binding of approximately 500 molecules of DDM per dimeric OpuA (16 predicted transmembrane segments). Thus, from these examples of different transporters, there seems to be no simple rule of thumb to assess the amount of detergent binding to the hydrophobic domain of the proteins.

The membrane reconstitution strategy outlined earlier (i.e., insertion of purified protein complexes into detergent-destabilized preformed liposomes) has been successful for several ABC transporters, that is, the MDR efflux systems LmrA (Margolles *et al*., 1999) from *L. lactis* and BmrA (Steinfels *et al*., 2004) from *Bacillus subtilis* (Fig. 1A), the oligopeptide transporter Opp (Doeven *et al*., 2004) (Fig. 1B2), the osmoregulatory glycine betaine uptake system OpuA (van der Heide *et al*., 2000) (Fig. 1C),

and the glutamine–glutamic acid uptake system GlnPQ (Schuurman-Wolters *et al*., 2005) (Fig. 1D) from *L. lactis*. The histidine uptake system $HisJQMP_2$ (Fig. 1B1) from *Salmonella typhimurium* (Ames *et al*., 2001) and the maltose transport system $MalEFGK_2$ (Fig. 1B1) from *E. coli* (Davidson *et al*., 1991) have been reconstituted via detergent dilution. In case of $HisJQMP_2$ and $MalEFGK_2$, the complexes were purified in *n*-decanoyl-sucrose and DDM, respectively, and membrane reconstitution was achieved after detergent exchange to OG, followed by detergent dilution (Ames *et al*., 2001; Chen *et al*., 2001; Davidson *et al*., 1991). Membrane reconstitution of P-gp has been achieved by dilution of OG-purified protein in the presence of lipids (Ramachandra *et al*., 1998). The cystic fibrosis transmembrane conductance regulator (CFTR), an ABC protein that acts as a chloride channel, has been reconstituted by extensive dialysis of lithium dodecyl sulfate- or sodium pentadecafluorooctanoic acid-solublized protein in the presence of sonicated lipids (Ramjeesingh *et al*., 1999). Given our experience with the membrane reconstitution of several ABC and non-ABC type of transporters, we would not generally recommend the use of these ionic detergents.

The proteoliposomes obtained after detergent removal by polystyrene beads can either be used directly for functional assays or frozen and stored in liquid nitrogen. ABC efflux systems that are reconstituted inside out can be studied by adding Mg-ATP to the external medium and monitoring the uptake of substrate. However, ABC uptake systems reconstituted right side out have the ATP-binding cassettes on the inside, and the ATP or ATP-regenerating system needs to be included in the vesicle lumen to determine substrate uptake. The inclusion of components in the vesicle lumen is often accomplished by multiple cycles of freezing and thawing, followed by extrusion of the proteoliposomes through polycarbonate filters. By freezing and slow thawing of (proteo)liposomes, the membranes fuse, with water-soluble components trapped in the lumen, but the vesicles also become multilamellar in this process. The multilamellar (proteo)liposomes can be made homogeneous and largely unilamellar by extrusion through polycarbonate filters (average pore diameter of 400, 200, or 100 nm; Avestin Inc., Ottawa, Canada). The smaller the pore diameter, the more homogeneous the vesicles are, but the more the specific internal volume decreases, which is disadvantageous for assaying transport reactions. In general, we use filters of 400 or 200 nm through which the (proteo)liposomes are extruded 11 times. A typical protocol for the reconstitution of ABC transport proteins is given in procedure 6.

PROCEDURE 6. A stock solution of liposomes at 20 mg/ml lipid concentration in 50 m*M* KPi, pH 7.0, is extruded through 400-nm pore-size polycarbonate filters, diluted to 4 mg/ml [final buffer composition 50 m*M*

KPi, pH 7.0, 20% (w/v) glycerol], and titrated using Triton X-100. The Ni^{2+}-NTA-purified ABC transporter at a protein concentration of 0.1–1 mg/ml in elution buffer [50 m*M* KPi, pH 7.0, 200 m*M* KCl, 20% (w/v) glycerol, 0.05% (w/v) DDM plus 200 m*M* imidazole] is mixed with the detergent-destabilized liposomes to give a protein:lipid ratio of 1:100 (w/w). The 20% (w/v) glycerol in the buffers is only used for ABC transporter complexes that disassemble in the absence of the cosolvent. In case imidazole has a destabilizing effect, it can be replaced by histidine. The protein and the liposomes are incubated for 30 min at room temperature while shaking gently. To remove the detergent, 40 mg/ml wet weight BioBeads SM2 are added, followed by a 15-min incubation at room temperature. BioBeads SM2 are added four more times, and the incubation times are 15 min, 30 min, overnight, and 1 h at 4°. After five times dilution with 50 m*M* KPi, pH 7.0 (to lower the glycerol concentration), the ABC transporter-containing proteoliposomes are collected by centrifugation for 1.5 h at 150,000*g* and 4°, resuspended to 20 mg/ml of lipid in 50 m*M* KPi, pH 7.0, flash frozen, and stored in liquid nitrogen.

Membrane reconstitution of LmrA, BmrA, OpuA, and GlnPQ has been performed in one or two steps, that is, the insertion of the translocator complex into the membrane either with or without freeze–thaw extrusion to incorporate ATP or an ATP-regenerating system into the (proteo) liposome lumen as appropriate (see later). For the reconstitution of Opp or equivalent systems, depicted schematically in Fig. 1B2, the procedure is somewhat more complex because of the requirement for lipid-anchored substrate-binding protein. Membrane reconstitution of the oligopeptide transporter was achieved via a three-step procedure (Doeven *et al.*, 2004). First, the purified translocator complex OppBCDF in DDM was incorporated into Triton X-100-destabilized liposomes. Thereafter, purified OppA was anchored to the outside of OppBCDF containing liposomes via its N-terminal lipid modification by absorbing the purified protein in 0.05% (w/v) DDM to the (proteo)liposomes, followed by removal of residual detergent with polystyrene beads. This resulted in proteoliposomes containing all five component proteins of the Opp system. Finally, ATP or an ATP-regenerating system was incorporated into the vesicle lumen by freeze–thaw extrusion.

ATP versus ATP-Regenerating System

ABC transporters are driven by ATP but are strongly inhibited by the hydrolysis product ADP. In experiments where ATP is added to the external medium of a proteoliposome suspension, the amount of ADP formed (micromolar) is often low compared to the amount of ATP present

(millimolar), and ADP inhibition may be low. However, when ATP is included in the vesicle lumen, the decrease in ATP and the accompanying increase in ADP are substantial and transport may halt after a few minutes, even when residual ATP is still present (Patzlaff *et al.*, 2003). Higher levels of uptake can be attained by incorporating an ATP-regenerating system in the vesicle lumen, but even then uptake will eventually level off due to the accumulation of ADP.

PROCEDURE 7. In a typical experiment (final concentrations are indicated), ATP[-Mg] at 3–10 m*M* and adjusted to pH 7.0 together with creatine kinase (2 mg/ml; Roche Diagnostics, Mannheim, Germany), creatine-monophosphate[-Na] (20–30 m*M*) plus 50 m*M* phosphate[-K or -Na salt] are mixed with the proteoliposomes (20 mg of lipid/ml; see procedure 6), and the mixture is frozen in liquid nitrogen and thawed slowly (tubes with 0.5 ml of proteoliposomes are placed in contact with the wall of a Styrofoam block) at room temperature. The freeze–thaw cycle is repeated two to five times (see later), after which the proteoliposomes are made homogeneous by extrusion through polycarbonate filters. It is critical to flush the extruder with buffer plus Mg-ATP or ATP-regenerating system before and after the final extrusion of the proteoliposomes through polycarbonate filters. Subsequently, external components are removed by ultracentrifugation (300,000*g* for 15 min), and the proteoliposomes are washed and resuspended in isotonic buffers, usually 50–100 phosphate[-K or -Na salt] at pH 7.0.

In many cases, two to three cycles of freezing and thawing are sufficient to trap ATP or ATP-regenerating system and other components in the vesicle lumen. It is our experience that many membrane proteins insert into preformed liposomes in a particular orientation (Knol *et al.*, 1996), predominantly inside out or right side out, and multiple cycles (five or more) of freezing and thawing are needed to obtain a random orientation of the protein (unpublished observations); an example is given in the Section ''ABC Transporters in Large Unicellular Vesicles,'' illustrates the advantages of a random protein orientation.

Choice of Lipids

Although mixtures of synthetic lipids can be used as sources of exogenous lipids, most bacterial transport systems tested to date show good activity when reconstituted into liposomes composed of a 3 to 1 mixture of *E. coli* total lipids and egg PC. The *E. coli* total lipid mixture contains ~75% phosphatidylethanolamine (PE), ~20% phosphatidylglycerol (PG) and ~5% cardiolipin (CL) or ~75% PE, ~5% PG plus ~20% CL, depending on the growth phase at which the cells were harvested for the isolation of

the lipids. Although purified *E. coli* lipids can be purchased from Avanti Polar Lipids Inc., it is our experience that one obtains a much better preparation by self-purifying the crude total lipid extract of Avanti with an acetone/diethylether wash following published procedures (Newman and Wilson, 1980). For systems that depend critically on a particular lipid composition, it is desirable to use synthetic lipids, as the quality of commercial total lipid extracts can vary from batch to batch. A good starting point for a mixture of synthetic lipids is 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC, bilayer-forming zwitterionic lipid), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol (DOPG, bilayer-forming anionic lipid), and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE, nonbilayer-forming zwitterionic lipid) in a ratio of 1:1:2 (van der Heide *et al*., 2001). In place of dioleoyl (18:1), one can also use dipalmitoleoyl (16:1) lipids.

ABC Transporters in Large Unilamellar Vesicles (LUVs)

Substrate Import versus Export

Proteoliposomes obtained after extrusion through 200-nm polycarbonate filters have an average diameter of 170 ± 50 nm and are often described as large unilamellar vesicles. In order to monitor uptake into these vesicles, one could have a preference for ABC efflux systems reconstituted ''inside out'' and ABC uptake systems ''right side out,'' but it is not possible to predict beforehand whether a system will be incorporated in a particular orientation. A random orientation has the disadvantage that half of the molecules do not participate in the transport reaction. However, one can also take advantage of a random orientation, as illustrated in the experiment presented in Fig. 6. After five cycles of freeze–thawing and subsequent extrusion through 200-nm polycarbonate filters, the OpuA molecules are oriented randomly. This results in half of the molecules having their substrate-binding domains on the outside and the ATP-hydrolyzing subunits on the inside (''*in vivo* or right-side-out orientation''), whereas the other half has the ''inside-out orientation.'' Because transport of glycine betaine by OpuA is unidirectional and dependent on access of the ABC subunits to the membrane-impermeant cosubstrate, ATP, molecules with the right-side-out and inside-out orientation can be studied separately. Figure 6A shows the uptake of glycine betaine via right-side-out reconstituted OpuA. After approximately 10 min, the uptake halted because of depletion of ATP and build up of ADP. At that point (Fig. 6B), Mg-ATP was added to the assay medium to effect the exit of glycine betaine from the proteoliposomes via inside-out reconstituted OpuA. Figure 6B shows that inside-out reconstituted OpuA was only activated when a high

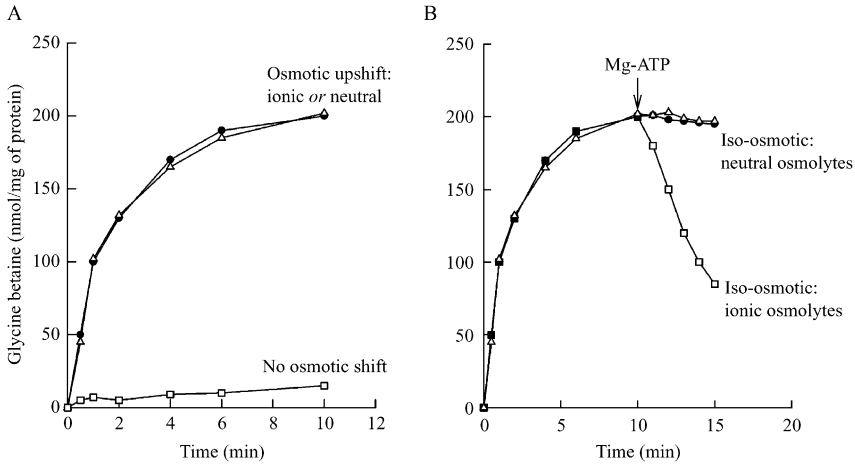

FIG. 6. Glycine betaine uptake and efflux in proteoliposomes containing OpuA. (A) Uptake of glycine betaine in proteoliposomes preloaded with ATP-regenerating system and resuspended in 50 m*M* KPi, 100 m*M* KCl, pH 7.0. Uptake assays were performed under isoosmotic (50 m*M* KPi, 100 m*M* KCl, pH 7.0, □) or hyperosmotic conditions (50 m*M* KPi plus 400 m*M* KCl, pH 7.0, ● 50 m*M* KPi plus 430 m*M* sucrose, pH 7.0, Δ). (B) After 10 min of uptake, the efflux of glycine betaine was stimulated by the addition of 50 m*M* KPi, pH 7.0, containing 9 m*M* ATP/Mg^{2+} in the presence of 430 m*M* sucrose (Δ) or 400 m*M* KCl (□). Modified after Patzlaff *et al.* (2003).

concentration of electrolyte was present externally and not with an equivalent amount of sugar, indicating that OpuA is in fact sensing ionic strength rather than osmotic stress (for details, see van der Heide *et al.*, 2001; Poolman *et al.*, 2004). The unidirectionality of transport and the two populations of OpuA molecules allowed us to distinguish between effects exerted by effector molecules at the external and cytoplasmic face of the transporter. In case of OpuA, studies of right-side-out and inside-out OpuA were important for elucidating the osmosensing mechanism of the transporter (Poolman *et al.*, 2004; van der Heide *et al.*, 2001) and determining the ATP/substrate stoichiometry (Patzlaff *et al.*, 2003) (next section).

ATP/Substrate Stoichiometry

In addition to monitoring transport in proteoliposomes, one can also follow the hydrolysis of ATP. With ATP inside the vesicles, the decrease in ATP (and increase in ADP), associated with substrate transport via right-side-out-oriented ABC uptake, can be determined by the luciferin-luciferase assay (ATPlite-M Packard Inc., Groningen, The Netherlands)

(McElroy and DeLuca, 1983). For inside-out-oriented ABC uptake systems or right-side-out-oriented efflux systems, the decrease in ATP concentration is too low to be determined by luciferin-luciferase due to the large external volume. An alternative method, not dependent on a decrease in ATP concentration, involves measurement of the appearance of inorganic phosphate (malachite green-based phosphate assay) (Hess *et al.*, 1975), associated with ATP-driven transport. In this experiment, the concentration of external ATP is kept constant (e.g., around 10 m*M*). This more sensitive detection method requires phosphate-free buffers. In the case of OpuA and some other transporters, we have noted that the transport activity in proteoliposomes after reconstitution from phosphate buffers is consistently higher than when phosphate-free media are used. In these cases, proteoliposomes were prepared in phosphate-based buffers, after which external phosphate was removed by extensive washing (gel filtration and/or ultracentrifugation) with an alternative isotonic buffer.

To date, there is no consensus on the ATP/substrate stoichiometry for members of the ABC transport family. Indeed, quite the opposite is true, with variations in stoichiometries ranging from 1 to 50 reported even when measured on the same ABC transporter (see Patzlaff *et al.*, 2003). In initial stoichiometry measurements performed with right-side-out-oriented OpuA, we observed variations in stoichiometries in different batches of proteoliposomes that were beyond our control. By sizing the proteoliposomes through 400-, 200-, and 100-nm polycarbonate filters, we could demonstrate a systematic increase in stoichiometry of right-side-out-reconstituted OpuA with decreasing size of the proteoliposomes. Measurement of ATP/substrate stoichiometries on the population of inside-out-oriented OpuA proved that the size of the proteoliposome did not affect the stoichiometry. In each sample, we observed an ATP/substrate stoichiometry of approximately two with no deviation with respect to proteoliposome size. With right-side-out OpuA, ATP/substrate stoichiometry was determined from the decrease in luminal ATP levels, and the ATP concentration (and ATP/ADP ratio) decreased significantly in the course of the experiment. With inside-out-oriented OpuA, the ATP was present on the outside and could be kept constant at approximately 10 m*M*, which apparently ensures a better coupling than when the ATP and the ADP concentration vary. As stated earlier, it is possible that some transporters hydrolyze one ATP per substrate translocated, whereas other systems such as OpuA use two molecules of ATP, but we feel that many of the stoichiometry values reported in the literature suffer from experimental artifacts, for example, poor coupling between ATP hydrolysis and transport, and substrate leakage (see next paragraph) (Patzlaff *et al.*, 2003), so that most of the earlier experiments may need to be reevaluated.

Fluorescent Substrates to Monitor Transport

Most transport assays are based on determining the distribution of radiolabeled substrates between the inner and the outer compartment of a cell or vesicle. External label is separated from accumulated label by rapid filtration, followed by the measurement of filter-bound radioactivity; the procedures and materials are essentially the same as described for the substrate-binding assays (procedures 2 and 3). However, the nature of the substrate can pose problems, particularly when hydrophobic substrates are used. Hydrophobic substrates, for example, those used by ABC efflux systems such as P-glycoprotein and homologues, leak out relatively easily (during the washing of the filters), resulting in underestimation of the transport activities (and overestimation of ATP/substrate stoichiometries). The leakage of hydrophobic substrates is very prominent in proteoliposomal systems, where the membrane–lipid surface area is relatively large and passive leakage is even more prominent than in native membranes. Fluorescent substrates can offer an alternative as measurements can be performed on-line and separation of internalized and external substrate is not necessary. Particularly powerful are substrates such as ethidium that become highly fluorescent when intercalated to DNA. These measurements, however, require inclusion of DNA in the proteoliposome lumen and removal of external DNA. Because ethidium is relatively hydrophobic, it is important to perform the appropriate controls and compare liposomes with and without ABC transporter (or use inactivated transporter). If DNA intercalation is not the rate-limiting process, one observes a difference in the rate of fluorescence increase when transporter-mediated ethidium uptake is faster than passive diffusion of ethidium.

Hoechst 33342, however, not only becomes highly fluorescent in the presence of DNA, but also when trapped inside the hydrophobic part of the membrane, and this compound is used frequently to assay the activity of MDR type transporters, not only in proteoliposomes, but also in intact cells (Lubelski *et al*., 2004; Lugo *et al*., 2005; Putman *et al*., 2000). It has been noted that the interpretation of transport data obtained with Hoechst 33342 is not as straightforward as often assumed because of the complex pH dependence of the fluorescence spectrum of the compound (Mazurkiewicz, 2004). When Hoechst 33342 is dissolved in aqueous media, the fluorescence quantum yield is much higher at pH 5.0 than at 8.0, but the opposite is true in the presence of liposomes. The increase of Hoechst 33342 fluorescence with increasing pH in the presence of membranes is most likely due to a more efficient partitioning of deprotonated probe into the lipid bilayer. As Hoechst 33342 can exist in four different

ionisation states, that is, from 0 to 3+, each with its own lipid-partitioning coefficient, the interpretation of fluorescence traces can thus be highly complex.

ABC Transporters in Giant Unilamellar Vesicles (GUVs)

Giant Unilamellar Vesicles

Giant unilamellar vesicles have been widely used for studies on lipid mobility, membrane dynamics, and lipid domain (raft) formation using single molecule techniques such as fluorescence correlation spectroscopy (FCS) (Kahya *et al*., 2003). Reports on membrane protein dynamics in these types of model membranes are by far less advanced due to the difficulty of incorporating proteins into GUVs in a functional state.

Protein-containing GUVs can be prepared by drying proteoliposomes (LUVs) followed by the addition of aqueous medium. Water penetrates the dried lamellar structures and GUVs are formed spontaneously due to membrane fusion processes, particularly when 10–25% (w/w) of anionic lipid is present or 10 m*M* of Mg^{2+} is added to neutral lipids after prewetting (Akashi *et al*., 1998). In addition, AC electrical fields have been reported to facilitate or impede GUV formation. The major bottleneck for direct incorporation of membrane proteins into GUVs is the dehydration step preceding the formation process. When (proteo)liposomes prepared from unsaturated lipids are dried, the transition temperature (T_M) increases by 70–80° (Ricker *et al*., 2003). This causes the lipids to go from a liquid crystalline to a gel phase, which induces lateral phase separation. These events may cause the protein to aggregate and lose activity. Kahya *et al*. (2001) circumvented this problem by using peptide-induced fusion of LUVs, containing the membrane protein of interest, with preformed GUVs. Although this method has been applied successfully to study the dynamics and aggregation state of bacteriorhodopsin in GUVs (Kahya *et al*., 2002), the method is laborious and requires the presence of an unusual lipid (not commercially available) and a fusogenic peptide in the model membranes. Alternative methods (Folgering *et al*., 2004; Girard *et al*., 2004) for incorporating polytopic membrane proteins into GUVs involve (partial) dehydration of LUVs containing (purified) membrane proteins, followed by rehydration in the presence of an AC electrical field (Angelova *et al*., 1992). This method is suitable for highly stable membrane proteins, but not for more labile complexes such as ABC transporters. For single molecule techniques such as FCS, fluorescence resonance energy transfer (FRET), and atomic force microscopy (AFM), it is essential that heterogeneities due to nonproductive protein conformations can be ruled

out and that 100% protein activity can be recovered. The following procedure takes advantage of the stabilizing properties of disaccharides on membranes and proteins to incorporate membrane protein(s) (complexes) into GUVs (Doeven *et al.*, 2005), including, among others, the lipid-anchored peptide-binding protein OppA and the translocator complex OppBCDF of the oligopeptide ABC transporter from *L. lactis*. Sucrose (and trehalose) prevents lateral phase separation and presumably protein aggregation during drying by maintaining the membrane in the liquid crystalline phase; the sugars keep the phase transition temperature, T_M, of the membrane low (Ricker *et al.*, 2003). In addition, sucrose may have a stabilizing effect during drying by direct interaction (hydrogen bonding) of the sugar with polar groups of the protein.

Conversion of LUVs into GUVs

In the following method, protein activity can be preserved by adding sucrose during drying of protein-containing LUVs; depending on the system, as little as 20–100 mg sucrose/g of lipid is sufficient for the recovery of full activity.

PROCEDURE 8. LUVs (10 μl of 20 mg/ml lipids), containing Alexa Fluor 488-labeled membrane protein at a given protein-to-lipid ratio in 50 m*M* NH_4HCO_3, pH 8.0, are dried overnight under vacuum at 4° on UV-ozone-cleaned glass or ITO-coated coverslips (custom coated by GeSim, Dresden, Germany) in a custom-built sample chamber. UV-ozone cleaning is not essential for GUV formation but increases the wetting properties of the coverslip surface, making it easier to dry liposomes from aqueous solution. Sucrose is added to stabilize the proteins during dehydration. A side effect of sugars during drying is that they inhibit membrane fusion (Hincha *et al.*, 2003). However, there appears to be an optimal sucrose concentration at which membrane protein activity is retained (20–100 mg sucrose/g lipid) and membrane fusion is still possible (<0.86 g sucrose/g lipid) (Doeven *et al.*, 2005). Rehydration is performed by adding 0.5 ml of 10 m*M* potassium phosphate, pH 7.0, at room temperature. For electroformation of GUVs (Angelova *et al.*, 1992), lipids are dried onto ITO-coated coverslips, and a Pt wire is assembled 1 mm above the ITO-coated slide in the sample chamber, after which an AC electric field is applied (10 Hz, 1.2 V). GUV formation is monitored by fluorescence microscopy.

Fluorescence Correlation Spectroscopy

The lateral mobility of OppA and OppBCDF in GUVs was determined by FCS measurements as described (Doeven *et al.*, 2005). Confocal images were made of GUVs containing fluorescent-labeled protein, and the focal

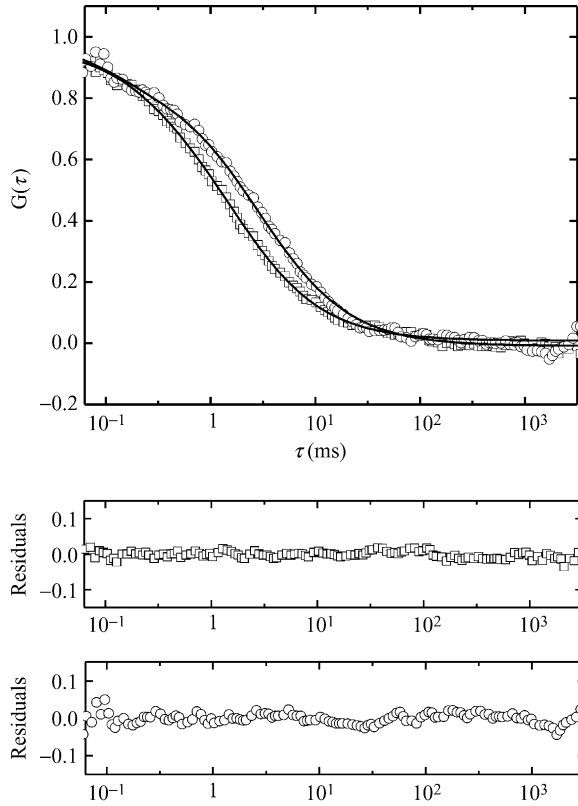

FIG. 7. Diffusion of Opp ABC transporter components in GUVs (unpublished result). Autocorrelation curves for OppA I602C (□) and OppBC(I296C)DF (○) in GUVs. Proteins were labeled with Alexa Fluor 488 as described (Doeven *et al.*, 2005). Curves were fitted with a one-component, two-dimensional diffusion model (solid lines) using Origin software (OriginLab Corporation, Northampton, MA); the residuals of the fits are shown below the figure.

volume was focused on the pole of the GUVs. Representative autocorrelation curves for OppA and OppBCDF are shown in Fig. 7. The lipid-anchored oligopeptide-binding protein OppA diffused with the same speed as fluorescent lipid probes, indicating that OppA does not have interactions with the membrane other than through its lipid anchor. The integral membrane protein complex OppBCDF diffused two times slower compared to OppA, which is in accordance with the Saffman and Delbruck model for diffusion in biological membranes (Saffman *et al.*, 1975). We are currently analyzing, at the single molecule level in GUVs by fluorescence

auto- and cross-correlation spectroscopy, the effects of substrate (peptides) and activation state (ATP or nonhydrolyzable nucleotides) on the interactions of OppA with OppBCDF.

Concluding Remarks

At present more than 10 different ABC transporters have been purified and reconstituted in artificial membranes in functional form, and the methods presented here should with minor, if any, modifications be applicable to many more systems. A concern with multisubunit membrane protein complexes is their instability in the detergent-solubilized state. As the functional state of ABC transporters is often characterized by measurements of nucleotide binding and/or hydrolysis, loss of functional and structural integrity of the membrane-embedded domain may go unnoticed. In fact, the relatively high ATPase activity of many ABC transporters in the absence of substrate may actually be caused by dissociation of the complexes in the presence of detergent. The high membrane protein concentrations employed in crystallization trials will necessarily involve significantly higher detergent concentrations bound to the protein (see earlier discussion), which could have profound consequences for the subunit–subunit associations and conformation of the membrane-spanning region. For example, the subunit arrangement reported for the MsbA lipid A exporter from *E. coli* may reflect a partial dissociation of the subunits, mediated by the detergent, during the crystallization process. By screening a range of detergent and (co-)solvent conditions, it has been possible, for the vast majority of membrane proteins studied since the mid-1990s, to obtain intact transporter complexes. The full functionality of the systems can only be assessed after insertion of the proteins into lipid vesicles, for which lipid to protein ratios of 5000–50,000 to 1 on mole basis are generally sufficient. In our hands, it has proven difficult to incorporate proteins into artificial lipid vesicles at lipid-to-protein ratios below 5000, that is as required for various types of biophysical analyses (ESR, FTIR, NMR). Although protein may seem associated with the lipid vesicles as assessed from density centrifugation experiments (unpublished observations), a significant fraction of the protein may not be inserted correctly into the membrane (and may not be translocation competent). In fact, the specific activity of the systems studied to date drops below a lipid-to-protein ratio of 5000. The recent achievement of incorporating ABC transporters and other membrane proteins into giant unilamellar vesicles offers opportunities to study the proteins not only in ensemble, but also at the single molecule level.

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