The ATP/Substrate stoichiometry of the ATP-binding cassette (ABC) transporter OpuA

Patzlaff, JS; Poolman, B; Patzlaff, Jason S.

Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.M304796200

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2003

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Patzlaff, JS., Poolman, B., & Patzlaff, J. S. (2003). The ATP/Substrate stoichiometry of the ATP-binding cassette (ABC) transporter OpuA. The Journal of Biological Chemistry, 278(32), 29546-29551. https://doi.org/10.1074/jbc.M304796200

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ATP-binding cassette (ABC) transport proteins catalyze the translocation of substrates at the expense of hydrolysis of ATP, but the actual ATP/substrate stoichiometry is still controversial. In the osmoregulated ABC transporter (OpuA) from Lactococcus lactis, ATP hydrolysis and substrate translocation are tightly coupled, and the activity of right-side-in and inside-out reconstituted OpuA can be determined accurately. Although the ATP/substrate stoichiometry determined from the uptake of glycine betaine and intravesicular ATP hydrolysis tends to increase with decreasing average size of the liposomes, the data from inside-out reconstituted OpuA indicate that the mechanistic stoichiometry is 2. Moreover, the two orientations of OpuA in proteoliposomes allowed possible contributions from substrate (glycine betaine) inhibition on the trans-side of the membrane and inhibition by ADP to be determined. Here we show that OpuA is not inhibited by up to 400 mM glycine betaine on the trans-side of the membrane. ADP is an inhibitor, but accumulation of ATP was negligible in the assays with inside-out-oriented OpuA, and potential effects of the ATP/ADP ratio on the ATP/substrate stoichiometry determinations could be eliminated.

ATP-binding cassette (ABC) transporters, found in both prokaryotes and eukaryotes, play an important role in various physiological processes ranging from uptake of nutrients, multidrug resistance, secretion of signal molecules or toxins, cell volume regulation, and other processes (reviewed in Refs. 1–4). Despite the diversity of functions and substrates of the ABC transporters, all members fuel the translocation process by ATP hydrolysis. Also, the basic architecture of ABC transporters is remarkably similar among members in the superfamilies. Always present are two cytoplasmic-exposed nucleotide-binding domains, the ATP-binding cassettes, and two hydrophobic domains that are predicted to span the membrane multiple times in an α-helical conformation (2). The conserved assembly and arrangement of these domains have been clearly demonstrated in the recently determined structure of BtuCD, the transporter, OpuA, from Lactococcus lactis which responds to osmotic stress, which is sensed at the cytoplasmic face as a change in ionic strength (7–9).

The issue of ATP/substrate stoichiometry for members of the ABC transport family is still a subject of debate. ATP/substrate stoichiometries for a variety of ABC transporters range from 1 to 50 (10–20). Given the similarity in architecture between different members of the ABC superfamily, it is likely that a universal mechanistic stoichiometry is applicable to all members. The large variations observed are most likely the result of experimental flaws, differences in the setup of the experiments, and/or the need to correct for uncoupled ATP hydrolysis and/or substrate leak pathways. The OpuA system affords optimal conditions for determination of ATP/substrate stoichiometries because ATP hydrolysis is tightly coupled to the osmotic signal and the presence of substrate. In this report, we perform stoichiometry measurements on proteoliposomes in which OpuA has been functionally reconstituted. We have performed two independent assays to derive the stoichiometries and conclude that 2 molecules of ATP are hydrolyzed per molecule of glycine betaine translocated.

EXPERIMENTAL PROCEDURES

Materials—M17 broth was purchased from Difco, nickel-nitrilotriacetic acid resin was obtained from Qiagen Inc., Biobeads SM-2 were from Bio-Rad, n-dodecyl-β-D-maltoside was purchased from Anatrace, Triton X-100 was obtained from Roche Applied Science, 1,2-Dioleoyl-sn-glycero-3-phospho-rac-1-glycerol)–sodium salt (DOPG) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids. Radiolabeled [N-methyl-14C]choline chloride (55 mCi/mmol) and [N-methyl-14C]choline chloride (80 Ci/mmol) were purchased from Amersham Biosciences and used as precursors to synthesize [N-methyl-14C]glycine betaine and [N-methyl-14C]glycine betaine, respectively, as described (21). Unlabeled glycine betaine was purchased from Sigma, and its purity was confirmed by proton-NMR spectroscopy. Creatine kinase, mono-phosphate creatine-disodium salt, tetracaine, ATP-disodium salt, and carnitine were reagent grade and obtained from commercial sources. ATP and Pi, commercial standards were from ATPliteTM_M (PerkinElmer Life Sciences) and Sigma, respectively.

Isolation of Membrane Vesicles and OpuA Purification—L. lactis strain NZ9000 (22) was grown semi-aerobically at 30 °C, and large scale isolation of membranes was performed as described (9, 23). OpuA was purified from membrane vesicles as described previously (9). The resulting protein fractions were assayed for purity and concentration via SDS-PAGE and A280 UV-visible spectroscopy, respectively.

Membrane Reconstitution of OpuA—OpuA was incorporated into liposomes consisting of synthetic DOPG and DOPE at a 1:1 ratio. Equal amounts of DOPG and DOPE in chloroform were mixed, dried in a
rotary evaporator, and, subsequently, resuspended to 20 mg/ml in 50 mM phosphate, pH 7.0. For optimal hydration, lipids were sonicated with a point tip sonicator (S&M Inc., Danbury CT) for three cycles of 15 s on and 45 s off. Sonicated liposomes were then subjected to three rounds of freezing in liquid nitrogen and thawing at 4 °C. Subsequently, the liposomes were extruded through 400-nm pore size polycarbonate filters (Avestin Inc., Ottawa, Canada), after which the extruded liposomes were destained by titration with Triton X-100. Destaining was monitored by measuring the turbidity at 540 nm, and Triton X-100 was added to just beyond the point of “detergent saturation” (24). At this point, n-dodecyl-β-D-maltoside solubilized, and purified OpuA was added to the lipids at 1:100 or 1:50 protein to lipid ratio (w/w), and the mixture was incubated for 30 min at room temperature with gentle agitation. The protein to lipid ratio of 1:100 (w/w) corresponds to 1 to ~30,000 on a molar basis. The proteoliposomes were formed by removal of detergent by the stepwise addition of polystyrene beads (Biobeads SM2, Bio-Rad). To the mixture of protein and lipid, 40 mg/ml polystyrene beads were added in a series of steps: 15 min at room temperature, 15 min at 4 °C, 30 min at 4 °C, overnight at 4 °C, and 60 min at 4 °C. The polystyrene beads were removed by filtration, and the proteoliposome mixture was diluted 4× with 50 mM phosphate, pH 7.0, after which the proteoliposomes were collected by ultracentrifugation at 150,000 × g for 90 min. The proteoliposomes were washed twice and finally resuspended to 20 mg/ml lipid weight and stored in liquid nitrogen.

**ATP-driven Glycine Betaine Uptake in Proteoliposomes**—For uptake or efflux, 9 mM ATP/Mg2⁺ or an ATP-regenerating system (2.4 mg/ml creatine kinase, 9 mM ATP/Mg2⁺, 24 mM sodium-mono-phosphate creatine) was enclosed in the proteoliposomes during three cycles of freezing in liquid nitrogen and thawing at 4 °C. Proteoliposomes were made homogeneous via extrusion through polycarbonate filters of various pore size (400, 200, or 100 nm) (Avestin Inc.). Following extrusion, the proteoliposomes were washed with 50 mM phosphate, pH 7.0, and pelleted by ultracentrifugation at 300,000 × g for 12 min to remove external ATP/Mg2⁺. In some experiments, a gel filtration column (Sephadex G-50 medium, 20 × 0.7-cm column) was used to wash proteoliposomes or to exchange potassium-phosphate for potassium-Hepes. Following extrusion, the proteoliposomes were diluted with an isotonic buffer to obtain an osmolality (iso-osmotic to hyper-osmotic) and containing [14C]glycine to initiate uptake, proteoliposomes were diluted into buffers of varying osmolality (iso-osmotic to hyper-osmotic) and containing [14C]glycine betaine. At the appropriate time points, samples of proteoliposomes were assayed for [14C]glycine betaine uptake at 30 °C. At 30 °C, the proteoliposomes were incubated for 30 min with 9 mM ATP/Mg2⁺ or ATP-regenerating system ([●]) and resuspended in 50 mM potassium P₄, 100 mM KCl, pH 7.0, at 30 °C. ATP/Mg₂⁺- and ATP-regenerating system (ARS)-stimulated uptake assays were performed under iso-osmotic ([●]) or hyperosmotic conditions (plus 400 mM KCl; [●]). As shown in B, after 10 min of uptake, efflux of glycine betaine was stimulated by the addition of 50 mM potassium P₄, pH 7.0, containing 9 mM ATP/Mg₂⁺ in the presence of 430 mM sucrose ([●]) or 400 mM KCl ([●]).

**Measurement of ATP Hydrolysis**—In addition to measurement of glycine betaine uptake and efflux, ATP hydrolysis was measured directly by determining the changes in concentration of ATP (luciferase-luciferase assay) or indirectly by determining the changes in concentration of phosphate (malachite green-based phosphate assay) (25). For measurement of ATP levels inside the proteoliposomes, identical proteoliposome samples were collected and treated as described above.

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**RESULTS**

**Transport and ATP Hydrolysis by Right-side-in- and Inside-out-oriented OpuA**—Assays of OpuA activity were made for right-side-in and inside-out reconstructed molecules (Fig. 1, A and B). Uptake of glycine betaine was performed with proteoliposomes containing ATP/Mg²⁺ or the ATP-regenerating system (Fig. 1A). In the absence of enclosed ATP/Mg²⁺, no glycine betaine uptake was observed (data not shown). The ATP-regenerating system allowed a higher level of uptake because the ATP level remained high (and the ADP level remained low) for longer a period than with ATP/Mg²⁺ alone. Under iso-osmotic conditions, little or no glycine betaine uptake was observed when OpuA was incorporated into liposomes composed of 50% DOPG/50% DOPE.
The transporter is in the inactive state under these conditions (7, 8). Osmotic upshift activated the OpuA transporter, and the final level of glycine betaine uptake was reached after ∼10 min.

Efflux of [14C]glycine betaine was observed upon the addition of ATP/Mg2+ to the proteoliposomes that had preaccumulated glycine betaine for 10 min (Fig. 1B). This efflux results from the stimulation of the inside-out-oriented OpuA molecules. Consistent with the osmotic activation mechanism, involving activation by ionic strength at the cytoplasmic face of the membrane (9), efflux of glycine betaine occurred with high (>200 mM) concentrations of KCl, NaCl, or methyl glucamine-chloride and not with alanine or sucrose. Efflux did not occur in the absence of ATP/Mg2+ or in the presence of the non-hydrolyzable ATP analogue AMP-PNP (data not shown). The apparent rate of glycine betaine efflux by inside-out-oriented OpuA (and ATP or an ATP-regenerating system inside the vesicle lumen), which reflects the non-random incorporation of the molecules with this reconstitution method (24). With different preparations of proteoliposomes, we observed that the fraction of right-side-in-oriented OpuA is in the range of 65–80%.

The changes in internal ATP and ADP concentrations as a result of glycine betaine uptake are shown in Fig. 2A. The data indicate that minimal ATP hydrolysis had occurred during the overnight preparation of the proteoliposomes. Fig. 2B clearly shows that both glycine betaine and ionic activation are needed for ATP hydrolysis, indicating that ATP hydrolysis and substrate translocation are tightly coupled. This tight coupling, generally not observed for other ABC transporters, enables a higher degree of certainty for interpretation of results regarding stoichiometry measurements.

**Inhibition of OpuA Substrate Translocation**—The limitation of glycine betaine uptake after 10 min (Fig. 1, A and B), despite a residual level of −3 mM ATP (Fig. 2A), is most likely due to depletion of ATP in proteoliposomes with right-side-out OpuA and a fraction of liposomes without OpuA and thus a constant ATP level. However, the inhibition of uptake could partly have a kinetic origin, that is, it could be a consequence of the accumulation of ADP and/or glycine betaine. ADP is known to inhibit the ATPase activity of ABC transporters, and inhibition by high concentrations of substrate, accumulated on the trans-side of the membrane, has been observed for glycine betaine uptake in Lactobacillus plantarum and Listeria monocytogenes (27, 28).

It is difficult to measure the effects of inhibition by ADP or glycine betaine with right-side-in-oriented OpuA because it would require manipulation of the lumen contents (and reconstitution conditions). Therefore, we measured possible inhibitory effects of ADP and substrate on the activity of the inside-out-oriented OpuA molecules. In Fig. 3A, we report the effect of ATP and different ADP concentrations on the rate of efflux of glycine betaine from the proteoliposomes. Without the addition of ATP, no efflux of glycine betaine was observed. Under typical conditions of efflux with 9 mM ATP/Mg2+, the rate of efflux was almost maximal. The apparent Kᵢ for ATP stimulation of efflux was ∼3 mM (data not shown). Importantly, there was a significant reduction of glycine betaine efflux when ADP/Mg2+ was added together with ATP/Mg2+ (Fig. 3A); the Kᵢ for ADP inhibition of ATP-stimulated (9 mM) efflux was ∼12 mM (data not shown). Thus, ADP has a limiting effect on the rate and extent of glycine betaine efflux, which, together with a reduc-
the presence of 9 mM ATP/Mg$^{2+}$ through polycarbonate filters of 400-, 200-, and 100-nm pore size in some of different average diameter and made by extrusion measurement of external ATP hydrolysis and efflux of glycine betaine. Indeed, the amount of ATP in the vesicles decreased with the pore size of the filters through which the proteoliposomes were extruded. Moreover, the ratio of ATP hydrolysis and glycine betaine uptake despite the presence of an apparent $\sim 3$ mM ATP in the ensemble of (proteo) liposomes. Similarly, the incomplete efflux of glycine betaine suggests that a significant fraction of the proteoliposomes that has accumulated glycine betaine (via right-side-in-oriented OpuA) does not have inside-out OpuA to expel the substrate.

**ATP/Substrate Stoichiometry Determination**—ATP/substrate stoichiometries can be obtained from comparisons of the rates and extent of ATP hydrolysis and glycine betaine uptake or efflux. In Fig. 4, we show the results of ATP hydrolysis (Fig. 4A) and glycine betaine uptake measurements (Fig. 4B), performed on proteoliposomes of different average diameter and made by extrusion through polycarbonate filters of 400-, 200-, and 100-nm pore size in the presence of 9 mM ATP/Mg$^{2+}$. We used proteoliposomes of different size with the anticipation that the internal volume may present a limitation on the amount of ATP that can be included, thereby limiting the amount of glycine betaine that can be taken up. Indeed, the amount of ATP in the vesicles decreased with the pore size of the filters through which the proteoliposomes were extruded. Moreover, the ratio of ATP hydrolysis and glycine betaine uptake, that is, the ATP/substrate stoichiometry, tended to be higher in proteoliposomes of smaller diameter and volume (Fig. 4C). In these proteoliposomes with a smaller volume to surface ratio, the concentration of ATP will decrease faster as a result of ATP-dependent translocation.

An alternative method for stoichiometry determination, not limited by a decrease in ATP or accumulation of ADP, involves measurement of external ATP hydrolysis and efflux of glycine betaine. In this experiment, the concentration of external ATP was kept constant (9 mM), and the ATP hydrolysis by inside-out-oriented OpuA was determined from the appearance of inorganic phosphate. This more sensitive detection method for measurement of phosphate, liberated as a function of ATP hydrolysis, required phosphate-free buffers. A series of control experiments, measuring OpuA activity in phosphate-free buffer, showed no significant differences in terms of activity, ATP hydrolysis coupled to glycine betaine transport, or ionic activation (data not shown). There was no detectable hydrolysis of ATP when glycine betaine was not present in the vesicle lumen or when the translocator was not osmotically activated. In Fig. 5, we show the phosphate release (Fig. 5A) and glycine betaine efflux (Fig. 5B) from proteoliposomes extruded through polycarbonate filters of 400-, 200-, and 100-nm pore size. The ratio of both measurements is presented in Fig. 5C and shows that the ATP hydrolysis/glycine betaine transport stoichiometry is approximately 2 and is now independent of the vesicle diameter and volume. The data presented in Fig. 5C (and Fig. 4C) represent the average of three independent experiments, carried out with three independent protein preparations/membrane reconstitutions.

**DISCUSSION**

In this report, we have addressed the issues of substrate translocation inhibition and ATP/substrate stoichiometry for the osmoregulated ABC transporter OpuA. The inhibition of substrate translocation is of importance for the control of glycine betaine levels, the major osmoregulator in *L. lactis*. OpuA accumulates compatible solutes such as glycine betaine to submolar intracellular concentrations (7, 8). However, thermodynamically, the hydrolysis of 1 or 2 ATP molecules per molecule of substrate transported would be sufficient to allow more than a 10$^{5}$-fold concentration of substrate, corresponding to unrealistically high cytoplasmic concentrations. The discrepancy between the theoretical and observed levels of substrate translocation can be explained by inhibition of the transport process. One possible mechanism involves blockage of the transporter by previously accumulated substrate. Studies on glucose transporters, a neutral amino acid transporter, and a transport system for leptin have shown that the corresponding transporters are inhibited by trans-substrate with apparent inhibition constants in the submolar range (29–31). Trans-inhibition by substrate has also been observed for glycine betaine uptake in the closely related transport systems from *L. plantarum* and...
L. monocytogenes (27, 28). Our findings here clearly eliminate trans-inhibition as a mechanism that limits substrate uptake. Glycine betaine present at submolar concentrations at the cytoplasmic face of OpuA has no effect on the transport activity. Data presented here suggest that accumulation of ADP plays a significant role in the inhibition of substrate translocation, at least in proteoliposomes. Whether ADP or the ATP/ADP ratio plays a role in the osmotic regulation of the glycine betaine pools in vivo remains to be established. We favor the hypothesis that cytoplasmic ionic strength is the main determinant of OpuA activity, which controls the glycine betaine accumulation levels (9).

To date, there is no consensus on the ATP/substrate stoichiometry for members of the ABC transport family. Quite the opposite is true, with variations in stoichiometries ranging from 1 to 50 even when measured on the same ABC transporter (10–20). The values determined representative or real variations of stoichiometries within the members of the ABC transporter family? Among the members of the ABC superfamily, there are differences in the number and location of substrate binding sites, and variations in the number of ATP binding sites are possible; therefore, one cannot rule out the possibility that different members of the ABC superfamily have different ATP/substrate stoichiometries. However, given the likelihood of conservation of biochemical mechanisms, it is likely that the large variations in stoichiometry are due to experimental artifacts.

We have explored possible sources of experimental error in previous estimates of ATP/substrate stoichiometries for ABC transporters. An early work compared the growth yields of bacteria on different sugars under anaerobic conditions, and this suggested that only one ATP was expended per maltose or maltodextrin transported (18). The disadvantage of this in vivo approach relates to the multiple biological processes that are difficult to monitor and control. Another potential error relates to poor coupling of ATP hydrolysis and substrate translocation. ATP hydrolysis without translocation of substrate will result in increased stoichiometries. One can correct for poor coupling by subtracting the value for ATP hydrolysis in the absence of substrate from the measured value in the presence of substrate, but this could lead to an underestimation of the ATP/substrate stoichiometry. The futile hydrolysis of ATP in the absence and presence of substrate is not necessarily the same. Most likely, the futile hydrolysis of ATP is diminished in the presence of substrate because the formation of a ternary enzyme-ATP-substrate complex will lower the fraction of the binary enzyme-ATP complex and thus lower the futile hydrolysis of ATP.

Poor coupling in P-glycoprotein resulted in findings of 50 ATP/molecule of Hoechst 33342 due to both a high basal rate of ATP hydrolysis and futile cycling of Hoechst 33342 between the lipid and aqueous phase (20). ATP hydrolysis with and without substrate was reported in uptake studies on the histidine permease of Salmonella typhimurium, and the stoichiometry of 5 was attributed to poor coupling of ATP hydrolysis to substrate translocation (17). For the maltose transport system from E. coli, ATP/substrate stoichiometries up to 17 were attributed to differences in protein subunit association, due to subtle variations in the preparation of proteoliposomes, that lead to increased futile cycling of the ATPase (12).

The nature of the substrate poses additional problems in the reported stoichiometry measurements. A hydrophobic substrate leaks out of a vesicle relatively easily, resulting in inflated stoichiometries. Moreover, the hydrophobic nature of the P-glycoprotein substrates has been shown to result in high non-specific binding to the membrane of proteoliposomes, leading to underestimation of the ATP/substrate stoichiometry (16).

In this work, we could avoid the above errors by taking advantage of the exceptional coupling of ATP hydrolysis to substrate translocation in the OpuA system (substrate and osmotic activation are necessary for ATP hydrolysis) and the absence of glycine betaine leakage on the time scale of the measurements. We strongly feel that membrane-reconstituted OpuA with its high, regulatable, and reproducible activity currently provides the best ABC system to assess ATP/substrate stoichiometry measurements.

In our initial stoichiometry measurements performed with right-side-out-oriented OpuA, we observed variations in stoichiometries in different batches of proteoliposome 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 with decreasing size of the proteoliposomes. Measurement of stoichiometries on the population of inside-out-oriented OpuA proved that the size of the proteoliposome does not affect the stoichiometry. In each sample, we observed an ATP/substrate stoichiometry of approximately 2 with no deviation with respect to proteoliposome size. With right-side-out OpuA, the 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 is present on the outside and could be kept constant at ~9 mM, and this apparently ensures a better coupling than when the ATP and the ADP concentrations vary. Although the internal glycine betaine decreased in the efflux assays, the concentration always stayed 3 orders of magnitude.
above the $K_d$ for glycine betaine binding ($K_d$ is $<$1 $\mu$m). At present, we cannot rule out the possibility that the higher curvature of the smaller proteoliposomes affects the coupling of right-side-in-oriented OpuA, resulting in the somewhat higher stoichiometry values.

Recent studies on P-glycoprotein are consistent with a mechanism of two ATPs/substrate translocated. Eytan et al. (13) have analyzed the transport of an ATP-driven, valinomycin-dependent, $^{86}$Rb$^+$-ionophore complex in proteoliposomes, which circumvented the problems posed by the hydrophobicity of most P-glycoprotein substrates. They simultaneously measured the ATPase and transport activities of P-glycoprotein under identical conditions and observed 1.3–2.0 ATP/ionophore molecule transported. Sauna and Ambudkar (14, 15), exploiting the vanadate-induced transition state conformation of P-glycoprotein, measured the effects of ATP binding and hydrolysis on substrate binding affinity. On the basis of these and previous studies, it was concluded that two ATP hydrolysis events occur in a single catalytic cycle, one for substrate translocation and one for resetting the protein for the next catalytic cycle (14, 15). They propose a catalytic cycle with the translocation of one substrate leading to an ATP/substrate stoichiometry of 2. However, this possibility cannot be ruled out that 2 molecules of substrate are translocated per catalytic cycle.

Our finding of a stoichiometry of two ATPs/substrate stoichiometry reported here will aid in the formulation of a kinetic mechanism for the function of the OpuA transporter and is most likely relevant for ABC transporters in general. A simple mechanism may involve binding of glycine betaine to the substrate binding domains followed by a two-step binding of ATP to the ATPase subunits. One ATP may be hydrolyzed to interconvert the OpuA from the cis to trans conformation, allowing the substrate to be released at the cytoplasmic face. The second ATP may be used to reset the system, that is, to convert OpuA from the trans to cis state, resulting in completion of the translocation cycle.

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