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In Vitro Functional Characterization of BtuCD-F, the *Escherichia coli* ABC Transporter for Vitamin B_{12} Uptake†

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ABSTRACT: BtuCD is an ATP binding cassette (ABC) transporter that facilitates uptake of vitamin B_{12} into the cytoplasm of *Escherichia coli*. The crystal structures of BtuCD and its cognate periplasmic binding protein BtuF have been recently determined. We have now explored BtuCD-F function in vitro, both in proteoliposomes and in various detergents. BtuCD reconstituted into proteoliposomes has a significant basal ATP hydrolysis rate that is stimulated by addition of BtuF and inhibited by sodium ortho-vanadate. When using different detergents to solubilize BtuCD, the basal ATP hydrolysis rate, the ability of BtuF to stimulate hydrolysis, and the extent to which sodium ortho-vanadate inhibits ATP hydrolysis all vary significantly. Reconstituted BtuCD can mediate transport of vitamin B_{12} against a concentration gradient when coupled to ATP hydrolysis by BtuD in the liposome lumen and BtuF outside the liposomes. These in vitro studies establish the functional competence of the BtuCD and BtuF preparations used in the crystallographic analyses for both ATPase and transport activities. Furthermore, the tight binding of BtuF to BtuCD under the conditions studied suggests that the binding protein may not dissociate from the transporter during the catalytic cycle, which may be relevant to the mechanisms of other ABC transporter systems.

ABC[1] transporters constitute a large family of membrane transport proteins that use energy from the binding and hydrolysis of ATP to pump substrates into or out of the cytoplasm, often against a concentration gradient. ABC transporters are found in bacteria, archaea, plants, and animals, where they facilitate nutrient uptake, osmotic regulation, antigen processing, and toxin/drug export (*1*−*4*). Clinically relevant examples are implicated in at least thirteen different genetic diseases in humans (*5*) or contribute to resistance of tumor cells to chemotherapeutic agents (*3*) and multi-drug resistance in bacteria (*6*).

ABC transporters consist of two membrane spanning domains (MSDs), that form a substrate translocation pathway, and two attached, cytoplasmic ATP-binding cassette (ABC) domains that bind and hydrolyze nucleotide (*1*, *7*). Bacterial ABC importers also require an extracellular substrate binding protein (SBP) that specifically recognizes and delivers the substrate to the translocation channel (*8*). Transported substrates range in size from single ions to polypeptides and include hydrophobic drugs as well as water-soluble nutrients, a diversity reflected in the sequences and architectures of MSDs (*9*). In contrast, the ABC domains feature several highly conserved sequence motifs, including the P loop (Walker-A), Walker-B, Q loop, and ABC signature motif, that bind and hydrolyze ATP (*10*).

At present, the only crystal structure of a bacterial ABC import system is for BtuCD, the vitamin B_{12} transporter of *Escherichia coli* (*11*). A role for the membrane-spanning BtuC subunit in B_{12} transport across the cytoplasmic membrane was elucidated (*12*) before both BtuC and the ABC subunit BtuD were cloned (*13*). After the B_{12} binding protein BtuF from *Salmonella typhimurium* was identified (*14*), the *E. coli* homologue was cloned, purified, and determined to bind B_{12} with an affinity of ∼15 nM (*15*).

We have previously determined the crystal structures of *E. coli* BtuCD and BtuF (*11*, *16*); structures of BtuF in the presence and absence of vitamin B_{12} have also been determined by Hunt and co-workers (*17*). In this study, we set out to characterize the function of the BtuCD-F transport system in vitro. ATP hydrolysis rates were analyzed both in detergent solution and in reconstituted lipid vesicles, and ATP-powered B_{12} transport was monitored in proteoliposomes. While our proteoliposome assays more closely reflect the in vivo situation, the studies in detergent solution assess

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[1] Abbreviations: ABC, ATP binding cassette; ARS, ATP regenerating system; DDM, dodecyl maltoside; FOS12, Fos-choline 12; LDAO, lauryl dimethylamine-*N*-oxide; MSD, membrane spanning domain; SBP, substrate binding protein; TNM, (Tris−NaCl−$MgCl_2$ buffer); TX-100, Triton X-100.

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protein activity under conditions similar to those used to obtain three-dimensional crystals.

MATERIALS AND METHODS

Protein Expression and Purification. BtuCD was prepared as described (*11*), and for reconstitution, the detergent was exchanged on the nickel affinity column from 0.1% lauryl dimethylamine-*N*-oxide (LDAO) to 0.14% Triton X-100 (TX-100). BtuF was expressed with an N-terminal decahistidine tag, using the vector pET22b (Novagen) and *E. coli* BL21(DE3) cells (Novagen). The protein was purified in the absence of vitamin B_{12} from periplasmic extract using Ni-NTA affinity chromatography (Qiagen). After elution from the nickel column, the buffer was exchanged to 20 mM Tris-HCl pH 7.5, 250 mM NaCl, using a HiPrep desalting column (Amersham Biosciences), and the protein was stored at 4 °C. For stability studies, the BtuCD-F complex was produced as described earlier (*16*) and the detergent was exchanged by gel filtration chromatography. All detergents were from Anatrace (Maumee, Ohio) and were Anagrade, except TX-100, which was purchased from Sigma.

Membrane Reconstitution of BtuCD. An overview of the general methodologies employed in this study for the reconstitution and functional analysis of ABC transporters may be found in ref *18*. Egg L-α-phosphatidylcholine and *E. coli* polar lipid extract (Avanti Polar Lipids) at 20 mg/mL in chloroform were combined in a 1:3 ratio (w/w). Chloroform was removed by rotary evaporation, and residual solvent was removed at reduced pressure (ca. 2 mmHg, 10 min). Dried lipids were hydrated in 50 mM Tris-HCl pH 7.5 at 20 mg/mL by incubation at room temperature with periodic vortexing or stirring. The solution was sonicated three times on ice (15 s on/45 s off) using a rod sonicator. The hydrated lipid solution was frozen in liquid nitrogen and stored at −80 °C. BtuCD was reconstituted essentially as described for the OpuA transporter (*19*). The lipids were thawed in a room temperature water bath, subjected to two rounds of freezing and thawing, and extruded through a 400 nm polycarbonate membrane using a Mini-Extruder (Avanti Polar Lipids). Protein, buffer, and detergent were added to the mixture to final concentrations of 10 mg/mL lipids, 50 mM Tris-HCl pH 7.5, 0.14% TX-100, with BtuCD at a 1:50 ratio (w/w) of protein/lipids. The mixture was equilibrated at room temperature for 30 min with gentle agitation. BioBeads SM2 (BioRad) (40 mg/mL wet weight) were added, and the solution was agitated at room temperature for 15 min. Similar aliquots of BioBeads were added four more times for the following incubation periods at 4 °C: 15 min, 30 min, overnight, and 60 min. BioBeads were then removed by filtration through a disposable spin filter column. The solution was diluted ∼5× with 50 mM Tris-HCl pH 7.5 and centrifuged at 150000*g* for 90 min in a TLA 100.3 rotor. The supernatant was removed and the lipids were washed two more times, centrifuging as above for 15 min. Proteoliposomes were finally resuspended in 50 mM Tris-HCl pH 7.5 at 20 mg/mL lipids and were stored in liquid nitrogen.

To incorporate components such as BtuF, vitamin B_{12}, or ATP-regenerating system (ARS) into the vesicle lumen, proteoliposomes plus additives were mixed, frozen in liquid nitrogen and thawed in a room temperature water bath. The freeze−thaw cycle was repeated three times, after which the proteoliposomes were made homogeneous by extrusion through polycarbonate filters as specified below.

ATPase Activity Assays. ATPase activity in proteoliposomes was measured in 300 μL reactions containing 1.7 mg/mL lipids (∼0.13 μM BtuCD), 50 mM Tris-HCl pH 7.5, and 150 mM NaCl. 50 μM vitamin B_{12} and various concentrations of BtuF were present during the freeze−thaw cycles (see figures and figure captions). Reactions were incubated in a 37 °C water bath for 3 min before adding 2 mM ATP and 10 mM $MgCl_2$ (or $MgSO_4$) to initiate the reaction. 50 μL samples were removed at various time points and were added to 50 μL of 12% SDS. Inorganic phosphate was then assayed by the modified molybdate method (*20*).

ATPase activity in LDAO was measured in 300 μL reactions containing 35 nM BtuCD, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% LDAO. 50 μM vitamin B_{12}, 2 mM sodium ortho-vanadate, and/or various concentrations of BtuF were present as indicated in the figures and figure captions. Reactions were incubated in a 37 °C water bath for 3 min before 2 mM ATP and 10 mM $MgCl_2$ (or $MgSO_4$) were added to initiate the reaction. 50 μL samples were removed at various times and were added to 50 μL of 12% SDS. Inorganic phosphate was assayed as above. ATPase activity in the detergent Fos-choline 12 (FOS12) was measured as above, except that 0.1% FOS12 and 175 nM BtuCD were used. ATPase activities in dodecyl maltoside (DDM) and TX-100 were measured as above except that 0.1% DDM or 0.14% TX-100 and 70 nM BtuCD were used. In all cases, the new detergent was added to BtuCD purified in LDAO, with the result that LDAO was diluted to subcritical micelle concentrations.

Vitamin B_{12} Transport Assays. To measure uptake of vitamin B_{12}, an ATP regenerating system (ARS) was added to BtuCD-containing proteoliposomes in the presence or absence of ATP (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 24 mM creatine phosphate, 2.4 mg/mL creatine kinase, and 0 or 2 mM ATP). In one set of experiments, the concentration of creatine phosphate in the ARS was varied between 0 and 50 mM. This mixture was frozen in liquid nitrogen and thawed in a room temperature water bath three times to incorporate all components into the vesicle lumen, and then extruded through a 400 nm polycarbonate membrane using a Mini-Extruder (Avanti Polar Lipids). The extruder was rinsed with ARS-containing buffer, and the rinse solution was added to the proteoliposome mixture. The proteoliposome mixture was diluted with TNM buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$) and centrifuged in a TLA 100.3 rotor at 60 000 rpm for 15 min at 4 °C. Proteoliposomes were washed once, resuspended in TNM buffer at 10 mg/mL lipid, and kept on ice to limit ATP hydrolysis by BtuCD. ^{57}Co-labeled vitamin B_{12} (MPBiomed), unlabeled B_{12}, and BtuF were premixed at various concentrations in TNM buffer and incubated for 2 min at 37 °C before ARS-loaded proteoliposomes were added to start the transport reaction. The final concentrations of lipids and BtuCD in the transport reaction were 4 mg/mL and ∼0.3 μM, respectively, in a total volume of 0.5 mL. 50 μL aliquots were removed at various times and diluted into 2 mL of cold stop buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 8% PEG-6000, 100 μM unlabeled B_{12}). Samples were then filtered through 0.2 μm cellulose acetate filters using a

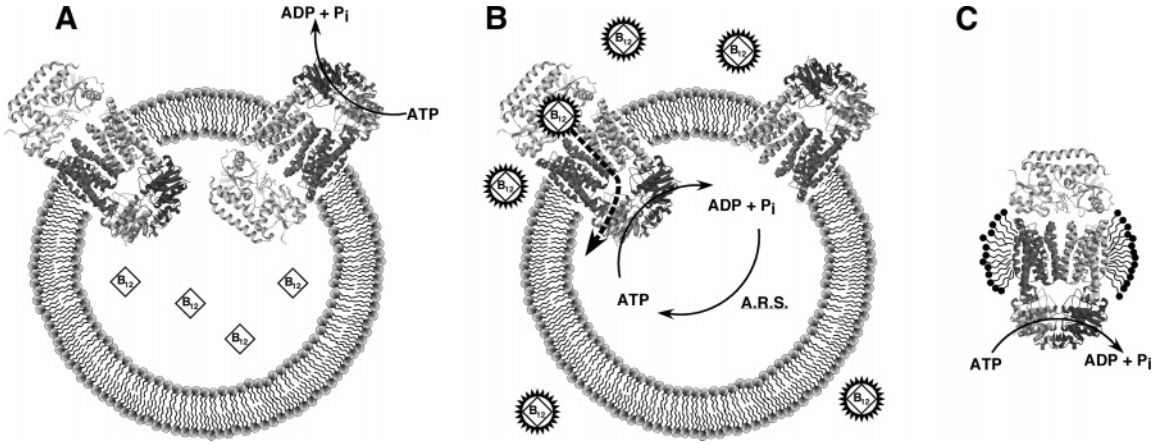

FIGURE 1: Schematic representation of the in vitro assay setups used in the present study. (A) BtuCD reconstituted in liposomes for ATP hydrolysis assays in the presence and absence of BtuF and vitamin B_{12}. (B) Transport of radioactive vitamin B_{12} into proteoliposomes. To keep the concentration of ATP constant for prolonged periods of time, an ATP regeneration system (ARS) was entrapped in the vesicle lumen. (C) ATP hydrolysis assays in detergent micelles.

vacuum filter manifold. Filters were washed twice with 2 mL of cold stop buffer and placed in test tubes. The amount of ^{57}Co-vitamin B_{12} retained on each filter was measured using a γ counter. The concentration of vitamin B_{12} inside the vesicles was determined from this quantity, using a value of 1 μL/mg phospholipid for the internal volume of vesicles (*21*).

RESULTS

The experimental approaches used to characterize the ATPase and transport activities of BtuCD are schematically depicted in Figure 1. Transporters reconstituted into proteoliposomes may be inserted in either right-side-in or inside-out orientations, depending on whether the ABC domains are facing the inside or the outside of the vesicle, respectively. The inside-out transporters may be used to monitor ATPase activity with exogenously supplied nucleotide (Figure 1A), while right-side-in transporters are correctly oriented to assay the transport activity of vitamin B_{12} into the vesicle, when ATP is incorporated into the interior (Figure 1B). Although detergent solubilized transporters cannot be used to measure substrate transport, their ATPase activity can be characterized (Figure 1C).

BtuCD Proteoliposomes. BtuCD containing proteoliposomes were formed by mixing together detergent-solubilized BtuCD and detergent-destabilized liposomes, followed by application of BioBeads to remove detergent. As judged by the intensity of protein bands on SDS−PAGE, the reconstitution efficiency of the transporter was approximately 50%, to give an overall incorporation level of 0.01 mg BtuCD/mg phospholipid.

Stability of BtuCD-F Complex in Various Detergents. The binding protein-transporter complex BtuCD-F was found to be stable in the detergents FOS12, LDAO, DDM, and TX-100 used for the ATPase assays. After mixing BtuCD and BtuF, excess BtuF was removed by gel filtration chromatography. The integrity of the complex was assayed by gel filtration chromatography after storing the protein for several days at either 4 °C or room temperature. A typical experiment is shown in Figure 2. Similar results were obtained when BtuCD and BtuF were incubated together with 2 mM MgATP for 8 min at 37 °C prior to gel filtration (not shown),

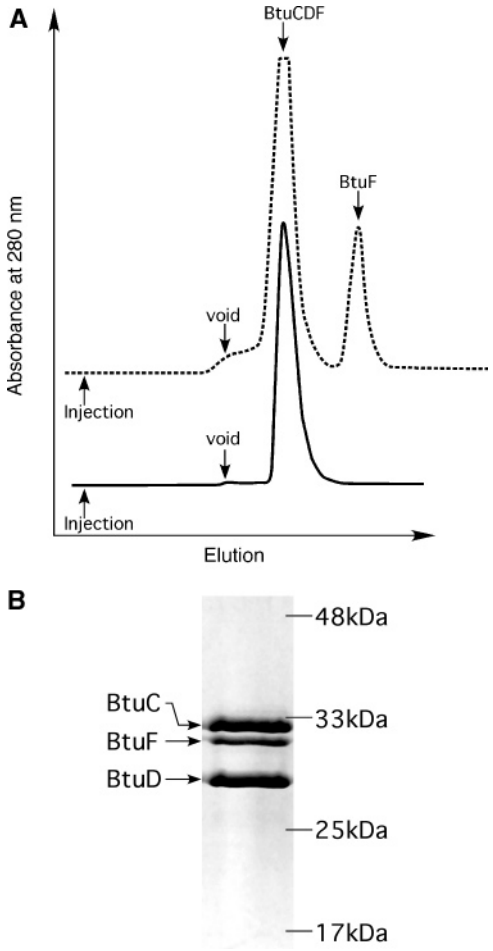

FIGURE 2: Stability of BtuCD-F in various detergents. A representative experiment is shown for dodecyl maltoside, but similar results were obtained for all other detergents used in this study. (A) BtuCD-F was generated in LDAO as described (*16*), and the detergent was exchanged to DDM using two consecutive gel filtration chromatography runs (Superdex 200, Amersham Biosciences). The first run is shown by the dashed line and demonstrates excess binding protein. The main peak was collected and applied again to the same column (solid line). A single peak is obtained that is stable for several days. (B) SDS−PAGE analysis of the BtuCD-F peak obtained in (A).

indicating that the complex remains stable while hydrolyzing ATP. Shorter chain detergents (alkyl chains of 10 carbons

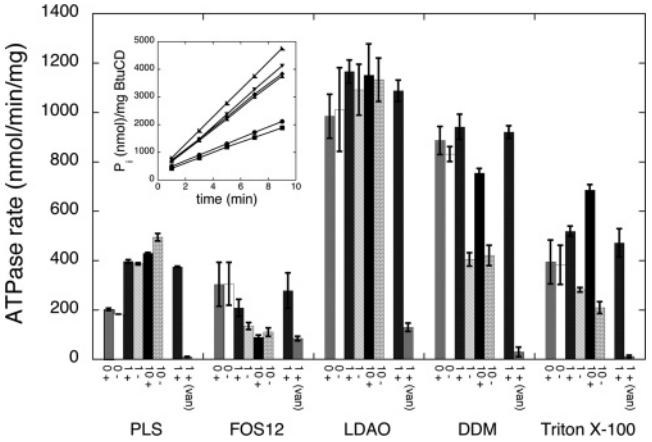

FIGURE 3: BtuCD ATPase activity in proteoliposomes (PLS) and various detergents. Rates were measured at 2 mM ATP, using various concentrations of BtuF as indicated by the numbers (0, 1, 10 (in μM)) under the bars, in the presence or absence of 50 μM vitamin B_{12} (+ or − under each bar) and using 2 mM sodium ortho-vanadate for inhibition (labeled "van"). The inset illustrates the specific hydrolysis of ATP over time by BtuCD proteoliposomes measured in the presence of 2 mM ATP at 0 (●, ■), 1 (◆, ▲), or 10 (▼, right triangle) μM BtuF, with (●, ◆, ▼) or without (■, ▲, right triangle) 50 μM B_{12} and is representative of all ATPase reaction time courses used to prepare this figure.

or less) were unable to maintain the structural integrity of the transporter (not shown).

ATPase Activity in Proteoliposomes and Detergents. The ATPase activity of BtuCD reconstituted in proteoliposomes was tested under various conditions (Figures 3 and 4A). In the absence of BtuF and vitamin B_{12}, BtuCD had a basal rate of hydrolysis of 180 nmol/min/mg (Figure 4A). The ATPase activity was stimulated by BtuF, reaching 440 nmol/min/mg at 20 μM BtuF (Figure 4A). The presence of vitamin B_{12} had little effect on the ATPase activity of BtuCD in proteoliposomes, regardless of how much BtuF was present (Figure 3).

ATP hydrolysis rates were also examined in four different detergents: LDAO, a zwitterionic detergent used for the crystal structure determination of BtuCD (*11*); TX-100, which was used for reconstitution (see Materials and Methods); DDM, the nonionic detergent most commonly used to solubilize ABC transporters (*18*, *22*); and the phosphocholine containing FOS12. LDAO was present at a subcritical micelle concentration with TX-100, DDM, and FOS12 under all conditions, since concentrated BtuCD purified in LDAO was diluted into the other detergents. It was demonstrated for TX-100 that ATPase rates were unaffected by residual LDAO by repeating the experiments after complete removal of LDAO (not shown).

ATPase rates varied widely in the different detergents (Figure 3). The highest rates were observed in LDAO, with a basal activity of 980 nmol/min/mg. While BtuF was found to moderately stimulate hydrolysis in LDAO (16% over the basal rate, Figure 4A), the presence of vitamin B_{12} had no effect. The lowest ATP hydrolysis rates were observed in FOS12 where the basal rate was 300 nmol/min/mg (Figure 3). In contrast to its stimulatory effect in proteoliposomes or LDAO, BtuF depressed the ATPase rate in FOS12 over 4-fold compared to the basal rate (Figure 4A). ATPase rates in DDM and TX-100 were intermediate between those found in LDAO and FOS12, and BtuF and vitamin B_{12} had different

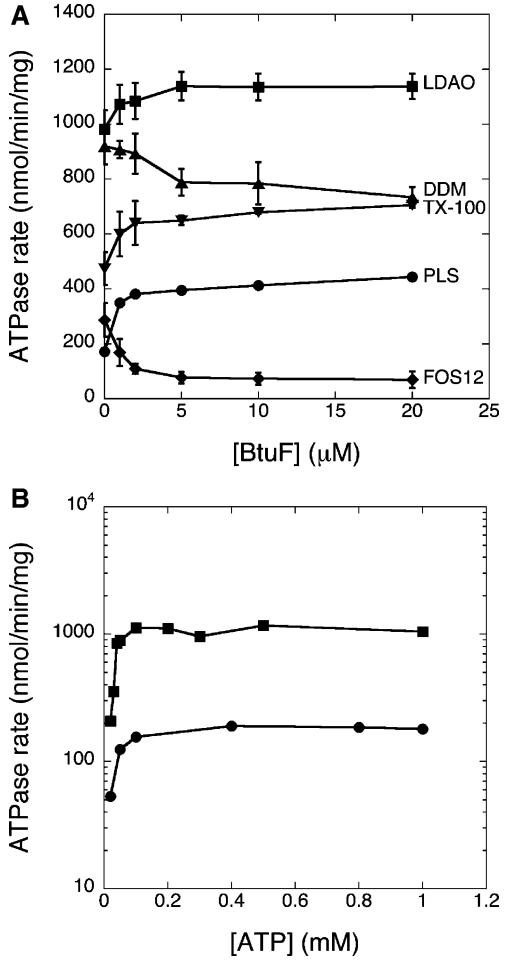

FIGURE 4: ATP hydrolysis rates at various ATP and substrate concentrations. (A) ATPase rates as a function of BtuF concentration in proteoliposomes (PLS) and various detergents at 2 mM ATP and 50 μM vitamin B_{12}. (B) ATPase rates as a function of ATP concentration at 50 μM vitamin B_{12} and 1 μM BtuF for proteoliposomes (●) and LDAO (■).

effects (Figure 3). In both DDM and TX-100, addition of apo-BtuF depressed the hydrolysis rate below the basal level (Figure 3). However, this decrease was not observed in the presence of vitamin B_{12}. Indeed, when vitamin B_{12} was present μM with BtuF in TX-100, hydrolysis was stimulated over 50% (Figures 3 and 4A).

The ATPase activity of BtuCD was measured at various ATP concentrations, both in proteoliposomes and in LDAO, in the presence of BtuF and vitamin B_{12} (Figure 4B). Although the K_m for ATP could not be accurately determined due to limitations of the assay, it was estimated to be below 50 μM, confirming that ATP was initially present at a saturating concentration (2 mM) in the ATPase reactions.

Inhibition of ATPase Activity by Sodium Ortho-vanadate. The ability of 2 mM sodium ortho-vanadate to inhibit BtuCD was tested in proteoliposomes and in each of the four detergents in the presence of BtuF and vitamin B_{12} (Figure 3). ATPase activity in proteoliposomes, DDM, and TX-100 was reduced by >97%. The ATPase activities in LDAO and FOS12 were less sensitive to the vanadate, being reduced by only 88% and 69%, respectively, under this condition.

Vitamin B_{12} Uptake by BtuCD Proteoliposomes. Transport by reconstituted BtuCD was monitored using radioactive, ^{57}Co-labeled vitamin B_{12}. To prevent fast depletion of internal

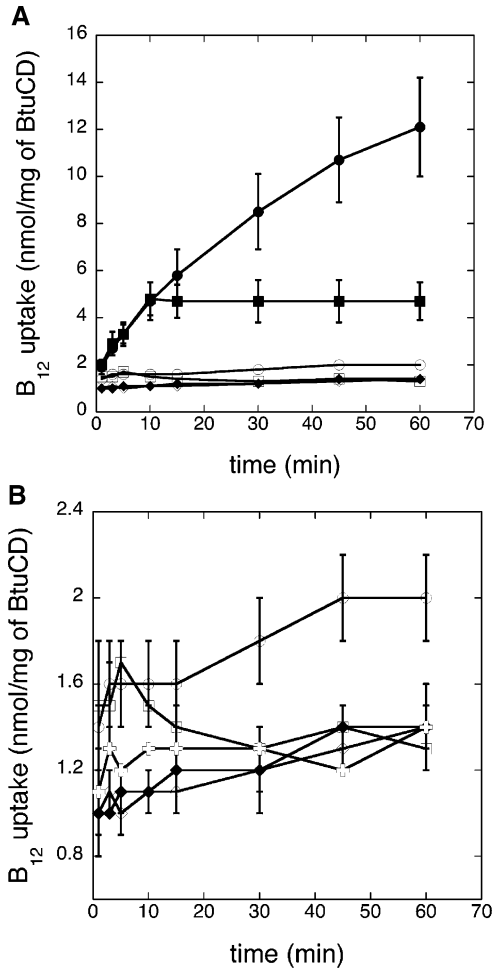

FIGURE 5: Time course of vitamin B_{12} transport into BtuCD proteoliposomes. (A) Uptake into BtuCD proteoliposomes was measured as described in Materials and Methods using 5 μM ^{57}Co-labeled vitamin B_{12} and (●) 1 μM BtuF and 2 mM ATP; (■) 1 μM BtuF, 2 mM ATP, and 100 μM unlabeled vitamin B_{12} chase; (○) 1 μM BtuF; (□) 1 μM BtuF and 100 μM unlabeled vitamin B_{12} chase; (◆) 2 mM ATP; (◇) no addition. Each point represents the average of 3 experiments. For clarity, error bars for only the two upper curves are indicated. (B) Magnified view of the lower curves of (A), with error bars, are depicted using the same symbols. In addition, liposomes devoid of BtuCD were used at 5 μM ^{57}Co-labeled vitamin B_{12} and 1 μM BtuF (cross); this background of ~1.2 nmol of B_{12} binding per mg of protein has not been subtracted from the uptake data.

ATP through the uncoupled hydrolysis catalyzed by BtuCD, an ATP regenerating system (ARS) was incorporated within the lumen of the proteoliposomes (Figure 1B). The proteoliposomes were then washed to remove external ATP and uptake was measured after addition of BtuF and radioactive B_{12} (Figure 5, closed circles). The B_{12} uptake rate during the first 5 min of transport under these conditions (1 μM BtuF, 5 μM B_{12}) was 0.3 nmol/min/mg. This value was somewhat variable between proteoliposome preparations and has been measured as high as 1.0 nmol/min/mg. This may reflect difficulties in accurately determining the protein concentration in liposomes. When excess unlabeled B_{12} was added to a parallel reaction after 10 min (Figure 5, closed squares), the uptake of labeled B_{12} stopped. The amount of radioactive substrate trapped within the proteoliposomes remained constant, demonstrating that B_{12} had indeed been transported into the liposome lumen and that there was no leakage or export.

Table 1: BtuCD-F Mediated Uptake at Various Initial Concentrations of Vitamin B_{12}[a]

initial $[B_{12}]$ (μM)	vitamin B_{12} uptake (pmol)	$[B_{12}]_{in}$ (μM)	$[B_{12}]_{out}$ (μM)	$[B_{12}]_{in}/[B_{12}]_{out}$
0.005	1.4	0.71	0.0022	330
0.01	2.9	1.5	0.0042	350
0.1	27	14	0.045	300
0.5	120	58	0.27	210
1	170	84	0.67	130
10	170	84	9.7	8.7

[a] Transport into proteoliposomes was measured in the presence of 0.5 μM BtuF and various concentrations of ^{57}Co-labeled vitamin B_{12}, with an ATP-regenerating system incorporated in the proteoliposome lumen. The concentration of phospholipids in the proteoliposomes was 4 mg/mL, in a total reaction volume of 0.5 mL. To calculate the final vitamin B_{12} concentration gradient, the internal volume of the proteoliposomes was estimated as 1 μL/mg lipids (*21*).

Uptake of vitamin B_{12} was found to be strictly dependent on the presence of BtuF on the outside and ATP within the lumen of the vesicles. When BtuF was present in the absence of ATP, a small amount of B_{12} became associated with the proteoliposomes (Figure 5, open circles). This radioactivity was not chased away by 100 μM unlabeled B_{12} in the reaction stop buffer (kept at 4 °C, see Materials and Methods). However, upon addition of 100 μM unlabeled B_{12} to the reaction and incubation at 37 °C, the amount of labeled B_{12} associated with the proteoliposomes decreased over a time scale of several minutes (Figure 5, open squares). The implications of this observation are discussed below.

The maximum amount of B_{12} uptake was measured at various initial concentrations of B_{12} (Table 1). At low B_{12} concentrations (5−100 nM), BtuCD-F was capable of establishing a >300-fold concentration gradient between the inside and outside of the proteoliposomes. At higher initial concentrations of B_{12} (0.5−10 μM), this value decreased, presumably due to a depletion of ATP and ATP-equivalents and/or the buildup of inhibitory concentrations of ADP. To determine the relationship between vitamin B_{12} uptake and the amount of nucleotide equivalents (ATP plus creatine phosphate) in the proteoliposome interior, the extent of B_{12} transport by BtuCD-F was measured in the presence of 2 mM ATP and variable concentrations of creatine phosphate ranging from 0 to 50 mM (Figure 6). As the creatine phosphate concentration increased, the total amount of B_{12} transported into the proteoliposomes (estimated from the amount of vitamin B_{12} accumulated at 120 min) also increased. For creatine phosphate concentrations less than 10 mM, the total uptake of B_{12} varied nearly linearly with the sum of the concentrations of adenine nucleotides (ADP + ATP) and creatine phosphate initially present.

DISCUSSION

The protocol used in this study for reconstituting purified, detergent-solubilized BtuCD is based upon that developed by Rigaud and colleagues (*23*, *24*). It differs from the original method by the application of freeze−thaw−extrusion steps to incorporate components (e.g. BtuF, ATP) into the vesicle lumen. The method has been successfully used for the functional reconstitution of a variety of transporters (*18*, *19*, *25*, *26*). The combination of *E. coli* phospholipids and chicken egg phosphatidylcholine is obviously not identical to the native lipid composition of *E. coli*, and our proteoli-

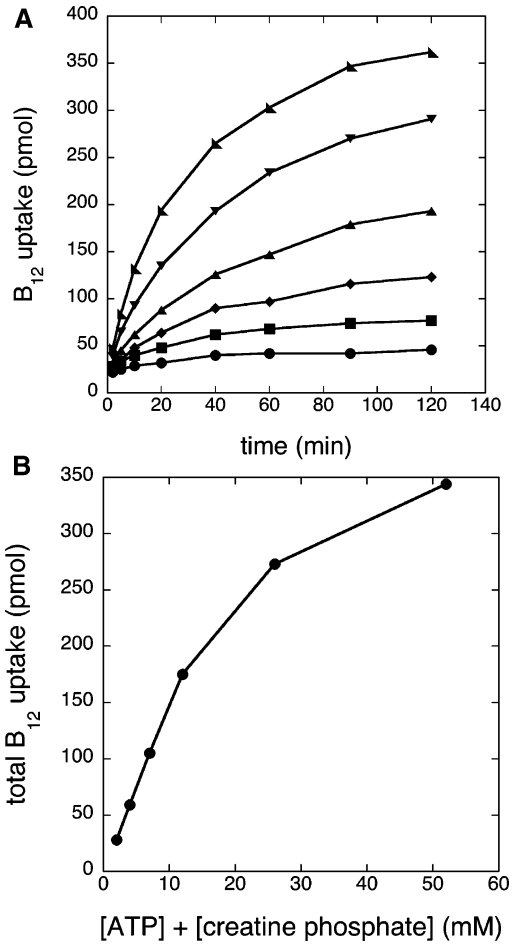

FIGURE 6: Dependence of the rate of vitamin B_{12} transport into BtuCD proteoliposomes on the internal concentration of creatine phosphate. (A) Vitamin B_{12} uptake into BtuCD proteoliposomes was measured as described in methods using 1 μM each of BtuF and vitamin B_{12}, with 2 mM ATP and creatine phosphate concentrations of 0 (●), 2 (■), 5 (◆), 10 (▲), 24 (▼), or 50 (right triangle) mM included in the proteoliposome reconstitution. The proteoliposome preparation contained 4 mg/mL phospholipid and 0.3 μM BtuCD (0.01 mg/mg phospholipid) in a total reaction volume of 0.5 mL. The lines connecting sequential time points are of no theoretical significance. (B) Dependence of the total accumulation of vitamin B_{12} into proteoliposomes (as measured at the 120 min time points) on the sum of the internal concentration of ATP and creatine phosphate. An extrapolated value of 18 pmol of B_{12} at $t = 0$ min has been subtracted from all these data points. The slope of the line fit to the four lowest concentration data points is 15 pmol of B_{12}/mM nucleotide equivalent.

posomes also do not have a transmembrane potential or a pH gradient. Although these deviations from the in vivo situation may influence the kinetics of the system, we find that the preparations of BtuCD and BtuF used in our crystallographic analyses (*11*, *16*) are functionally competent for both ATPase and transport activities in our in vitro system.

ATPase Activity. Both in proteoliposomes and in detergent solution, BtuCD exhibits a significant basal rate of ATP hydrolysis. In proteoliposomes, the addition of BtuF (with or without vitamin B_{12}) stimulates the basal activity only 2-fold (Figure 4). The presence of BtuF also stimulates the ATPase activity of BtuCD solubilized in either LDAO (used in the crystallization study) or TX-100, while decreasing the activity of the transporter reconstituted into either DDM or FOS12. For comparison, a significant basal ATPase activity (1700 nmol/min/mg) was described for DDM-solubilized maltose transporter that could be stimulated nearly 3-fold by addition of the maltose binding protein (*22*), although a separate study reported only a limited ability of the maltose binding protein to stimulate the ATPase activity of a maltose transporter preparation solubilized in the same detergent (*27*). These apparently conflicting observations highlight the sensitivity of the properties of ABC transporters to the detailed experimental protocols.

While the ATPase rates measured for BtuCD are comparable to those observed for other ABC importers such as the histidine (*28*) and maltose transporters (*29*), with maximal ATPase rates approaching 1000 nmol/min/mg (*10*), the insensitivity to the presence of the ligand, vitamin B_{12}, is striking. In the maltose, histidine, and glycine betaine transport systems, substrate-bound binding protein stimulates ATPase activity much more efficiently than free binding protein (*8*, *19*, *30*, *31*). One potentially significant difference between these systems and BtuCD is that BtuF does not undergo large conformational changes upon binding B_{12} (*17*), in contrast to the maltose binding protein that exhibits a significant hinge-bending motion upon ligand binding (*32*). Furthermore, the binding of BtuF to BtuCD is virtually irreversible (*16*), unlike the equivalent complex of the maltose transporter, which could only be trapped using vanadate as an inhibitor (*27*). Apparently, unliganded BtuF complexed to the transporter may mimic B_{12}-bound BtuF sufficiently well to stimulate ATPase activity.

Vanadate is a potent inhibitor of ligand translocation, and the ability of this species to inhibit the ATPase activity of ABC transporters has been interpreted as indicating that the ABC and MSD domains are functionally coupled (*33*). As with the kinetic characteristics of the ATPase reaction, the specific details of vanadate inhibition depend upon the lipidic environment surrounding BtuCD. In proteoliposomes, DDM, and TX-100, ATPase activity was virtually eliminated at 2 mM vanadate, whereas hydrolysis in FOS12 and LDAO was inhibited less effectively (69−88%, respectively) under these conditions (Figure 3). This behavior of BtuCD contrasts with previous studies reporting that the ATPase activities of DDM-solubilized maltose transporters from *E. coli* (*22*) and *Thermococcus litoralis* (*34*) are insensitive to vanadate. While the mechanistic basis underlying the sensitivity of the ATPase activity of ABC transporters to the solubilizing detergents is not understood, it seems plausible that they alter the equilibria between different conformational states of the transporter, in a manner analogous to that proposed for binding protein independent mutants of the maltose transporter (*29*, *35*). Both BtuCD and these mutant forms of the maltose transporter have high basal ATPase rates, although they differ significantly in their requirement for the binding protein component to productively translocate ligand.

Vitamin B_{12} Uptake. As was observed in the histidine, maltose, and glycine-betaine transporter systems (*21*, *36*−*39*), B_{12} uptake by BtuCD-F is strictly dependent on the presence of ATP in the vesicle lumen and BtuF on the vesicle exterior (Figure 5). With an ATP regenerating system in the proteoliposome lumen, an ~300-fold concentration gradient of B_{12} could be established (Table 1). This level of accumulation was observed even at an initial external B_{12} concentration of 5 nM, a concentration 3 times lower than the K_d of vitamin B_{12} binding to BtuF (*15*). Although we

were unable to quantitate the K_m for B_{12}-bound BtuF to BtuCD in the transport reaction due to experimental limitations, we observed maximal transport rates at 1 μM BtuF with saturating concentrations of vitamin B_{12} (data not shown), implying that the K_m of BtuF in the B_{12} transport reaction is significantly lower than that observed in the maltose (25−50 μM) (*40*), histidine (8−65 μM) (*38*, *41*), or oligopeptide transport systems (~50 μM) (*42*).

The apparent B_{12} transport rate of BtuCD-F reported above (0.3 nmol/min/mg) does not take into account such factors as the vectorial reconstitution ratio (the relative proportions of right-side-in and inside-out-facing transporters (Figure 1B)), the possible presence of multilamellar proteoliposomes, the inhibitory potential of accumulated ADP, and nonfunctional transporters (*18*). We have estimated the extent of accessible, transport competent BtuCD molecules by using data from the transport experiment illustrated in Figure 5 as follows: When BtuF is present in the absence of ATP, a small amount of radioactive vitamin B_{12} becomes associated with the proteoliposomes (Figure 5B, compare open circles and open diamonds), but not with proteoliposomes devoid of BtuCD (open crosses). This small amount of B_{12} can be displaced, albeit slowly, by unlabeled B_{12} added directly to the transport reaction at 37 °C (Figure 5B, open squares). These observations indicate that neither is this "extra" radioactive B_{12} nonspecifically bound to the lipids or BtuCD nor has it been transported into the proteoliposomes. Thus, it has likely been sequestered between BtuF and BtuCD and can be exchanged over the course of several minutes at 37 °C, but not in the reaction stop buffer at 4 °C. This provides a potentially accurate method to quantitate the number of accessible and functionally competent BtuCD in proteoliposomes, assuming that the amount of radioactive B_{12} that can be displaced directly correlates to the number of functional BtuCD transporters. Based on the results of three experiments, this calculation establishes that 7 ± 2% of the BtuCD in our reconstitution system is properly oriented and accessible to BtuF, thereby yielding a corrected transport rate of 4.3 nmol/min/mg under these experimental conditions. With an ATPase rate in proteoliposomes of 440 nmol/min/mg, an apparent stoichiometry of ~100 ATP per B_{12} transported can be obtained.

A second estimate of the apparent stoichiometry between ATP hydrolysis and B_{12} uptake that is independent of the fraction of properly oriented and accessible BtuCD transporters may be obtained from the dependence of the total amount of B_{12} transported on the nucleotide equivalents in the interior of the proteoliposome (Figure 6). In this experiment, the uptake of vitamin B_{12} into proteoliposomes is measured as a function of internal creatine phosphate concentration, fixing the concentration of ATP used in the reconstitution at 2 mM. While the amount of vitamin B_{12} taken up increases with the internal creatine phosphate concentration, the overall rate of uptake also increases with the creatine phosphate concentration, even though the concentration of ATP was held constant during the reconstitution of all the samples. This suggests that the activity of creatine kinase may have been impaired during the reconstitution process (*43*). At the lower creatine phosphate concentrations (<~10 mM), the total amount of vitamin B_{12} transported into the proteoliposomes varies in a nearly linear fashion with the sum of the initial ATP and creatine phosphate concentrations, such that the uptake of ~150 pmol of B_{12} requires an effective nucleotide pool of ~10 mM inside the proteoliposomes. Since the interior volume of the proteoliposomes is estimated to be 2 μL under the assay conditions, 10 mM corresponds to 20 nmol of nucleotide equivalent. From the ratio of the internal nucleotide pool to the total amount of B_{12} transported (20/0.15), an apparent stoichiometry of 130 ATP per B_{12} may be obtained, which is in reasonable agreement with the value of 100 ATP per B_{12} obtained from a direct comparison of the ATPase and transport rates.

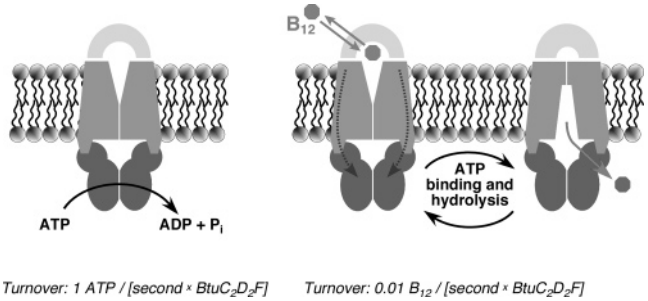

FIGURE 7: Schematic and kinetics of (left-hand side) BtuCD-F-mediated ATP hydrolysis and (right-hand side) B_{12} transport under the conditions described in this study. BtuC, BtuD, and BtuF are represented in medium, dark, and light shades of gray, respectively. See text for further explanation.

Apparent Stoichiometry and Revised Model of BtuCD-F-Mediated B_{12} Transport. In our in vitro system (Figure 1), the initial ATPase rate of BtuCD in the presence of BtuF and B_{12} is ~100 times faster than the uptake rate, with corresponding turnover numbers of 1 ATP per second for ATPase activity and 0.01 molecule of B_{12} transported per second for the uptake activity. The apparent stoichiometry of ~100 ATP per B_{12} reflects the high basal ATPase rate of BtuCD in the absence of BtuF. While the mechanistically relevant stoichiometry for ABC transporters is likely 2 ATP per substrate transported (*18*), this ratio has only been reliably observed in an in vitro system for the tightly coupled *Lactococcus lactis* OpuA transporter (*19*). More typically, the ATPase and transport rates differ by one to three orders of magnitude (*18*, *27*, *37*), which has been attributed to experimental differences between the two types of assays (*27*, *43*), including orientation effects and accumulation of ADP. Detailed analyses of the mechanistic relationship between ATP hydrolysis and substrate translocation by ABC transporters clearly require development of more robust schemes for purification, reconstitution, and functional assay.

The results described in this paper suggest a revised model for the transport cycle of BtuCD-F (Figure 7). In previous models, we proposed that the B_{12} binding protein is released from the transporter during the transport cycle (*44*). While this may be valid for certain transporters (e.g. maltose and histidine transporters), BtuF remains bound to BtuCD in our present model throughout the transport cycle (Figure 7), and vitamin B_{12} has access to the binding site of BtuF during this time. Our model reflects (i) the extremely tight binding observed between BtuF and BtuCD in LDAO (*16*) and in all other detergents used in this study, (ii) the low K_m of BtuF for BtuCD in the transport reaction, and (iii) the ability of the BtuCD-F complex to sequester B_{12} in the absence of ATP. BtuF attached to the transporter simulates ATP hydrolysis cycles, which in turn switches the transporter between two states with cytoplasmic and periplasmic gates

alternatively opened. This model may be relevant to the mechanism of those transporters with binding proteins featuring a rigidifying backbone α-helix connecting the two lobes of the SBP (*45*), or more generally to those that are tethered to either the transporter or the membrane (*46*, *47*), which should stabilize the ternary complex by preventing the three-dimensional diffusion of the binding protein away from the complex.

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