Chapter 3

Initial Functional Characterization of BtuCD-F, the Binding Protein-Dependent ABC Transport System for Vitamin B_{12} in *E. coli*

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Abstract

BtuCD-F is a binding protein-dependent ABC transporter system that uses the power of ATP hydrolysis to pump vitamin B_{12} into the cytoplasm of *E. coli*. Both the inner membrane associated transporter, BtuCD, and the periplasmic substrate binding protein, BtuF, have been characterized by x-ray crystallography. We now show that BtuCD can be reconstituted into proteoliposomes and mediate uptake of vitamin B_{12} in the presence of ATP and BtuF. In the absence of ATP, B_{12} appears to become sequestered between BtuF and BtuCD. Vitamin B_{12} can be concentrated at least 300-fold by BtuCD-F. The ATPase activity of BtuCD was examined in proteoliposomes as well as in detergent solution. BtuCD has a significant basal rate of hydrolysis under all conditions tested, making an estimate of the stoichiometry of ATP hydrolyzed to B_{12} transported impossible. In proteoliposomes, both B_{12}-bound and apo-BtuF stimulates the ATPase rate. Interestingly, the rate of ATP hydrolysis, as well as the effect of BtuF, vitamin B_{12} and sodium ortho-vanadate on that rate, is different in each detergent. These results indicate that ABC transporters are very sensitive to their environment and underline the importance of detergent or lipid choice in membrane protein crystallization and

functional reconstitution experiments. Our results lead us to propose a revised model of the ABC transport cycle.

Introduction

ABC transporters are a very large family of transport proteins that move substrates across cellular and organelle membranes. Using the power of ATP binding and hydrolysis, these proteins can pump substrates both into and out of the cytoplasm, often against a concentration gradient. Nearly ubiquitous in nature, ABC transporters are found in bacteria, Archaea, plants and animals. They have roles in nutrient and micronutrient uptake, osmotic regulation, antigen processing, and toxin/drug export (1-4). ABC transporters are very clinically relevant. Currently, they are implicated in thirteen different genetic diseases in humans (5), as well as the resistance of tumor cells to chemotherapeutic agents (1) and multiple antibiotic-resistance in bacteria (4).

All ABC transporters possess a similar molecular architecture: two membrane spanning domains (MSDs) that form a substrate translocation pathway and two peripherally associated ATP-binding cassette (ABC) domains that bind and hydrolyze nucleotide (1, 2). The substrates transported by this family range from single ions to polypeptides and hydrophobic drugs to sugars (6), a diversity which is reflected in the poor sequence similarity between the membrane spanning domains of various transporters (7). The ABC domains, on the other hand, are characterized by several highly conserved sequence motifs, including the P loop (Walker-A) motif, Walker-B, Q loop, and ABC signature

sequence, all of which are involved in the binding and hydrolysis of nucleotide (4). Most bacterial ABC transporters are nutrient importers that, in addition to two MSDs and two ABC domains, have a substrate binding protein (SBP) that delivers substrate to the translocation channel (2, 8, 9). SBPs are an important part of their cognate transport system and uptake by wild type transporters is absolutely dependent upon them. SBPs bind their substrates with high affinity and selectivity, helping ensure, and in some cases determine, the specificity of its cognate transporter (8, 10). This conservation of architecture suggests that there is also a conservation of function and mechanism in the family of ABC transporters.

ATP hydrolysis is required for substrate transport by all ABC transporters, presumably to energize and coordinate conformational changes within the ABCs and MSDs that permit substrate translocation (2, 4, 6). In the maltose (MalFGK2-MBP) and histidine (HisQMP-J) transport systems, both liganded and unliganded SBPs have been shown to bind to their cognate transporters and stimulate ATP hydrolysis by the cytoplasmic ABC domains (11, 12, 13). However, both histidine-bound HisJ and maltose-bound MBP stimulate to a greater extent than HisJ or MBP alone. Analysis of the transition state for ATP hydrolysis, trapped using vanadate, revealed that, in the maltose transport system, MBP remains stably bound to MalFGK until ADP, P_i or both are released (14). In this transition state complex, maltose has been released while MBP likely remains in an open conformation (15). The binding protein is thought to then be released from the transporter in order to bind another substrate molecule once ADP and P_i are released. These results suggest that both the SBP and ATP work to keep transport unidirectional.

The SBP, tightly bound during ATP hydrolysis, acts like a gate closing the pathway back into the periplasm while the forward gate into the cytoplasm (formed by the membrane spanning domains) is opened to allow substrate translocation.

The structure of one complete bacterial import system has been solved by x-ray crystallography: BtuCD-F, the vitamin B_{12} transporter of *E. coli* (16-18 and Chapter 2). The structure of BtuCD reveals a translocation pathway formed at the interface of two BtuC membrane-spanning domains with a single closed gate near the cytoplasmic surface. Two BtuD ABC subunits are positioned below the BtuC subunits and arranged "head to tail" to form two ATP binding sites at the dimer interface. Residues in the helical sub-domain of BtuD make contact with the cytoplasmic L-loop of BtuC at the transmission interface. There is a large, water-filled hole in the middle of the four subunits through which the transported B_{12} molecule probably escapes after passing through the translocation channel. BtuF is a bi-lobed protein and B_{12} is bound in the "base-on" conformation in a deep cleft formed at the interface of the two lobes (Chapter 2).

A role for BtuC in vitamin B_{12} transport across the cytoplasmic membrane was first elucidated in 1980 (19). Both BtuC and BtuD were cloned in 1985 (20) and their similarity to other SBP-dependent importers was recognized in 1986 (21). BtuF was identified *in S. typhimurium* in 1999 (22) and was cloned from *E. coli* in 2002 (23). *E. coli* BtuF protein was purified and determined to bind B_{12} with a K_d of 15 nM. Prior to the work described here, there has been no *in vitro* biochemical characterization of

vitamin B_{12} uptake or ATP hydrolysis by the BtuCD-F system. Because BtuCD-F has been extensively characterized by x-ray crystallography (16-18), it is important to study the function of this ABC transport system.

Materials and Methods

Purification of BtuF and BtuCD. The *btuF* coding sequence with an N-terminal 10-His tag was sub-cloned into pET22b. BtuF protein was expressed in BL21 DE3 cells and purified from periplasmic extract using Ni-affinity chromatography. After elution from the nickel column, protein was immediately desalted into 20 mM Tris pH=7.5, 250 mM NaCl and stored at 4 ºC. BtuCD was prepared essentially as described (16) except that the bulk detergent was exchanged on the nickel affinity column from LDAO to 0.14% Triton X-100. After nickel affinity purification, the protein was immediately applied to a Sephacryl S200 or S300 gel filtration column equilibrated in 20mM Tris pH=7.5, 600 mM NaCl, 0.14% TX-100. Peak fractions were combined and protein was stored at 4 ºC.

Reconstitution of BtuCD into proteoliposomes. *E. coli* polar lipid extract and Egg L-α-phosphatidylcholine (Avanti Polar Lipids) (20 mg/mL in chloroform) were combined at a 3:1 ratio (w/w). Chloroform was removed by rotary evaporation and residual solvent was removed at reduced pressure (ca. 2 mm Hg, 10 min). Dried lipids were hydrated in 50 mM Tris pH=7.5 at 20 mg/mL by incubation at room temperature with periodic vortexing or stirring. Once homogenous, the solution was sonicated 3 times on ice (15 s on/45 s off). The hydrated lipid solution was aliquoted, frozen in liquid nitrogen and

stored at –80 ºC. BtuCD was reconstituted into proteoliposomes (PLS) essentially as described (24). Frozen hydrated lipids were thawed in a room temperature water bath, subjected to two more rounds of freeze/thaw and extruded through a 400 nm polycarbonate membrane using a Mini-Extruder (Avanti Polar Lipids). A mixture of 10 mg/mL liposomes, purified BtuCD (1:50 ratio (w/w) protein/lipids), 50 mM Tris pH=7.5 and 0.14 % TX-100 was equilibrated at room temperature for 30 minutes with gentle mixing. BioBeads SM2 (BioRad) (40 mg/mL (wet weight)) were added and the solution was incubated at room temperature for 15 min with gentle mixing. Similar aliquots of BioBeads were added four more times for the following incubation periods at 4 ºC: 15 min, 30 min, overnight and 60 min. BioBeads were removed by filtration through a Poly-Prep disposable column (BioRad). The PLS solution was diluted ~5x with 50 mM Tris pH=7.5 and centrifuged at 150,000 x g for 90 minutes in a TLA 100.3 rotor. The supernatant was removed and the lipids washed 2 times more, centrifuging as above for 15 minutes. PLS were resuspended in 50 mM Tris pH=7.5 at 20 mg/mL, aliquoted and stored under liquid nitrogen.

Vitamin B_{12} transport assays. To measure uptake of vitamin B_{12}, frozen BtuCD PLS were thawed and combined with an ATP regenerating system (ARS) or ARS minus ATP (50 mM Tris pH=7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0 or 2 mM ATP, 24 mM creatine phosphate and 2.4 mg/mL creatine kinase). 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 an Mini-Extruder (Avanti Polar Lipids). The membrane was rinsed with ARS-

containing buffer and the rinse solution was added to the PLS mixture. The PLS mixture was diluted with TNM buffer (50 mM Tris 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 ºC. PLS were washed once, resuspended in TNM at 10 mg/mL lipid and kept on ice to limit ATP hydrolysis. ^{57}Co-Vitamin B_{12} (MPBiomed), unlabeled B_{12} and BtuF were combined at various concentrations in TNM and incubated for 2 minutes at 37 ºC before ARS-loaded PLS were added to start the transport reaction. The final concentration of lipids and BtuCD in the transport reaction was 4 mg/mL and ~ 0.3 μM, respectively. 50 μL aliquots were removed at various times and diluted into 2 mL of cold stop buffer (50 mM Tris pH=7.5, 150 mM NaCl, 8% PEG-6000, 100 μM unlabeled B_{12}). Samples were then filtered through 0.2 mM cellulose acetate filters using a vacuum filter manifold. Filters were washed twice with 2 mL cold stop buffer and placed in test tubes. The amount of ^{57}Co-Vitamin B_{12} retained on each filter was measured using a gamma counter. The fold concentration of B_{12} in the uptake assay was calculated using a PLS internal volume of 1 μL per mg of total lipid (25).

BtuCD ATPase activity in PLS and detergent solution. ATPase activity in PLS was measured in 300 μL reactions containing 1.7 mg/mL PLS (~ 0.13 μM BtuCD), 50 mM Tris pH=7.5 and 150 mM NaCl. 50 μM vitamin B_{12} and/or various concentrations of BtuF were present as indicated in the figures and figure legends. After the addition of PLS, reaction mixtures were frozen in liquid nitrogen and thawed in a room temperature water bath three times to incorporate all components into the vesicle lumen. Reactions were incubated in a 37 ºC water bath for 3 minutes before ATP and $MgCl_2$ (or $MgSO_4$)

were added to 2 and 10 mM to initiate the reaction. 50 μL time points were removed at various times and added to 50 μL of 12 % SDS. Inorganic phosphate was assayed as described (26).

ATPase activity in LDAO was measured in 300 μL reactions containing 35 nM BtuCD, 50 mM Tris 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 legends. Reactions were incubated in a 37 ºC water bath for 3 minutes before ATP and $MgCl_2$ (or $MgSO_4$) were added to 2 and 10 mM, respectively to initiate the reaction. 50 μL time points were removed at various times and added to 50 μL 12 % SDS. Inorganic phosphate was assayed as described (26). ATPase activity in FOS12 was measured as above except that 0.1 % FOS12 and 175 nM BtuCD was used. ATPase activity in DDM and TX-100 was measured as above except that 0.1 % DDM or 0.14 % TX-100 and 70 nM BtuCD was used.

Dynamic light scattering. BtuCD PLS prepared as for the transport reaction were diluted in buffer and the average diameter and overall size distribution were analyzed using a DynaPro Light Scattering device.

Results

Reconstitution of BtuCD into proteoliposomes. BtuCD was purified essentially as described (16), except that the bulk detergent was exchanged from LDAO to Triton X-

100 on the nickel affinity column. Proteoliposomes (PLS) were formed by mixing Triton-solubilized BtuCD and Triton-destabilized liposomes with subsequent detergent removal using BioBeads SM2 adsorbent, following the published method (24). Based on SDS-PAGE analysis, approximately 50% of the BtuCD protein remains at the end of the reconstitution procedure. Using dynamic light scattering, we observed the population of BtuCD PLS to be relatively homogenous with an average diameter of 270 nm.

Vitamin B_{12} uptake by BtuCD proteoliposomes. We show below that BtuCD has a high basal ATPase rate in PLS (in the absence of BtuF and vitamin B_{12}). Therefore an ATP regenerating system was enclosed within the lumen of BtuCD PLS before uptake was assayed. PLS were washed to remove unenclosed ATP and uptake was measured after addition of BtuF and ^{57}Co-labeled vitamin B_{12} (Figure 1A, closed circles). To be certain that B_{12} is being transported into the PLS and is not non-specifically bound, an excess of unlabeled vitamin B_{12} was added to a parallel reaction after 10 minutes (Figure 1A, closed squares). The amount of radio-labeled substrate trapped within the PLS remains constant after the chase, showing that B_{12} has indeed been transported into the PLS and that there is no leakage or export of B_{12} from the vesicles. The B_{12} uptake rate during the first 5 minutes of transport under these conditions (1 μM BtuF, 5 μM B_{12}) is 0.3 nmol/min/mg. This value is somewhat variable between PLS preparations and transport experiments, and has been measured at up to 1.0 nmol/min/mg (1 μM BtuF, 10 μM B_{12}) (data not shown). Liposomes that do not contain BtuCD, show no B_{12} uptake (Figure 1B, crosses).

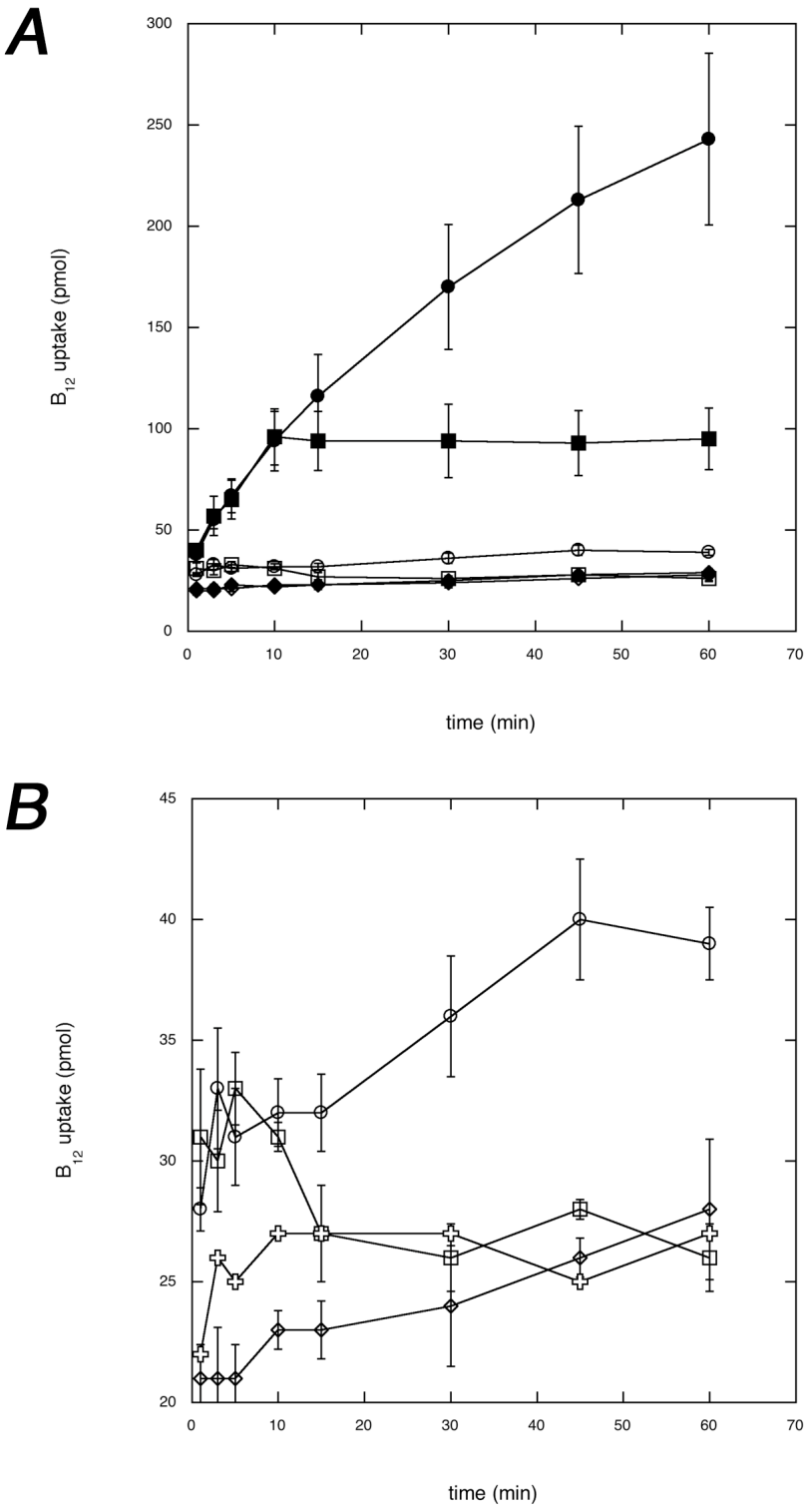

Figure 1. B_{12} Uptake by BtuCD proteoliposomes. (A) Uptake into BtuCD PLS was measured in the presence of 5 μM ^{57}Co-labeled vitamin B_{12} and (●) 1 μM BtuF and 2 mM ATP; (■) 1 μM BtuF, 2 mM ATP and 100 μM unlabeled B_{12} chase; (◆) 2 mM ATP; (○) 1 μM BtuF; (❒) 1 μM BtuF and 100 μM unlabeled B_{12} chase; or (◇) no addition. (B) Curves showing B_{12} uptake in the absence of ATP are shown with symbols as in (A). In addition, uptake into liposomes without incorporated BtuCD was measured in the presence of 5 μM ^{57}Co-labeled vitamin B_{12} and 1 μM BtuF (✞).

Uptake of vitamin B_{12} by BtuCD PLS is absolutely dependent on the presence of BtuF (Figure 1A, compare closed circles and closed diamonds). Import also requires the

presence of ATP within the vesicle lumen (Figure 1A, compare closed and open circles). When BtuF is present in the absence of ATP, a small amount of B_{12} becomes associated with the PLS (Figure 1, open circles). This radioactivity is not chased away by 100 μM unlabelled B_{12} present in the reaction stop buffer (see Materials and Methods). However, upon addition of 100 μM unlabelled B_{12} to the reaction and incubation at 37 ºC, the labeled B_{12} associated with the PLS decreases (Figure 1, open squares), indicating that no uptake has occurred in the absence of ATP. The possible significance of this phenomenon is discussed below.

B_{12} uptake was measured at various initial concentrations of B_{12} (Table 1). At low B_{12} concentrations (5-100 nM), BtuCD is capable of establishing a > 300-fold concentration gradient between the inside and outside of the PLS. At higher initial concentrations of B_{12} (0.5-10 μM), this value decreases, probably due to a depletion of ATP and ATP-equivalents and/or the buildup of inhibitory concentrations of ADP within the PLS. The half-time for maximum B_{12} uptake in the low initial B_{12} concentration range (where fold

	60 minutes			
initial $[B_{12}]$ (μM)	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

Table 1. Uptake at various initial concentrations of vitamin B_{12}. Uptake into BtuCD PLS was measured in the presence of an ATP-regenerating system inside the PLS, and 0.5 μM BtuF and various concentrations of ^{57}Co-labeled vitamin B_{12} outside. The amount of B_{12} inside the PLS after 60 minutes was used along with an estimate of the PLS internal volume to calculate the concentration of B_{12} inside.

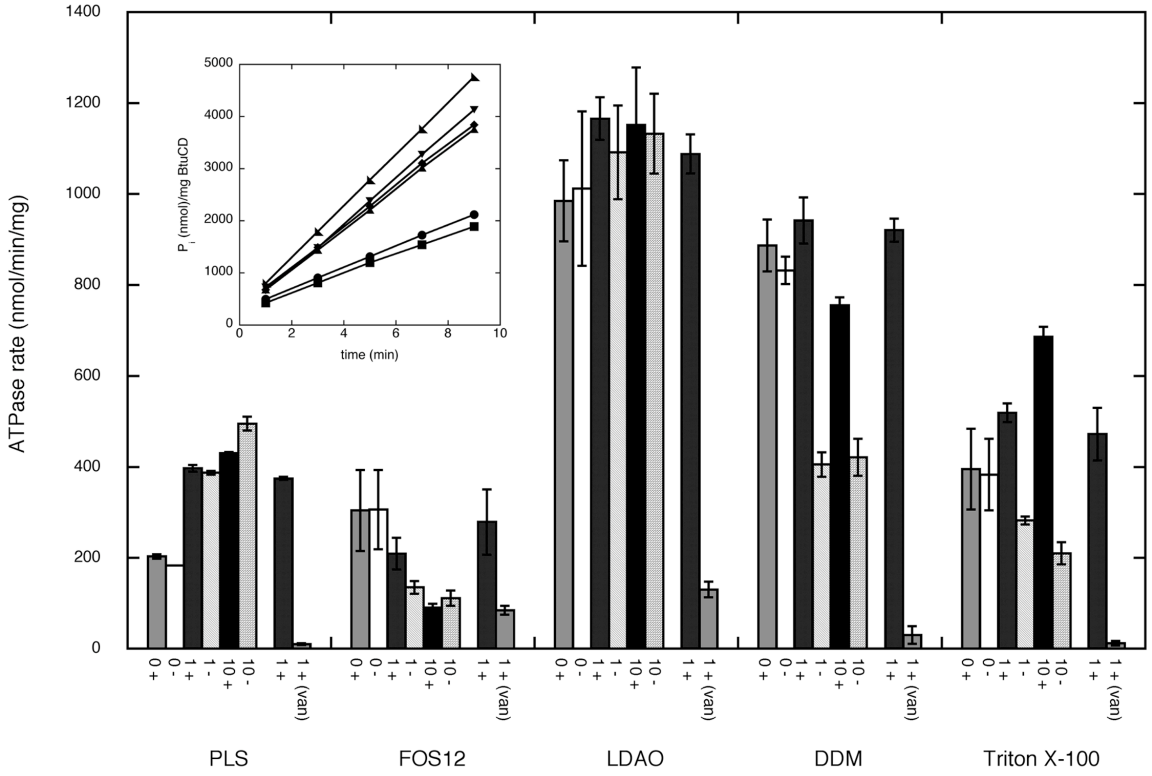

Figure 2. BtuCD ATPase activity in PLS and detergent solution. The ATPase activity of BtuCD was measured in the presence of 2 mM ATP and various concentrations of BtuF, vitamin B_{12} and sodium ortho-vanadate. The number (0, 1, 10) under each bar indicates the concentration of BtuF in μM while a (+) or (-) indicates the presence or absence of 50 μM B_{12}. Reactions containing 2 mM sodium ortho-vanadate are labeled (van). The inset illustrates the specific hydrolysis of ATP over time by BtuCD PLS measured in the presence of 2 mM ATP, 0 (●, ■), 1 (◆, ▲) or 10 (▼, ◣) μM BtuF, with (●, ◆, ▼) or without (■, ▲, ◣) 50 μM B_{12} and is representative of all ATPase reaction time courses.

accumulation is maximal) is 12 minutes.

ATPase activity of BtuCD in PLS. We have tested the ATPase activity of BtuCD reconstituted into PLS under various conditions (Figures 2 and 3). In the absence of BtuF and vitamin B_{12}, BtuCD has a basal hydrolysis rate of 180 nmol/min/mg (Figure 2). When BtuF is present, the ATPase activity is stimulated, reaching 440 nmol/min/mg at

20 μM BtuF (Figure 3). The presence of vitamin B_{12} had no significant effect on the ATPase activity of BtuCD in our assay, regardless of how much BtuF was present (Figure 2).

ATPase activity of BtuCD in detergent solution. We examined the ATPase activity of BtuCD in four detergents: lauryl dimethyl-amine oxide (LDAO), a nonionic detergent that is used to extract BtuCD from the *E. coli* membrane during purification and was also the detergent used for crystallization (16); Triton X-100 (TX-100), a polyoxyethylene detergent that is used during the purification of BtuCD for reconstitution purposes (see Materials and Methods); dodecyl maltoside (DDM), a nonionic detergent with a sugar-based head-group; and Fos-choline 12 (FOS12), an ionic "lipid-like" detergent.

ATPase rates vary widely in the different detergent solutions (Figure 2). LDAO has the highest rates overall and a basal activity of 980 nmol/min/mg (Figures 2 and 3). As in PLS, BtuF stimulates hydrolysis in LDAO (though only by 16% over the basal rate) and the presence of vitamin B_{12} has no effect.

The lowest ATP hydrolysis rates overall are observed in FOS12 where the basal rate is 300 nmol/min/mg (Figures 2 and 3). In contrast to its stimulatory effect in PLS and LDAO, apo-BtuF depresses the ATPase rate in FOS12 more than four fold compared to the basal rate. However, vitamin B_{12} now stimulates hydrolysis in the presence of 1 μM BtuF by ~35% compared to the depressed rate in the absence of B_{12}. The rate in the presence of 10 μM BtuF, though, is unaffected by B_{12}.

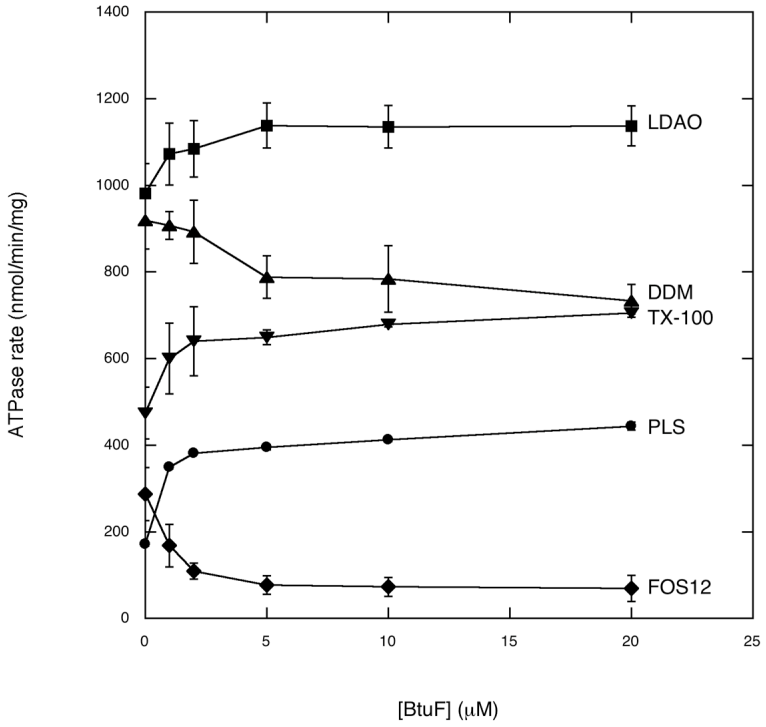

Figure 3. BtuCD ATPase activity at various concentrations of BtuF. The ATPase activity of BtuCD was measured in the presence of 2 mM ATP, 50 μM B_{12} and various concentrations of BtuF in: (●) PLS; (■) LDAO; (◆) FOS12; (▲) DDM; and (▼) Triton X-100.

ATPase rates in DDM and TX-100 are intermediate between those found in LDAO and FOS12, and BtuF and B_{12} have different effects (Figures 2 and 3). The basal rates of hydrolysis in DDM and TX-100 are 830 and 380 nmol/min/mg, respectively. In both detergents, addition of apo-BtuF depresses the hydrolysis rate below the basal level. When B_{12} is present with 1 μM BtuF in DDM, hydrolysis is then stimulated over two-fold, but this stimulatory effect decreases when more BtuF is present in the reaction. When B_{12} is present with 1 μM BtuF in TX-100, on the other hand, hydrolysis is stimulated a little less than two-fold, but now the stimulatory effect increases when more BtuF is present in the reaction. Overall, addition of BtuF up to 20 μM in the presence of B_{12} results in a continuous increase in hydrolysis rates in TX-100. However, addition of BtuF above 2 μM in DDM reduces the hydrolysis rate (Figure 3).

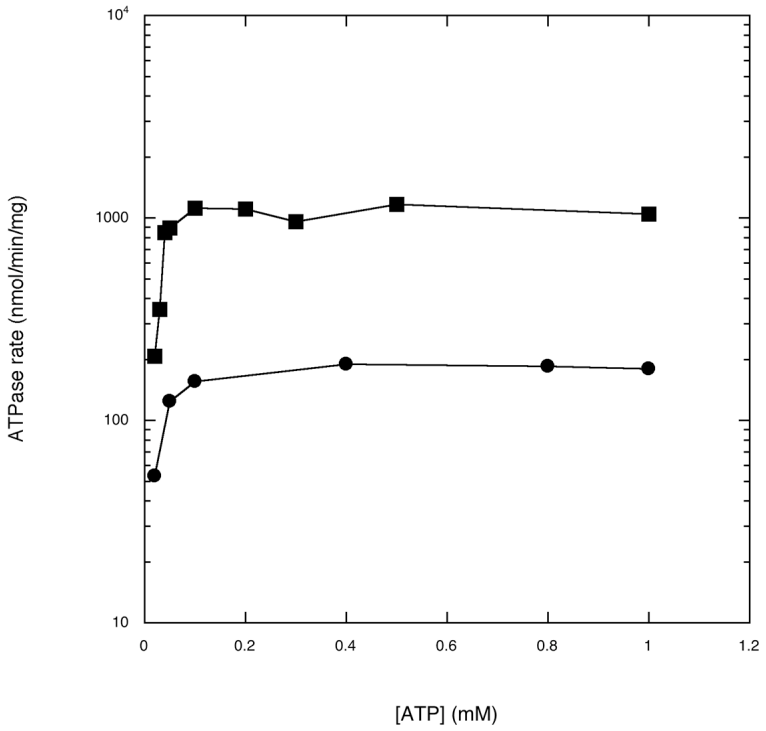

Figure 4. ATP dependence of BtuCD ATPase activity. The ATPase activity of BtuCD was measured in the presence of 50 μM B_{12}, 1 μM BtuF and various concentrations of ATP in (●) PLS and (■) LDAO.

ATP dependence of BtuCD ATPase activity. We have tested the ATP concentration dependence of the ATPase activity of BtuCD in the presence of BtuF and vitamin B_{12} in PLS (Figure 4, circles) and in LDAO detergent solution (squares). The K_m for ATP in the ATPase reaction appears to be below 0.1 mM in both PLS and LDAO, confirming that the 2 mM concentration of ATP used in all the transport and ATPase reactions described in this work is saturating.

Inhibition of ATPase activity by sodium ortho-vanadate. The ability of 2 mM sodium ortho-vanadate to inhibit BtuCD ATPase activity in the presence of BtuF and vitamin B_{12} was tested in PLS and in each of the four detergents (Figure 2). Activity in PLS, DDM and TX-100 was reduced 97% by vanadate. ATPase rates in LDAO and FOS12, in contrast, were reduced by 88% and 69%, respectively.

Discussion

Functional reconstitution of B_{12} transport. Here we present a first *in vitro* biochemical characterization of BtuCD-F, the only complete SBP-dependent ABC transport system characterized by x-ray crystallography (16-18). When a protein, especially a protein machine, is studied by x-ray crystallography, the question of validity is often raised. Exactly how much does the model based on data from highly purified protein locked into a crystal lattice reflect *in vivo* reality? Or even *in vitro* reality? The crystal structure of BtuCD is consistent with a majority of the available biochemical data on ABC transporters (16) but, until now, BtuCD itself has not been functionally tested. Because the crystal structure of BtuCD was solved using highly purified protein in detergent solution, we thought the starting point for our functional assessment must be this same extracted and purified material. Thus, we sought out conditions under which BtuCD, purified in a manner similar to that used for crystallization, could be inserted into liposomes and its ATPase and transport activity assayed. Our reconstitution procedure, in which purified, detergent-solubilized protein is inserted into pre-formed, detergent-saturated liposomes, is based upon that described by Rigaud and colleagues (27, 28), and used previously for the reconstitution of the OpuA ABC transporter (24, 29). The combination of *E. coli* phospholipids and chicken egg phosphatidylcholine used successfully in this study has also been used to reconstitute highly active OpuA and other transport proteins from lactic acid bacteria (29, 30), but it is obviously not identical to the native environment of BtuCD. Furthermore, our liposomes contain no other membrane-

bound proteins and do not have the transmembrane potential or Δ pH carried by the cytoplasmic membrane of *E. coli*. Our successful demonstration of ATPase and transport activity by reconstituted BtuCD shows that none of these factors are critical for basic transporter function, though it remains possible that one or more of them could influence the kinetics or thermodynamics of B_{12} transport. While the results described here may only represent a first approximation of BtuCD-F's transport and ATPase capabilities, they nevertheless suggest that the structural model of BtuCD obtained by x-ray crystallography is valid and that we may confidently analyze current and forthcoming functional data using this structure as a framework.

Vitamin B_{12} uptake. The *E. coli* vitamin B_{12} uptake system has the same fundamental properties as other well-characterized SBP-dependent uptake systems including the maltose transporter of *E. coli* (MalFGK-MBP), the histidine permease of *S. typhimurium* (HisQMP-J), and the glycine-betaine transporter OpuA of *L. lactis* (31-35). That is, B_{12} transport is absolutely dependent on the presence of ATP in the vesicle lumen and BtuF on the vesicle exterior (Figures 1A and 5B). Furthermore, BtuCD-F is capable of establishing a large (> 300-fold) concentration gradient of B_{12} (Table 1). This level of concentration was observed at initial external B_{12} concentrations down to 5 nM, a concentration 3 times lower than the K_d of vitamin B_{12} binding to BtuF (23).

We were unable to quantitatively determine the K_m of BtuCD for B_{12}-bound BtuF in the transport reaction due to experimental limitations. However, we have 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), histidine (8 μM) or oligopeptide transport systems (OppA from *L. lactis*; ~50 μM) (10, 35, 36).

The vitamin B_{12} uptake rate of BtuCD-F (0.3 to 1.0 nmol/min/mg) is close to that of the maltose and the oligopeptide transport systems (1.2 and 0.8 nmol/min/mg, respectively) (10, 14), while it is significantly slower than uptake of histidine and glycine-betaine (7.5 and 325 nmol/min/mg) (14, 33, 35). Some of the differences in these specific activities may be due to estimating protein concentration, reconstitution efficiency, and the orientation of the transporters in the liposome membrane. The rate of uptake by BtuCD-F was corrected for reconstitution efficiency (as judged by SDS-PAGE), but not for the ratio of BtuCD molecules that are "inside-out" vs. "right-side-in" (uptake is measured using only right-side-in transporters). The reconstitution method used in this study has been shown to preferentially yield asymmetric distributions of protein in the target membrane (28). For OpuA reconstituted in a manner nearly identical to BtuCD-F, the ratio is estimated to be 65% to 80% right-side-in (24). If BtuCD-F behaves similarly to OpuA during reconstitution, the B_{12} uptake rate is close to the 0.3 to 1.0 nmol/min/mg value reported here (within a factor of 2).

However, BtuCD may not behave like OpuA because, while OpuA has soluble domains on both the periplasmic and cytoplasmic sides of the MSD, BtuCD has only the cytoplasmic ABC domains (and no covalently attached SBP). While the orientation of BtuCD in the PLS membrane has not been addressed directly, an interesting phenomenon

observed in the transport reaction might give us a clue to the right-side-in vs. inside-out ratio for our reconstituted BtuCD system. When BtuF is present in the transport reaction in the absence of ATP, a small amount of radio-labeled vitamin B_{12} becomes associated with the PLS (Figure 1B, compare open circles and open diamonds). This level of B_{12} is not observed in the presence of BtuF if BtuCD is absent from the vesicles (Figure 1B, crosses). Even though the PLS are exposed to an excess of unlabelled B_{12} in the reaction stop buffer before they are filtered and the radioactivity associated with the PLS is counted, this small amount of radioactivity can only be chased away by unlabelled B_{12} added directly to the transport reaction incubated at 37 ºC (Figure 1B, open squares). These observations suggest that the B_{12} has neither been transported into the PLS nor non-specifically bound to the lipids. Because this phenomenon is observed only in the presence of BtuF *and* BtuCD, we suggest that the B_{12} has become sequestered between BtuF and BtuCD. In the absence of ATP, translocation cannot be completed, but since BtuF binds tightly to BtuCD (17), the sequestered B_{12} cannot be readily exchanged for another molecule from the surrounding solution either (Figure 5B). We propose that the exchange of sequestered labeled B_{12} for an unlabelled molecule from the surrounding solution happens very slowly in the cold temperature of the reaction stop buffer and is thus not observed during the course of the experiment. However, the exchange of B_{12} is faster at the warm temperature of the transport reaction and thus can be observed over the reaction time course upon the addition of the chase. If we assume that one BtuF (and one B_{12} molecule) is bound to every right-side-in BtuCD in the reaction in the absence of ATP, then the amount of labeled B_{12} that can be chased away by unlabelled B_{12} at 37 ºC directly correlates to the number of right-side-in BtuCD transporters. Calculating in this

way, BtuCD in our reconstitution system is 5.6% right-side-in and 94.4% inside-out. Assuming that only 5.6% of the BtuCD in each reaction is capable of transporting B_{12} into the vesicle lumen, the transport rate increases more than an order of magnitude to from 0.3 to 5.4 nmol/min/mg. Since the vast majority of transporters are inside-out, the ATPase rates reported here are virtually unaffected and have not been corrected.

ATPase activity in proteoliposomes. BtuCD in PLS has a basal rate of ATP hydrolysis very similar to that exhibited by the histidine permease in the absence of HisJ and histidine (37). The maltose transporter, on the other hand, displays virtually no ATPase activity in the absence of MBP, whether or not maltose is present (12). B_{12} alone does not stimulate the ATPase activity of BtuCD, though both B_{12}-bound and apo-BtuF can. Interestingly, both liganded and unliganded BtuF appear to stimulate hydrolysis to the same extent in our assay (~ 2 fold) (Figures 2 and 5A).

It is not surprising that apo-BtuF can stimulate BtuCD ATPase activity. It has been shown that unliganded MBP and HisJ can bind to their respective transporters (11, 13) and stimulate the ATPase activity of MalK and HisP (12, 37). Furthermore, it is clear that the structures of B_{12}-bound and apo-BtuF are quite similar (17, 18). This indifference to B_{12} is, however, unexpected because liganded binding protein is able to stimulate ATPase activity far more efficiently than the unliganded form in both the maltose and histidine transport systems (12, 37). ATPase activity in the glycine-betaine transport system is also highly coupled to the presence of substrate (24).

Why doesn't B_{12}-bound BtuF stimulate the ATPase activity of BtuD more efficiently than unliganded BtuF? ATPase activity is measured on inside-out oriented BtuCD molecules only and so BtuF and B_{12} must be incorporated into the lumen of the vesicles by cycles of freezing and thawing. One possibility is that B_{12} is not efficiently trapped inside the PLS by this method. A second possibility is that B_{12} is exported from the vesicles through the inside-out oriented BtuCD transporters so quickly upon addition of ATP to the outside of the PLS that there is no B_{12} left inside the vesicles during the reaction time course. This, of course, depends on the ratio of right-side-in to inside-out transporters, an uncertain quantity (discussed above). A third possibility is that B_{12} is simply not recognized by BtuCD. As noted above, BtuF does not undergo large hinge or twist motions between the B_{12}-bound and apo states, probably due to its rigid inter-domain helix (17, 18). If BtuC does not directly interact with the B_{12} molecule, BtuF may look so similar in the B_{12}-bound and apo states that the same signal is sent to the ATPase domains in either case. However, this does not seem likely for several reasons. First, B_{12} *does* stimulate the ATPase activity of BtuCD in both Triton X-100 and DDM detergent solution. Secondly, we know from the structure of B_{12}-bound BtuF (17, 18) that B_{12} is held in a large slot between the N- and C-terminal sub-domains of BtuF where it is exposed to solvent and well-positioned to directly contact BtuC. Finally, while the changes between B_{12}-bound and apo-BtuF may be subtle, there *are* clear differences including the partial unwinding of an alpha helix near the B_{12} binding site, and the swinging free of a tryptophan residue that is positioned like a cap over the binding site when B_{12} is present. Whether BtuCD-F's indifference to the presence of its substrate is physiologically relevant or simply an artifact of our assay system must be addressed in future studies.

In our assay systems, ATPase rates are measured only on inside-out oriented BtuCD molecules and transport only on right-side-in molecules. As noted above, the ratio of inside-out to right-side-in transporters has not been directly measured, but when our estimate based on sequestration of B_{12} in the transport assay is used, the ATPase rate in the presence of BtuF and B_{12} is 70 times faster than the uptake rate. However, because BtuCD has a high level of basal ATPase activity and is not stimulated by B_{12} in our assays, an real evaluation of the stoichiometry of ATP molecules hydrolyzed per B_{12} transported is virtually impossible. There may also be differences between the transport and ATPase assay systems that have yet to be characterized since a similar discrepancy between uptake and ATP hydrolysis rates was noted in the maltose transport system (14).

ATPase activity in detergent solution. Since the crystal structure of BtuCD was solved using protein in detergent solution, we wanted to study the activity of BtuCD-F in detergent. Obviously, transport cannot be measured without a membrane, but, somewhat unexpectedly, BtuCD has measurable ATPase activity in a wide variety of detergents. This is in contrast to the maltose transporter that is unable to hydrolyze ATP prior to reconstitution into PLS (12).

While BtuCD is active in all four detergents tested (LDAO, FOS12, DDM and TX-100), ATP hydrolysis rates and the effect of BtuF and B_{12} on those rates is quite variable. BtuCD in LDAO has a very high basal hydrolysis rate that can be stimulated slightly by BtuF, but does not respond to the presence of B_{12}. Under similar conditions, BtuCD can

bind to BtuF and form a complex that is extremely stable (17). It is interesting to note that this extremely tight interaction only produces a 16% increase in ATPase activity, while BtuF can stimulate the activity in PLS by 100%. This may mean that the ATPase activity of BtuD observed in LDAO is almost completely uncoupled from transmembrane signaling through BtuC. We have also observed, however, that once the BtuCD-F complex formed in LDAO is reconstituted into PLS, efficient B_{12} uptake can be observed (data not shown).

BtuCD ATPase activity in the lipid-like detergent FOS12 is very different from that in LDAO. The basal activity is much lower and addition of BtuF without B_{12} actually depresses the basal rate. At 1 μM BtuF, B_{12} is observed to stimulate hydrolysis ~35% over the depressed rate but at 10 μM BtuF, B_{12} has no effect at all. The basal ATPase rate in DDM is almost as high as that in LDAO, while the basal rate in TX-100 is about half as high. Like in FOS12, unliganded BtuF depresses the ATPase rates in both DDM and TX-100. When B_{12} is present with 1 μM BtuF in DDM, hydrolysis is stimulated over two-fold, but this stimulatory effect decreases when more BtuF is present in the reaction. When B_{12} is present with 1 μM BtuF in TX-100, on the other hand, hydrolysis is stimulated a little less than two-fold, but now the stimulatory effect increases when more BtuF is present in the reaction.

Intriguingly, while sodium ortho-vanadate inhibits ATP hydrolysis by BtuCD in every lipid and detergent environment examined, it does not do so equally well in all cases. In PLS, DDM and TX-100, ATPase activity was basically eliminated. The same

concentration of vanadate, however, was unable to completely shut down hydrolysis in LDAO and FOS12, the detergents in which the highest and lowest ATPase rates for BtuCD were recorded. These results may reflect subtle differences in the BtuD dimer interface in each detergent environment.

Implications of variable BtuCD ATPase activity. What do these variable effects of BtuF and B_{12} on the ATPase activity of BtuCD mean (Figure 5A)? Are they merely artifacts of a transmembrane protein functioning in detergent solution or reconstituted into an artificial membrane? In some ways, this is obviously true, but we believe that these results still tell us something about BtuCD-F as a protein machine. For example, the ATP hydrolysis rate is under no circumstances changed by the presence of B_{12} alone. Only the presence of BtuF gives B_{12} the power to stimulate BtuCD ATPase activity, implying that BtuF is presenting B_{12} to BtuCD. Thus, it seems logical that B_{12}-bound BtuF can also stimulate the ATPase activity of BtuCD in PLS, even though we were unable to detect this in our current assay.

A second important question is why does BtuCD hydrolyze ATP in the absence of binding protein or substrate? SBP-dependent ABC transporters must stand ready to use the power of ATP hydrolysis to import nutrients whenever a substrate-loaded binding protein interacts with the membrane spanning domain. Perhaps a basal level of ATPase activity keeps some transporters like BtuCD primed and ready for the appearance of substrate while in other transport systems this is not required. Perhaps we have not replicated in *in vitro* some level of regulation present *in vivo*. Or perhaps our purification

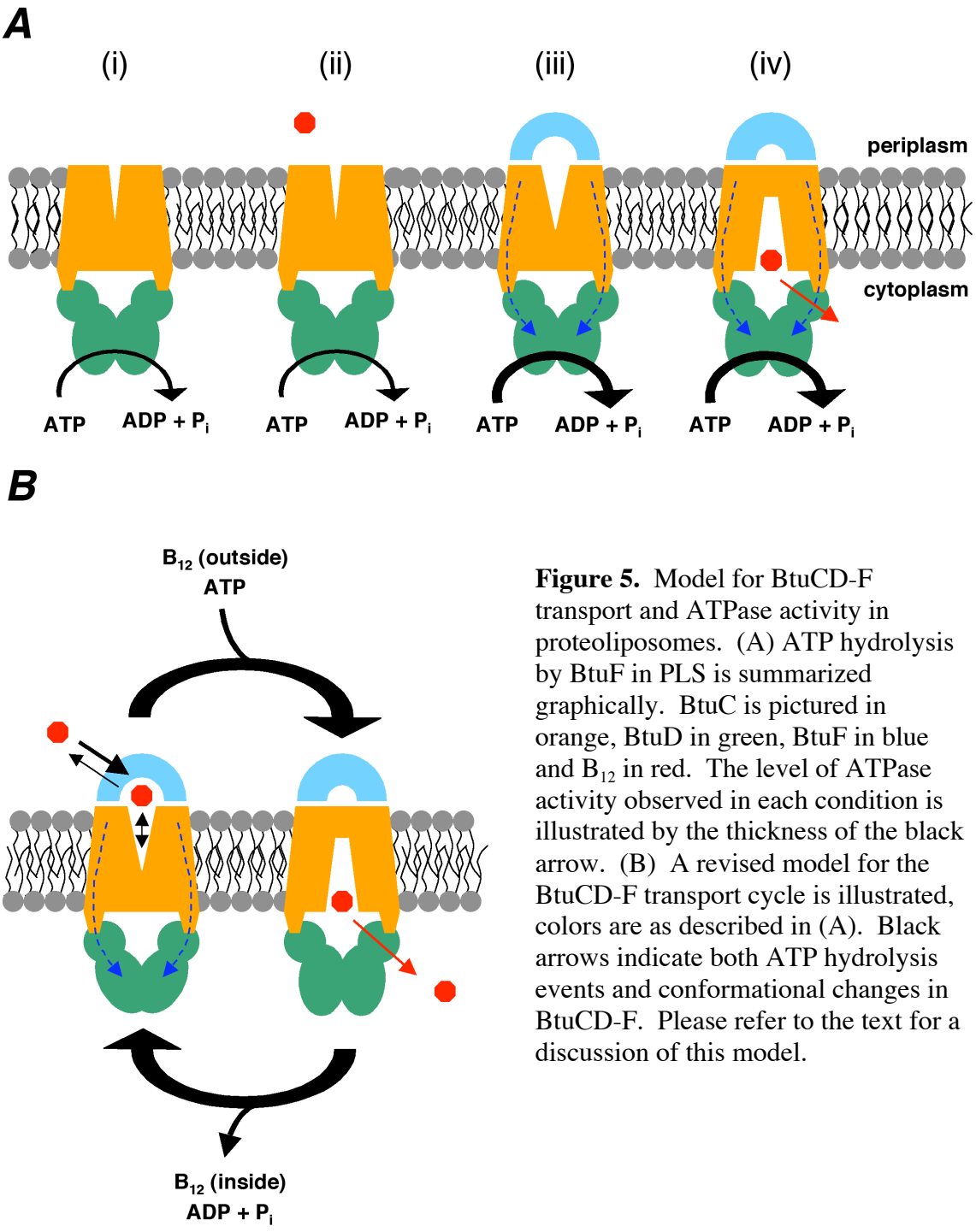

Figure 5. Model for BtuCD-F transport and ATPase activity in proteoliposomes. (A) ATP hydrolysis by BtuF in PLS is summarized graphically. BtuC is pictured in orange, BtuD in green, BtuF in blue and B_{12} in red. The level of ATPase activity observed in each condition is illustrated by the thickness of the black arrow. (B) A revised model for the BtuCD-F transport cycle is illustrated, colors are as described in (A). Black arrows indicate both ATP hydrolysis events and conformational changes in BtuCD-F. Please refer to the text for a discussion of this model.

procedure slightly alters the structure of the transporter, permitting continuous ATP hydrolysis in the absence of a signal from BtuF and B_{12}. At this time, the answer is not at all clear. However, all SBP-dependent ABC transporters may have the same inherent

capability. HisQMP has a basal hydrolysis rate in the absence of HisJ and histidine very similar to that exhibited by BtuCD (37). A mutation in the membrane spanning domain of the MalFGK transporter causes basal hydrolysis in that system (12). The OpuA system remains tightly coupled to both the presence of substrate and the osmotic signal upon purification from *L. lactis* and reconstitution into PLS. Perhaps mutants will one day be found that can uncouple the ATPase activity of this transporter from glycine-betaine and/or the osmotic signal. Solving the structure of other bacterial SBP-dependent ABC transporters along with more biochemical characterization of mutant and wild type complexes should shed light on why import systems have different properties *in vitro* and perhaps *in vivo*, and reveal a common mechanism for all ABC transporters.

BtuCD was reconstituted using a mixture of phospholipids (phosphatidylethanolamine, phosphatidylglycerol, cardiolipin and phosphatidylcholine). Given the profound effect of the detergent environment on the ATPase activity of BtuCD, future investigations into the effects of various lipid mixtures and reconstitution procedures on both the transport and ATPase activities could also be extremely interesting.

A revised model of the BtuCD-F transport cycle. Our results have led us to propose a revised model for the transport cycle of BtuCD-F (Figure 5B). Previous models propose that the SBP is released from the transporter during the transport cycle. In our model, BtuF remains bound to BtuCD, at least partially, even in the "resting state" (left cartoon) and vitamin B_{12} has access to the binding site of BtuF during this time. This change is based upon the extremely tight binding observed between BtuF and BtuCD (17), the low

K_m of BtuF for BtuCD in the transport reaction (see above), and the ability of the BtuCD-F complex to sequester B_{12} in the absence of ATP (see above). Once a B_{12} molecule enters the BtuF binding pocket and becomes sequestered, its diffusion back into the periplasmic space is inhibited. In the presence of ATP, the B_{12} molecule in the BtuCD-F complex binding site sends a signal to the ABC domains (blue dashed arrows) and transport is initiated. During the transport event, the cytoplasmic gate of BtuCD is opened, giving B_{12} access to the cytoplasm and presumably the pathway back into the periplasm is blocked by BtuC and/or BtuF (right cartoon). ATP hydrolysis is required at some point for transport of B_{12} into the cytoplasm. However, because BtuCD appears to hydrolyze ATP at a constant, high basal rate that is stimulated by BtuF but not B_{12}, it is unclear at what stage of the transport event it is required or how tight the coupling between hydrolysis and transport actually is. Nevertheless, the net result of the BtuCD-F transport cycle is clear: periplasmic B_{12} molecules and cytoplasmic ATPs are converted to cytoplasmic B_{12} molecules, ADP and inorganic phosphate.

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