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In Vitro Folding and Assembly of the *Escherichia coli* ATP-binding Cassette Transporter, BtuCD*[S]

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Studies on membrane protein folding have focused on monomeric α-helical proteins and a major challenge is to extend this work to larger oligomeric membrane proteins. Here, we study the *Escherichia coli* (*E. coli*) ATP-binding cassette (ABC) transporter that imports vitamin B_{12} (the BtuCD protein) and use it as a model system for investigating the folding and assembly of a tetrameric membrane protein complex. Our work takes advantage of the modular organization of BtuCD, which consists of two transmembrane protein subunits, BtuC, and two cytoplasmically located nucleotide-binding protein subunits, BtuD. We show that the BtuCD transporter can be re-assembled from both prefolded and partly unfolded, urea denatured BtuC and BtuD subunits. The *in* vitro re-assembly leads to a BtuCD complex with the correct, native, BtuC and BtuD subunit stoichiometry. The highest rates of ATP hydrolysis were achieved for BtuCD re-assembled from partly unfolded subunits. This supports the idea of cooperative folding and assembly of the constituent protein subunits of the BtuCD transporter. BtuCD folding also provides an opportunity to investigate how a protein that contains both membrane-bound and aqueous subunits coordinates the folding requirements of the hydrophobic and hydrophilic subunits.

In recent years substantial efforts have been made toward tackling the membrane protein folding problem (1–3). Pioneering investigations have led to a steady increase in the number of membrane proteins that can be refolded *in vitro* (4–10). With these studies important guidelines for handling and folding these highly hydrophobic proteins are emerging.

To date detailed biophysical folding studies on α-helical membrane proteins have been dominated by work on monomeric proteins (5) or relatively small oligomers (4, 11). The comparative simplicity of these proteins makes them attractive starting points for folding studies, however, they represent a very small portion of all proteomes (12); many membrane proteins function as part of multi-subunit complexes. It is therefore important to extend folding studies to encompass these larger and more complex oligomers, for which there is currently a dearth of information available regarding their folding and assembly. Furthermore, mutations that result in mis-assembly are common in membrane proteins (13) and in some cases not only result in loss of function but in the onset of human diseases such as cystic fibrosis (14).

To further our understanding of the assembly of oligomeric membrane proteins and the factors that stabilize them it is important to be able to recreate their folding and assembly *in vitro*. There are few reported examples of the *in vitro* refolding of multi-subunit α-helical membrane proteins, which include the homo-oligomers, bacterial diacylglycerol kinase (DGK) (4, 11), and the potassium channel, KcsA (7, 8). However, many membrane proteins function as assemblies of membrane embedded and extrinsic domains or subunits, as is the case for the large family of ATP-binding cassette (ABC)[2] transporters. These transporters possess extrinsic, aqueous subunits that hydrolyze ATP to drive transport. ABC proteins transport diverse substrates across cellular membranes (15, 16) and are found in all organisms and in humans their mis-folding and mis-assembly is implicated in several genetic diseases (17–21). Other ABC transporters contribute to the resistance of cancer cells to chemotherapeutic agents (22) and to antibiotic resistance in bacterial cells (23, 24). Here, we use the *Escherichia coli* ABC transporter, BtuCD as a model system for investigating the *in vitro* folding and assembly of a multi-subunit membrane protein complex. This provides the opportunity to investigate how a protein complex balances the requirements of its hydrophobic membrane subunits and hydrophilic aqueous subunits during folding and assembly.

A typical ABC transporter is composed of four core domains: two hydrophobic transmembrane domains (TMDs) and two cytoplasmic nucleotide-binding domains (NBDs) (15, 25). In prokaryotic ABC importers these building blocks are generally assembled from up to four separate protein subunits whereas in export systems they frequently consist of two polypeptide chains in prokaryotes or a single chain in eukaryotes. The vitamin B_{12} import system in *E. coli* encompasses the periplasmically located vitamin B_{12} binding protein, BtuF, and the membrane-associated complex, BtuCD. BtuCD is composed of two copies of the TMDs, BtuC, and two copies of the NBDs, BtuD, which assemble into a functional heterotetramer. Several high

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Table S1.

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[2] The abbreviations used are: ABC, ATP-binding cassette; TMD, transmembrane domain; NBD, nucleotide binding domain; DDM, *n*-dodecyl-β-D-maltopyranoside; LDAO, lauryldimethylamine-*N*-oxide; NEM, *N*-ethylmaleimide.

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resolution crystal structures have been solved for BtuCD both with and without bound BtuF (26, 27).

The modular structural organization of prokaryotic ABC importers provides an ideal paradigm for studying the assembly and intersubunit interactions of membrane protein complexes (28–31). Urea has been used to extract the NBDs of the histidine and maltose ABC transporters from *Salmonella typhimurium* and both transporters re-assembled to a functional state in proteoliposomes or inside-out membrane vesicles (IOVs) (29, 31).

In this work we investigate the refolding and re-assembly of the *E. coli* BtuCD transporter in *n*-dodecyl-β-D-maltopyranoside (DDM) detergent micelles from individual subunits. BtuCD belongs to the type II class of ABC importers which have a distinct TMD fold and mechanism from those of type I ABC importers such as the histidine and maltose transporters (25). The TMDs of type I importers generally contain 12 TM helices whereas each TMD of type II importers have 10 TM helices giving a total of 20 in the complete ABC importer. To our knowledge, this is the first folding study of a type II ABC importer.

The NBD subunits, BtuD, were prepared by the over-expression and subsequent purification as a soluble protein using a plasmid containing only the *btud* gene. A similar method for purifying the TMD subunits, BtuC, in detergent was unsuccessful due to protein aggregation. BtuC was therefore prepared from the complete BtuCD complex by removing the majority of BtuD with urea. The ability of this preparation of BtuC to interact with purified BtuD and re-assemble back into BtuCD, with subunits in correct stoichiometric amounts, is described from three different conditions (i) folded BtuC and BtuD (ii) folded BtuC and urea-unfolded BtuD, and (iii) urea-unfolded BtuC and BtuD. This work extends studies of ABC transporters to assembly from unfolded subunits and a transporter with a known high resolution structure. This gives insight into the amount of pre-existing structure, or key folds that are required to re-assemble an ABC transporter.

EXPERIMENTAL PROCEDURES

Purification of BtuD—BtuD was expressed with an N-terminal decahistidine tag (His tag) followed by a thrombin cleavage site using the pET-19b vector (Novagen) and *E. coli* BL21 (DE3) cells (Novagen). Cells carrying this modified construct were grown at 20 °C and 250 rpm in Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin. Cells were induced at an $A_{650\ nm}$ of ~0.6 with 1 mM IPTG and growth continued overnight. A slow overnight expression at 20 °C significantly improved the amount of BtuD expressed in a soluble cytoplasmic form. Cells were harvested by centrifugation and resuspended in 60 ml of Buffer A (300 mM NaCl, 50 mM Tris, pH 7.5, 5 mM ATP, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), and 20 mM imidazole) supplemented with 1 protease mixture inhibitor tablet (Roche) and a trace amount of DNase I. Cells were lysed by a single passage through a French pressure cell (Spectronic Instruments) at 1000 psi and centrifuged for 40 min at 39,000 × *g* at 4 °C. The supernatant was applied to a 5-ml HisTrap HP nickel column (GE Healthcare) pre-equilibrated with Buffer A. Protein was eluted with an imidazole gradient created using Buffer A and Buffer B (Buffer A with 1 M imidazole). The protein was further purified using a Superdex 200 gel filtration column (HiLoad 16/60; bed volume 120 ml) (GE Healthcare) equilibrated and eluted with Buffer C (300 mM NaCl, 50 mM Tris, pH 7.5, 4 mM ATP, 0.1 mM EDTA, 1 mM EDTA). Fractions containing BtuD were identified by SDS-PAGE, combined and concentrated through a 10 kDa MWCO Vivaspin 15 centrifugal concentrator and stored at −20 °C. For re-assembly the His tag was cleaved by a 3.5h incubation with thrombin and 5 mM $CaCl_2$ after the nickel column step. Imidazole was also removed during the incubation by dialysis into 150 ml of Buffer D (Buffer A + 5 mM $CaCl_2$,). Cleaved BtuD was separated by passage through a nickel column equilibrated with Buffer A and the flow-through containing tagless protein applied to the gel filtration column. BtuD was estimated to be >98% pure, by densitometric scanning of an SDS-PAGE gel, giving a yield of 4–6 mg BtuD per 6-liter culture.

Purification of BtuCD—The expression of BtuCD was carried out in *E. coli* BL21 (DE3) cells in a fermentor. The purification of the BtuCD complex from the cell pellets was essentially as previously described (26) and the detergent exchanged from 0.1% lauryldimethylamine-*N*-oxide (LDAO) to 0.1% *n*-dodecyl-β-D-maltopyranoside (DDM) on the nickel affinity column. BtuCD was >98% pure from SDS-PAGE gel, giving 6–9 mg of pure BtuCD from 10–15 g of frozen cell pellets.

Preparation of BtuD-depleted BtuC—BtuC was prepared by dis-assembling BtuCD in 5 M urea during nickel affinity purification. BtuC of BtuCD has an N-terminal decahistidine tag whereas BtuD does not. BtuD was eluted from the column by washing with Buffer E (500 mM NaCl, 50 mM Tris, pH 7.5, 0.1% DDM, and 5 M urea). The column was washed in buffer without urea and BtuC was subsequently eluted in 500 mM imidazole. Fractions with BtuC were confirmed by SDS-PAGE, pooled, and the imidazole removed by dialysis into 150 ml of Buffer F (300 mM NaCl, 25 mM Tris, pH 7.5, 0.1% DDM) for 45 min and at 4 °C. The dialysis was repeated three more times and BtuC concentrated through a Vivaspin 15 centrifugal concentrator MWCO 10 kDa and stored at −80 °C. BtuC was ~63% pure from an SDS-PAGE gel, and produced yields of around 6 mg of protein.

Urea-induced Unfolding of BtuC and BtuD—BtuC was added to buffer containing 150 mM NaCl, 25 mM Tris, pH 7.5, and 0.1% DDM and incubated for 10 min at room temperature in buffer with various concentrations of urea. The final protein concentration for CD was 0.5 mg/ml in a total volume of 80 μl. BtuD was unfolded as for BtuC in buffer containing 150 mM NaCl, 50 mM Tris pH 7.5 and 2 mM ATP. The final protein concentration was 50 μg/ml in a volume of 80 μl for fluorescence experiments and 2 μM in 300 μl for ATPase activity assays.

Re-assembly of BtuCD—Purified, His tagless BtuD in 2 mM ATP and 0.1% DDM was added in approximately equal amounts to BtuD-depleted BtuC and incubated on ice for 30 min prior to adding 20 mM imidazole. (Urea-unfolded BtuC and/or BtuD, were unfolded beforehand, followed by a ~16-fold dilution into buffer containing 300 mM NaCl, 50 mM Tris, pH 7.5, 0.1% DDM, 2 mM ATP prior to incubation with imidazole.) The mixture was incubated for 1 h at 4 °C with a 50% slurry of Ni-NTA agarose beads (Qiagen) pre-equilibrated in

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buffer containing 300 mM NaCl, 50 mM Tris, pH 7.5, 0.1% DDM, and 20 mM imidazole. The beads were recovered by centrifugation, the supernatant discarded, and the beads washed several times in the above buffer. Re-assembled BtuCD was eluted in buffer with 500 mM imidazole and dialyzed for 1 h at 4 °C into buffer containing 300 mM NaCl, 50 mM Tris, pH 7.5, and 0.1% DDM. This was repeated three times.

ATPase Activity Assays—300-μl reaction mixtures were incubated for 3 min at 37 °C, and ATP hydrolysis initiated by the addition of 10 mM $MgCl_2$ and 2 mM ATP. 5-μl samples were removed at various times and added to 50 μl of stop buffer containing 12% sodium dodecylsulfate (SDS). ATP hydrolysis rates were determined from the production of inorganic phosphate (P_i) using the modified molybdate method (32). Rates are given in terms of either nmol of P_i liberated per min per mg protein (nmol/min/mg) or as turnover per second (s^{-1}), the latter calculated assuming that BtuD functions as a dimer. Protein concentrations were determined form the absorbance at 280 nm (ϵ = 219800 M^{-1} cm^{-1}, estimated from the number of tryptophan, tyrosine, and cysteine residues (33)) and a Bradford assay, with good agreement between the two. ATP hydrolysis rates are given in the absence of BtuF, as no stimulation of activity was seen for BtuCD in the presence of BtuF (see supplemental Fig. S1), as previously reported (34).

Circular Dichroism Spectroscopy—CD spectra were collected at Birkbeck College, London, UK using an Aviv 62ds CD machine. Spectra were recorded at 25 °C using a 0.1 mm cell in 0.5-nm intervals with a 0.5 s integration time and a bandwidth of 1 nm. Data were analyzed using the CDtool software (35).

Fluorescence Spectroscopy—Fluorescence spectra were recorded on a FluoroMax-2 (Jobin Yvon) at room temperature using an excitation wavelength of 280 nm and measuring fluorescence emission between 300–450 nm with 5 nm emission and excitation slit widths. Data were fit using Microcal Origin 6.0 software.

Gel Filtration Analysis of Re-assembled Complexes—Gel filtration was carried out using a Superdex 200 gel filtration column (PC 3.2/30; bed volume 2.4 ml) mounted on an ÄktaPrime system using a precision column holder and at a flow rate of 0.1 ml/min. The mobile phase of the gel filtration column contained 300 mM NaCl, 50 mM Tris, pH 7.5 and 0.1% DDM, and the elution profiles were monitored by absorbance at 280 nm.

RESULTS

Characterization of Pure, Soluble BtuD—BtuD was overexpressed in *E. coli* with an N-terminal His tag and purified from the cytoplasmic fraction as a soluble protein. Analysis by SDS-PAGE shows that BtuD is >98% pure and has an apparent molecular mass of 31 kDa, consistent with the weight predicted from the amino acid sequence (Fig. 1*A*). SDS-PAGE and Western blotting with an anti-His antibody were also used to demonstrate the successful cleavage of the His tag (Fig. 1*B*). Only ~2% of His-BtuD remained and was removed by re-application to a nickel column. BtuD displayed a strong propensity to precipitate but was soluble in the presence of 5 mM ATP. The solubility of BtuD deteriorated upon ATP hydrolysis following addition of Mg^{2+}. Thus an ATP-bound conformation maintains isolated BtuD in solution, as previously reported for the NBD, HisP, of the histidine permease transporter (36).

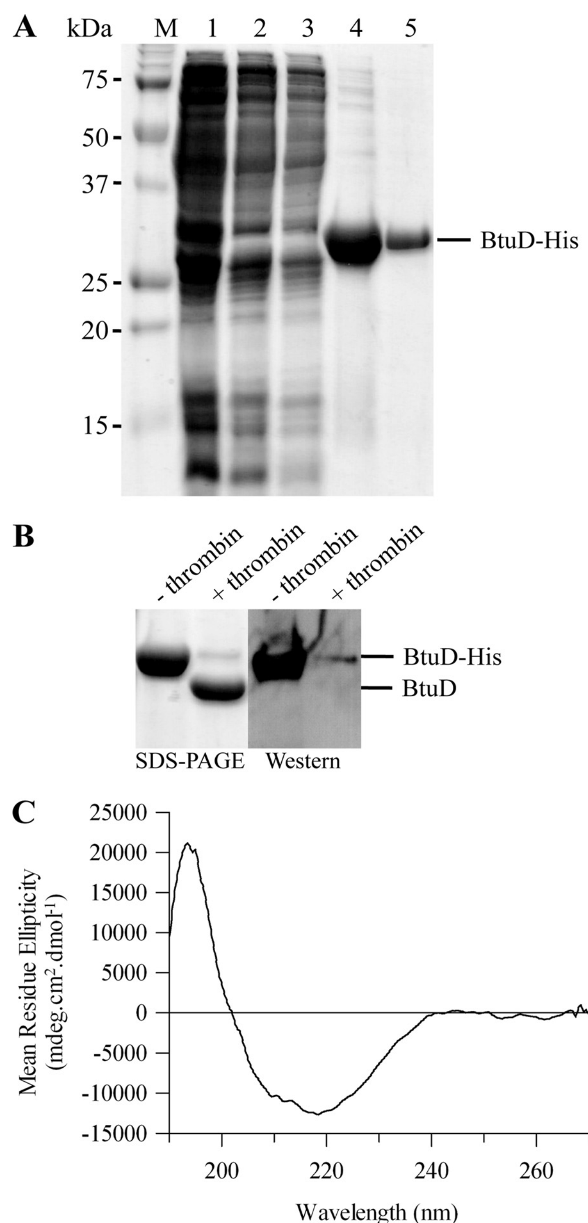

FIGURE 1. **Purification of the BtuD subunit.** *A*, SDS-PAGE analysis of fractions collected during the purification of BtuD. Cytoplasmic fraction derived from the crude cell extract (*lane 1*), flow-through from Ni-NTA affinity column (*lane 2*), wash-step (*lane 3*), eluate from the imidazole gradient (*lane 4*), and pooled peak fractions following gel filtration (*lane 5*) are shown. *B*, SDS-PAGE gel (*left*) and Western blot (*right*) showing efficient cleavage of the His tag of BtuD. Samples of uncleaved BtuD eluted from the Ni-NTA affinity column (−thrombin) and BtuD with a cleaved His tag following a 3.5-h incubation with thrombin (+ thrombin His) are shown. *C*, Far-UV CD spectrum of purified BtuD in 150 mM NaCl, 25 mM Tris, pH 7.5, 0.1 mM EDTA, 1 mM DTT, and 2 mM ATP and at a protein concentration of 0.4 mg/ml.

CD spectroscopy was used to determine the secondary structure content of purified BtuD. Deconvolution of the far-UV CD spectrum of this protein subunit (Fig. 1*C*) gave a secondary structure content of 35% α-helix and 18% β-sheet, similar to values from the BtuCD crystal structure (PDB IL7V) of 36% α-helix and 17% β-sheet. The ATPase activity of purified BtuD was compared with that of the complete BtuCD complex in DDM (supplemental Fig. S1 and Table S1). Pure BtuD hydro-

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lyzed Mg-ATP with a V_{max} of ~280 nmol/min/mg and K_m of 77 μM; similar to the BtuCD complex values V_{max} of ~640 nmol/min/mg and K_m of 53 μM, although the V_{max} of BtuD is approximately half that of BtuCD. These V_{max} and K_m values agree with those for other complete ABC transporters and their isolated NBDs (37). The *E. coli* maltose importer, $MalFGK_2$, for example has a reported V_{max} of 860 nmol/min/mg and K_m of 53 μM while the isolated NDB, MalK, have a V_{max} of ~700 nmol/min/mg and K_m of 70 μM. Turnover rates were 0.30 s^{-1} and 1.39 s^{-1} for BtuD and BtuCD, respectively. The ATPase activity of BtuD was also similar to that of the complete complex with respect to the sensitivity to glycerol, ADP and sodium orthovanadate and the insensitivity to *N*-ethylmaleimide (NEM). The presence of glycerol inhibited ATP hydrolysis in both BtuCD and BtuD by similar extents with 20% glycerol resulting in ~50% reduction in activity in both cases. This inhibition was reversible for BtuD upon the dilution of glycerol. The activities of BtuCD and BtuD were also inhibited by ADP, with 1 mM ADP required for half-maximal inhibition in both cases. In the presence of 2 mM sodium orthovanadate, the ATPase activity of BtuCD and BtuD was reduced by >96%, while 10 mM NEM had no effect. No cooperativity could be detected for BtuCD, but the Hill coefficient of BtuD was ~2, indicative of positive cooperativity between two ATP binding sites.

Preparation of BtuC by Dis-assembly of BtuCD in Urea—Overexpression and purification of isolated BtuC in DDM from a plasmid containing only the *btuC* gene resulted in aggregated protein. This suggests that BtuC either adopts a non native structure in DDM or that the correct folding requires the presence of BtuD. BtuC was therefore prepared by removing the BtuD subunits from BtuCD in urea. A large portion of BtuD was separated from BtuC in 5 M urea, as shown by an intense BtuD band at ~27 kDa (Fig. 2*A*, *lane 7*) in the eluate of the nickel affinity column (BtuC containing a His tag, and BtuD without). This urea treatment also resulted in additional protein bands in the eluate with BtuD: notably just below the 37 kDa marker, labeled band A. BtuD-depleted BtuC was then eluted from the column and ran as a doublet band at ~33kDa (Fig. 2*A*, *lane 9*) A doublet band for BtuC has previously been observed in BtuCD preparations (38) (see below). Some BtuD, ~20%, eluted with BtuC, supporting the notion that BtuD aids BtuC folding. The contaminating protein bands which migrated above the 50 kDa molecular marker were also present in the purification of BtuCD where they could be removed by gel filtration. Contaminating protein bands were also present just above the BtuC doublet band and just below the BtuD band. The final purity of BtuC, as estimated from the SDS-PAGE gel was ~63%, with BtuD as the main contaminant, and was less than that achieved for either purified BtuCD or BtuD (~97 and 98%, respectively). Importantly, this partially pure preparation of BtuC exhibited no ATPase activity showing that the small amount of BtuD present was nonfunctional in terms of ATP hydrolysis. The relative amounts of BtuC and BtuD present in this BtuD-depleted preparation of BtuC were 82 and 18%, respectively. From here on, this preparation of BtuC will be referred to as BtuC- or BtuD-depleted BtuC.

The far-UV CD spectrum of isolated BtuC in DDM (after removal of urea) was characteristic of a largely α-helical protein with ~80% α-helix, akin to the native secondary structure content of BtuC estimated from the known structure of BtuCD (75% α-helix and 0% β-sheet) (Fig. 2*B*). Thus, treatment with 5 M urea did not appear to cause any permanent damage to BtuC secondary structure. Treatment of BtuCD with a higher concentration of 8 M urea resulted in a slightly cleaner preparation of BtuC of 72% purity, with less BtuD (only 6%). There is also a reduced amount of the contaminating protein band A (about 43% less band A in 8 M than in the 5 M urea preparation). However, this higher urea concentration of 8 M also resulted in partial unfolding of BtuC, as shown by a reduction in helicity (Fig. 2*B*). Refolding of BtuC required the presence of BtuD. Thus, both urea depletion and expression of BtuC alone suggest that it is not possible to obtain an isolated, stable and folded form of BtuC; rather some BtuD is required to stabilize BtuC in detergent solution. It was possible to re-assemble BtuCD from the 8 M BtuC preparation and BtuD in 8 M urea, where both BtuC and BtuD are partly unfolded. The fact that there was less of the band A in the 8 M preparation but the complex still re-assembles also suggests that this contamination does not play a key role in the assembly.

We focus here on re-assembly using the 5 M urea BtuC preparation as this gives an intact BtuC subunit and thus allows us to assess re-asssembly of BtuCD from both pre-assembled BtuC and BtuD subunits (where the former BtuD-depleted BtuC has

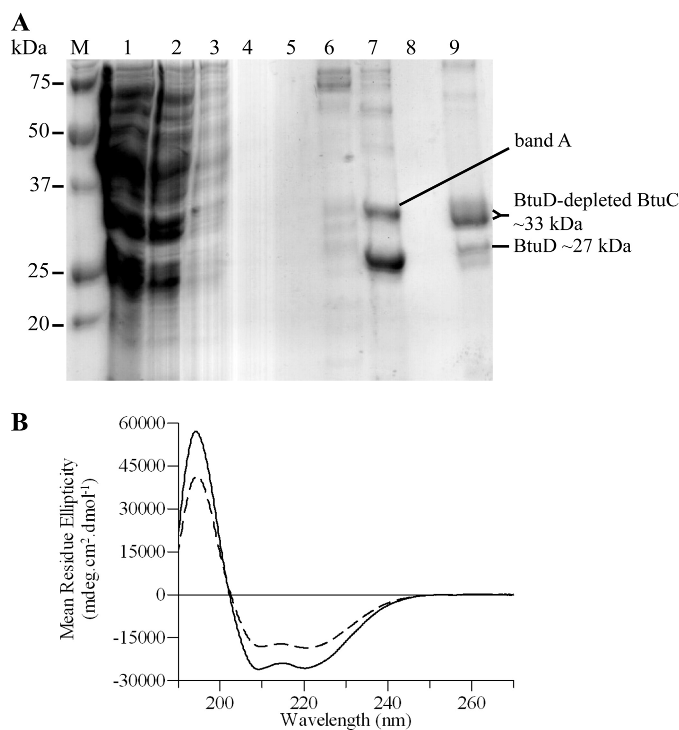

FIGURE 2. **Purification of the BtuC subunit.** *A*, SDS-PAGE gel following the preparation of BtuC by removal of BtuD in 5 M urea. Cytoplasmic and membrane fractions from whole cell extract (*lane 1*), flow-through from Ni-NTA affinity column (*lane 2*), LDAO wash (*lane 3*), LDAO/DDM wash for detergent exchange (*lane 4*), DDM wash (*lane 5*), 100 mM imidazole wash (*lane 6*), 5 M urea wash (*lane 7*), wash with urea omitted (*lane 8*), and 500 mM imidazole eluate (*lane 9*) are shown. Molecular mass markers are indicated in kDa (*lanes M*), and bands corresponding to BtuC and BtuD are labeled accordingly. *B*, Far-UV CD spectra of BtuC in either 5 M urea (*solid line*) or 8 M urea (*dashed line*) and in buffer containing 150 mM NaCl, 25 mM Tris, pH 7.5 and 0.1% DDM. The protein concentration was 0.5 mg/ml and 0.84 mg/ml for BtuC in 5 M or 8 M urea, respectively.

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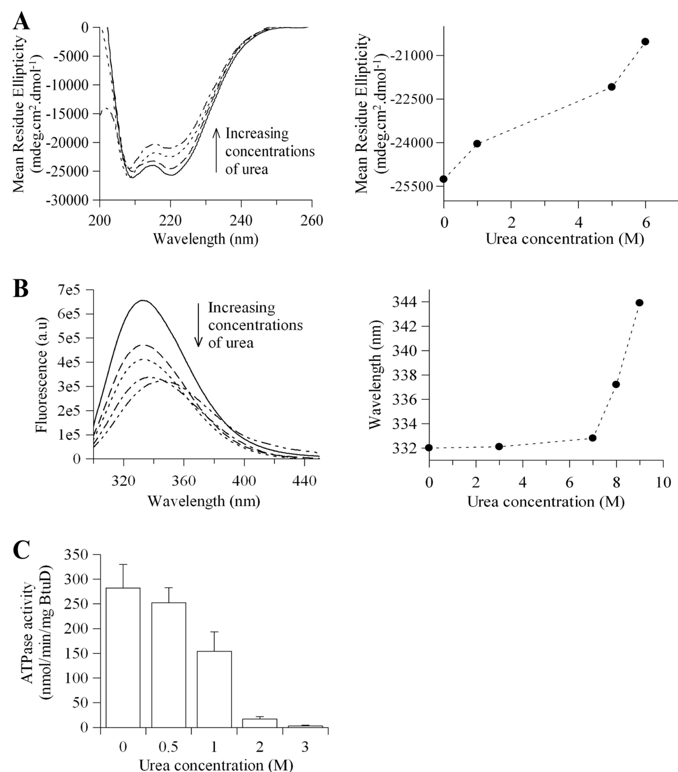

FIGURE 3. **Urea unfolding of isolated BtuC and BtuD subunits.** *A*, *left*: Far-UV CD spectra of BtuC in the presence of 0 M (*solid line*), 1 M (*dashed line*), 5 M (*dotted line*), and 6 M urea (*dashed-dotted line*). *Right*, plot of CD signal intensity at 222 nm *versus* urea concentration. *B*, *left*: fluorescence emission spectra of BtuD in the presence of 0 M (*solid line*), 3 M (*dashed line*), 7 M (*dotted line*), and 9 M urea (*dashed-dotted line*). *Right*: plot of the fluorescence wavelength maxima *versus* urea concentration. *C*, ATPase activity of BtuD measured in urea concentrations between 0 and 3 M.

18% BtuD associated with it), as well as partly unfolded BtuD and BtuC.

Urea Denaturation of BtuC—The unfolding of the isolated BtuC and BtuD subunits by urea was investigated by changes in far-UV CD and protein fluorescence, as well as ATPase activity for BtuD. A decrease in the 222 nm CD band, indicative of a reduction in helical structure, was observed upon the addition of urea to BtuC (Fig. 3*A*). In 6 M urea the 222 nm intensity was reduced by ~20%. Urea caused only small changes in BtuC fluorescence (data not shown). The overall shift in the fluorescence maximum between 0–9 M urea was less than 1 nm and there was little decrease in fluorescence intensity. BtuC contains 14 tryptophans, this could therefore suggest that either there is little change in tertiary structure or solvent accessibility of tryptophans or that decreases in fluorescence due to tryptophan exposure to the aqueous phase during unfolding is offset by a loss of tryptophan quenching.

Urea Denaturation of BtuD—Unfolding of BtuD was carried out in the presence of ATP to maintain solubility. No significant changes in the CD spectrum of BtuD were observed for urea concentrations up to 8 M. However, significant changes were observed in protein fluorescence. BtuD has four tryptophan residues and a fluorescence maximum at 332 nm (Fig. 3*B*). Although little shift in the emission maximum was observed in urea concentrations up to 7 M, a large red-shift accompanied by a significant decrease in emission intensity, was observed in higher concentrations (344 nm in 9 M urea). This result is characteristic of tryptophan side chains moving to a more hydrophilic environment during unfolding. Low urea concentrations affected ATPase activity, with activity being negligible in 3 M urea (Fig. 3*C*). Thus, loss of functionality of BtuD is not accompanied by significant structural losses. Because 2 ATPs bind across the BtuD dimer interface (39–44), this suggests that the BtuD dimer dissociates in low urea concentrations (0.5–3 M), with little structural changes in the monomers. In contrast high urea (7–9 M) causes partial BtuD monomer unfolding.

TABLE 1

ATPase activities of re-assembled BtuCD complexes

BtuCD was re-assembled from (i) pre-folded BtuC and pre-folded BtuD (C_FD_F), (ii) pre-folded BtuC and partly unfolded BtuD (C_FD_U), and (iii) BtuC and BtuD, both partly unfolded (C_UD_U). The starting points for re-assembly under each of these conditions are summarized below. In each case, the ATPase activity of the resulting re-assembled complex was measured and compared to that of native BtuCD. Standard errors of the mean are given.

Reassembled BtuCD complex	Starting point for re-assembly		ATPase activity of re-assembled complexes (s^{-1})	% ATPase activity of native, purified BtuCD
	BtuC subunit	BtuD subunit		
C_FD_F	Folded BtuC with native-like secondary structure	Folded BtuD with native-like secondary structure and functional in ATP hydrolysis	0.78 ± 0.13	55
C_FD_U	Folded BtuC	Partially unfolded BtuD as detected by fluorescence spectroscopy with a red shifted fluorescence emission maximum and inability to hydrolyse ATP	0.93 ± 0.07	67
C_UD_U	Partially unfolded BtuC as detected by CD spectroscopy as a reduction in α-helicity	Partially unfolded BtuD	1.01 ± 0.10	72

Re-assembling a BtuCD Transporter Complex—The ability of BtuD-depleted BtuC to re-associate with pure BtuD to give a complex with correct BtuCD stoichiometry was investigated. Re-assembly was investigated from: (i) folded BtuC and folded BtuD (C_FD_F), (ii) folded BtuC and BtuD partly unfolded in 7.5 M urea (C_FD_U), and (iii) BtuC and BtuD, both partly unfolded in 8 M urea (C_UD_U) (see Table 1). Several methods were used to compare the re-assembled complexes to pure, functional BtuCD, including SDS-PAGE analysis to determine the stoichiometry of the BtuC and BtuD subunits, functional ATPase assays, gel filtration experiments, and CD and fluorescence.

(i) Re-assembly from folded BtuC and folded BtuD *(C_FD_F)*. BtuD-depleted BtuC was incubated with a molar equivalent of BtuD in the presence of ATP and applied to a nickel affinity column where BtuC binds via its His tag. The His tag of BtuD was removed prior to re-assembly. Thus BtuD cannot bind itself to the column and any binding of BtuD to the column is

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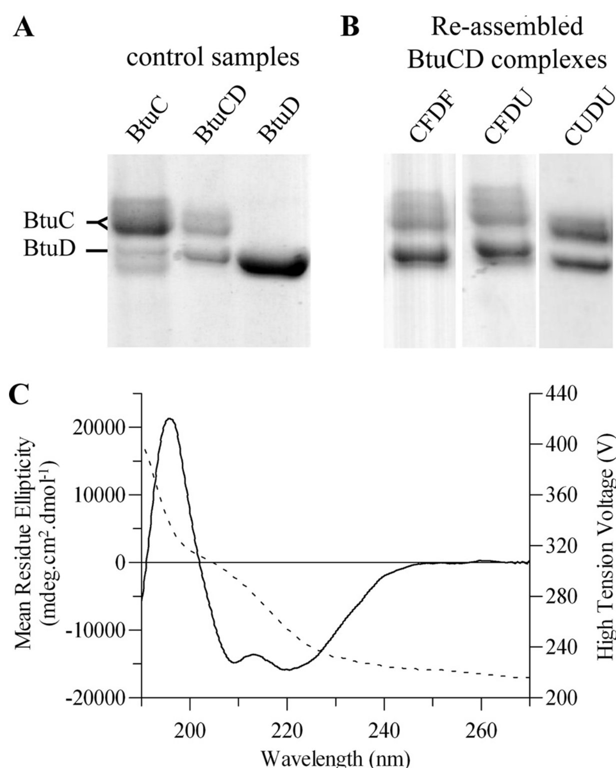

FIGURE 4. **Re-assembly of BtuCD from individual BtuC and BtuD subunits in various states of denaturation.** *A*, SDS-PAGE gel of purified BtuC (*i.e.* BtuD-depleted BtuC), BtuCD, and purified BtuD. *B*, SDS-PAGE gels of BtuCD re-assembled from C_FD_F, C_FD_U, and C_UD_U following elution from the Ni-NTA column. *C*, Far-UV CD spectrum of re-assembled BtuCD from C_FD_F and in buffer containing 150 mM NaCl, 50 mM Tris, pH 7.5 and 0.1% DDM. *Left axis*, mean residue ellipticity (*solid line*); *right axis*, HT (*dotted line*).

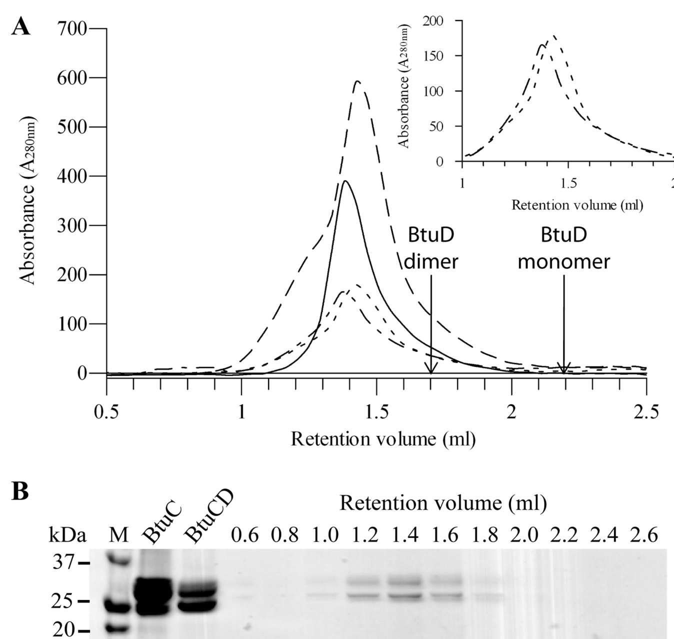

FIGURE 5. **Gel filtration analysis of re-assembled BtuCD complexes.** *A*, BtuCD re-assembled from C_FD_F (*dashed line*), C_FD_U (*dotted line*), and C_UD_U (*dashed-dotted line*). Purified BtuCD is also shown (*solid line*). The *arrows* show where pure BtuD runs on the gel filtration column in both a dimeric or monomeric conformation. BtuD-depleted BtuC ran at a similar position to BtuCD with a shoulder at 1.2 ml retention volume. *B*, SDS-PAGE analysis of the gel filtration fractions collected for BtuCD reassembled from C_FD_F. Molecular mass markers are indicated in kDa (*lane M*) and purified BtuC and BtuCD control samples are also labeled accordingly. Because of a lower protein concentration for BtuCD complexes re-assembled from C_FD_U and C_UD_U, a smaller amount of these samples was loaded onto the column than for BtuCD re-assembled from C_FD_F.

due to the re-association of additional BtuD with BtuC. The corresponding peak eluted from the column therefore reflects the BtuC and BtuD subunit stoichiometry of this re-assembled complex, where BtuD has associated with the BtuC preparation. Re-assembly is determined by an increase in the amount of BtuD present, over that in the initial BtuC preparation that has some BtuD already bound (~18% BtuD). In contrast the intact BtuCD complex will have ~50% BtuD and 50% BtuC. The proportions of BtuD bound to BtuC were determined by SDS-PAGE.

Fig. 4*A* shows an SDS-PAGE gel of the starting BtuC and BtuD preparations, (the latter with the His tag removed) as well as the purified complete BtuCD complex. This purified BtuCD complex remains intact in DDM solution as shown by the fact that it elutes as a defined single peak upon gel filtration corresponding to a complex with two BtuC and two BtuD subunits (38). The same subunit stoichiometry is also observed upon electrospray mass spectrometry of the BtuCD-DDM species, which remains as an intact complex in the mass spectrometer (45). On a denaturing SDS-PAGE gel the BtuC and BtuD subunits dissociate and pure, intact BtuCD migrates as three bands; a doublet band corresponding to BtuC (as described above with respect to Fig. 2*A*), and a single band at a lower molecular weight corresponding to BtuD (38). The BtuC doublet band is in part due to the N-terminal His tag, which can result in multiple bands if the tag is lost during membrane targeting. The relative BtuC (doublet) and BtuD band intensities were determined by densitometric scanning of the gel and used to estimate the proportions of BtuC and BtuD present in pure, intact BtuCD, giving 53% BtuC and 47% BtuD. Both bands of the doublet were used to estimate the amount of BtuC. The same doublet BtuC and single BtuD bands were also observed for BtuCD re-assembled from pre-folded BtuC and BtuD subunits (C_FD_F), as shown in the SDS-PAGE gel in Fig. 4*B*. Furthermore the intensities of these bands corresponded to 47% BtuC and 53% BtuD. These intensities match closely to those of purified, intact BtuCD. Thus more BtuD has associated with BtuC than in the BtuD-depleted BtuC starting point (18% BtuD in the latter *versus* 47% BtuD in re-assembled BtuCD). This implies that re-assembled BtuCD has formed from pre-folded subunits (C_FD_F) with correct stoichiometry of its constituent protein subunits. Two additional protein bands above and below the BtuC and BtuD bands are also observed, as seen in purified BtuC.

The CD spectrum of the re-assembled complex was also characteristic of a protein with a large percentage of α-helical secondary structure, with a large positive band at 190 nm and two smaller negative bands centered at 208 and 222 nm (Fig. 4*C*). Upon deconvolution of this CD spectrum, the secondary structure content was estimated to be 57% α-helix and 5% β-sheet, almost identical to that predicted from the known structure of BtuCD (59% α-helix and 5% β-sheet).

Re-assembled BtuCD, C_FD_F, also displayed behavior on a small-scale gel filtration column that was similar to that of pure

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BtuCD. The elution profiles in Fig. 5*A* show that in both cases a major peak is observed with a retention volume around 1.4 ml. SDS-PAGE analysis of the gel filtration fractions collected for the re-assembled complex confirms that this major peak contained both BtuC and BtuD (Fig. 5*B*). Furthermore, the percentage amounts of BtuC and BtuD, as determined by densitometric analysis, were found to be similar to that of pure BtuCD (45% BtuC and 55% BtuD). For the re-assembled complex there is a shoulder in the elution profile around 1.2 ml that is not observed for pure BtuCD. This is however, also observed for the preparation of BtuD-depleted BtuC.

A convenient tool in these studies for identifying native-like functional properties of the BtuD subunits in re-assembled complexes is the recovery of ATPase activity. Table 1 shows the rate of ATP hydrolysis for BtuCD re-assembled from C_FD_F ($0.78\ s^{-1} \pm 0.13$) is greater than that for BtuD alone ($0.30\ s^{-1} \pm 0.01$), but lower than that of intact native BtuCD ($1.39\ s^{-1} \pm 0.06$). This suggests that the re-assembled BtuCD complex has partially recovered ATPase activity, but has native BtuCD subunit stoichiometry.

Similar unfolding properties were also observed for re-assembled BtuCD, C_FD_F, and intact native BtuCD; the same changes in fluorescence were seen upon the addition of increasing concentrations of urea in either the absence or presence of ATP (data not shown). In both cases, a progressive red-shift in the emission maximum was observed that was larger in the absence of ATP than in its presence, suggesting that ATP might moderate the urea-unfolding of BtuCD. Collectively, the results are consistent with the overall structural organization of re-assembled BtuCD being very similar to that of pure BtuCD.

(ii) Re-folding and re-assembly from folded BtuC and folded BtuD partly unfolded in 7.5 M urea (C_F:D_U). The SDS-PAGE gel in Fig. 4*B* shows re-assembled BtuCD, C_FD_U, contains 46% BtuC and 54% BtuD, similar to pure BtuCD. The ATPase activity of the re-assembled complex of $0.93\ s^{-1} \pm 0.07$ was higher than that of $0.78\ s^{-1} \pm 0.13$ observed following re-assembly from two folded subunits C_FD_F (Table 1).

(iii) Re-folding and re-assembly from BtuC and BtuD, both partly unfolded in 8 M urea (C_U:D_U). The SDS-PAGE gel in Fig. 4*B* also shows that BtuCD with 2 BtuC and 2 BtuD subunits can be re-assembled when both subunits are partly unfolded (C_UD_U) to give a re-assembled complex with 49% BtuC and 51% BtuD, without the contaminating protein bands observed for complexes from C_FD_F and C_FD_U. These contaminating bands are from the preparation of BtuC and are likely to have been removed by the treatment of the BtuC subunits in 8 M urea prior to re-assembly. Re-assembly from two partly unfolded subunits also resulted in the re-assembled complex with the highest rate of ATP hydrolysis ($1.01\ s^{-1} \pm 0.10$) at 72% of the ATPase activity of purified BtuCD (Table 1).

The BtuCD complexes re-assembled from one or more partly unfolded subunits (C_FD_U and C_UD_U) had analogous properties to purified BtuCD with respect to elution on a gel filtration column (Fig. 5*A*). Moreover, the changes in fluorescence upon unfolding re-assembled BtuCD (from C_FD_U or C_UD_U) in urea, in both the absence and presence of ATP, were similar to purified BtuCD. However high quality CD spectra were difficult to obtain for complexes re-assembled from denatured subunits. The absorbance (high tension (HT) values for these samples exceeded 600 V at ≤ 215 nm and therefore the spectra below this wavelength were unreliable (see HT value <280V at 215 nm for C_FD_F in Fig. 4*C*). The high absorbance may suggest the presence of aggregated protein in the C_FD_U or C_UD_U complexes or that the high urea used in unfolding was not completely removed.

DISCUSSION

We show that the *E. coli* ABC transporter, BtuCD, can be re-assembled from its separate BtuC and BtuD subunits that are either pre-folded or partly denatured in urea. Furthermore, we have shown that unfolded subunits also re-assemble to give a full transporter with native BtuC and BtuD subunit stoichiometry and partial recovery of ATPase activity. Re-assembled BtuCD has the greatest ATPase activity, ~72% of pure, functional BtuCD in DDM, if re-assembled from unfolded subunits (C_UD_U). Our findings also suggest that the correct fold of BtuC is stabilized by the presence of at least some BtuD, with high urea concentrations being required to completely dissociate BtuD from BtuC. This could imply that, at least *in vitro*, a degree of folding has to occur for the individual BtuC and BtuD subunits prior to assembly. This is in line with previous work suggesting a partly folded helical core is necessary for correct folding of the monomeric membrane protein bacteriorhodopsin (46–48).

This *in vitro* study provides insights into the folding and assembly of oligomeric membrane protein complexes and supports previous findings that ABC transporters can be re-assembled from their individual subunits *in vitro* (30, 31). Moreover, we find evidence for co-operative subunit folding and assembly. This study, however, has focused on assembly in detergent micelles. The successful refolding and assembly gives information on the factors stabilizing the protein complex, but additional features are at play in biological membranes. A lipid bilayer is likely to stabilize membrane-embedded regions. Moreover, the native lipid composition could be important in correct folding and assembly of the protein complex. *E. coli* inner membranes contain predominantly phosphatidylethanolamine lipids and anionic lipids. This composition has been shown to be required for correct membrane protein domain topology, with an incorrect, inverted domain topology being observed in the absence of phosphatidylethanolamine (49). Additionally there may be specific and nonspecific lipid interactions involved in stabilizing the complex (49, 50). Unraveling the assembly pathways of membrane proteins in the cell is nontrivial and as a result, there is only limited knowledge on the *in vivo* assembly of multi-subunit complexes. Most studies aimed at deciphering membrane protein complex assembly have involved isolating assembly intermediates and, in general, have concluded that the process follows a unique and ordered pathway (51, 52). In the cell, the folding and assembly of multi-subunit membrane proteins is also likely to benefit from the presence of molecular chaperones. It is thought that the binding of these chaperones to proteins may be required to prevent undesirable interactions such as those leading to aggregation (51, 53). This could be important in coordinating the folding and assembly of different subunits.

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The unfolding of BtuC and BtuD to a state of loss of structure and/or structural function has been achieved in urea, but urea can only partially unfold both subunits. 5 M and 6 M urea reduce the helix content of BtuC by ~12 and 20%, respectively. Low urea concentrations dissociate the BtuD dimer, while partial unfolding of the BtuD monomer, as shown by changes in fluorescence, occurs in urea >7 M urea together with a depletion in ATPase activity, but little detectable structural change in secondary structure by CD in up to 8 M. This high resistance of BtuD to urea denaturation is due to the presence of ATP, which stabilizes the subunits in a dimeric state. In the absence of ATP BtuD displays susceptibility to urea-unfolding that is more typical of a water-soluble protein; BtuD is partly unfolded in 3 M urea. These findings are also consistent with gas-phase dissociation pathways observed by mass spectroscopy of the BtuCD complex (45).

All re-assembled BtuD complexes contained individual domains in correct stoichiometric amounts and behaved similarly to the original BtuCD complex upon gel filtration chromatography. In addition, the intrinsic fluorescence properties of all re-assembled complexes upon urea denaturation, both with and without ATP, were similar to the original complex. This latter result implies that the overall structural organization of the re-assembled complexes is similar to that of purified BtuCD. The secondary structure content of the CD spectrum collected for the complex re-assembled from pre-folded subunits also matched that of purified BtuCD. The ATPase rates of the re-assembled complexes increased in the order of folded BtuC and folded BtuD (C_FD_F) < folded BtuC and partly unfolded BtuD (C_FD_U) < partly unfolded BtuC and BtuD (C_UD_U). The latter gives the maximal activity for a re-assembled complex; 72% of the ATPase activity of pure BtuCD. Thus, concomitant subunit folding and assembly seems to result in the most active BtuCD complex. Necessary inter-subunit interactions may therefore form at an essential point during the folding of these subunits and thus that folding and assembly may be coupled processes, as previously described for KcsA (8).

There are few studies reporting the effects of interactions between subunits during the folding of oligomeric proteins, even for water-soluble proteins, and dimeric proteins have been a presiding focus (54–57). There have been more studies of multi-domain, water-soluble proteins (*i.e.* a single polypeptide chain that folds into more than one domain) with a recent analysis proposing that the nature of the domain interface dictates whether the domains fold independently or cooperatively (58); small, loosely packed or unstructured interfaces result in independent domain folding. Conversely, larger, more densely packed, hydrophobic interfaces correlate with an increased dependence of domain folding. The Fab fragment for example, consists of one light chain (L) and one heavy chain (H) that each fold into a constant (C) and variable (V) domain. Folding studies have shown that the domains fold dependently at the inter-chain domain interfaces, V_L-V_H and C_L-C_H, and independently at the intra-chain interfaces, V_L-C_L and V_H-C_H (59). Structural analysis confirmed that the shared interface was large (~1800 Å^2), densely packed (local atomic density ~40) and hydrophobic for the domains whose folding was mutually dependent. However, for the domains that folded independently, the shared interface was smaller (400 Å^2) and loosely packed (local atomic density ~18) (58). The interface buried in BtuCD between the BtuC and BtuD subunits is large, with a total surface are of ~1500 Å^2 buried between them (26). Furthermore, experimental evidence collected in our laboratory suggests that hydrophobic interactions are important in forming the BtuC-BtuD interface.[3] In these experiments, dis-assembly of BtuCD and removal of BtuD in urea was less efficient in increasing concentrations of NaCl, presumably due to its ability to stabilize hydrophobic interactions. That hydrophobic interactions are significant at the BtuC-BtuD interface, is also supported by an examination of the BtuCD complex (Fig. 6). On searching for all residues at the BtuC-BtuD interfaces with an atom within 5 Å of a residue in the other subunit, two clear hydrophobic patches are observed (Fig. 6*A*). The first of these is formed between Leu^{96} located in helix 3 of BtuD and Leu^{216} and Leu^{224} located in L1 and L2 of BtuC, respectively. The second is formed between Leu^{107} of helix 4 and Leu^{120} located just before the ABC signature motif of BtuD and Leu^{2} and Leu^{4} located in transmembrane helix 1 of BtuC. The space-filling models in Fig. 6*B* show the surface of each subunit involved in forming the BtuC-BtuD interface and are colored according to the nature of the residues found at this surface. Although the surface of BtuC is considerably more hydrophobic, there are clear, matching regions of hydrophobicity on the surface of BtuD. Thus, our BtuCD results fit with the current hypothesis that the inter-domain interfaces determine the folding dependence of these domains; there is a large hydrophobic subunit interface between BtuC and BtuD leading to a cooperative folding and assembly of these subunits.

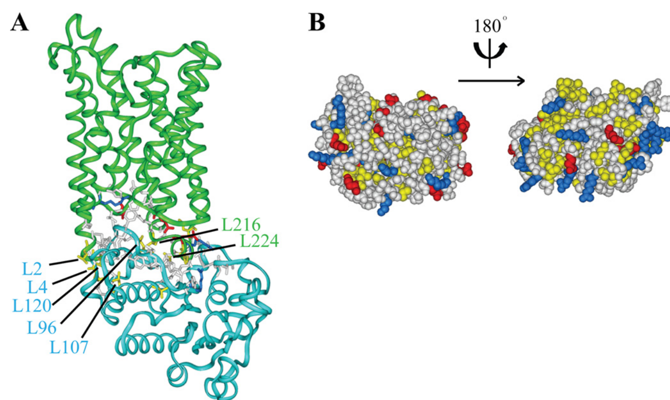

FIGURE 6. **The BtuC-BtuD transmission interface of BtuCD.** *A*, only one of the transmission interfaces of BtuCD is shown with BtuC and BtuD subunits shown in *green* and *cyan*, respectively. Residues with an atom within 5 Å of a residue in the other subunit and thought to be involved in forming one of the two hydrophobic patches at the BtuC-BtuD interface are labeled. *B*, space-filling models looking down onto the surface of each subunit involved in forming the transmission interface. Hydrophobic, positively charged, and negatively charged residues are colored in *yellow*, *blue*, and *red*, respectively. All other residues are in *white*. The figure was created using PBD entry 1L7V.

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[3] P. J. Booth, unpublished observation.

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***In Vitro* Folding and Assembly of the *Escherichia coli* ATP-binding cassette Transporter, BtuCD**

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SUPPLEMENTAL DATA

	BtuCD	BtuD
V_{max} (nmol/min/mg)	652 ± 16	301 ± 13
K_m	53	77
n	1.1 ± 0.2	1.8 ± 0.4
Inhibitors and stimulators	% Inhibition	
2 mM Na_3VO_4	97	98
10 mM NEM	0	0
[Glycerol] %		
0	0	0
2	19	14
5	26	9
10	18	27
15	47	38
20	54	47
[ADP] mM		
0	0	0
0.25	21	22
0.5	17	35
1	53	55
2	85	57
4	100	77

Table S1. Summary and comparison of the ATPase activities of BtuCD and BtuD under various conditions. ATPase activity assays were carried out at a protein concentration of 140nM for BtuCD and 1 μM for BtuD. For BtuCD assays were performed in the presence of 150mM NaCl, 50mM Tris pH 7.5, 0.1% DDM and 2mM ATP and for BtuD in the presence of 300mM NaCl, 50mM Tris pH 7.5, 0.1mM EDTA, 1mM DTT and 2mM ATP (unless specified otherwise). To determine the K_m for ATP and cooperativity (where n=the hill coefficient) assays were performed by varying the concentration of ATP

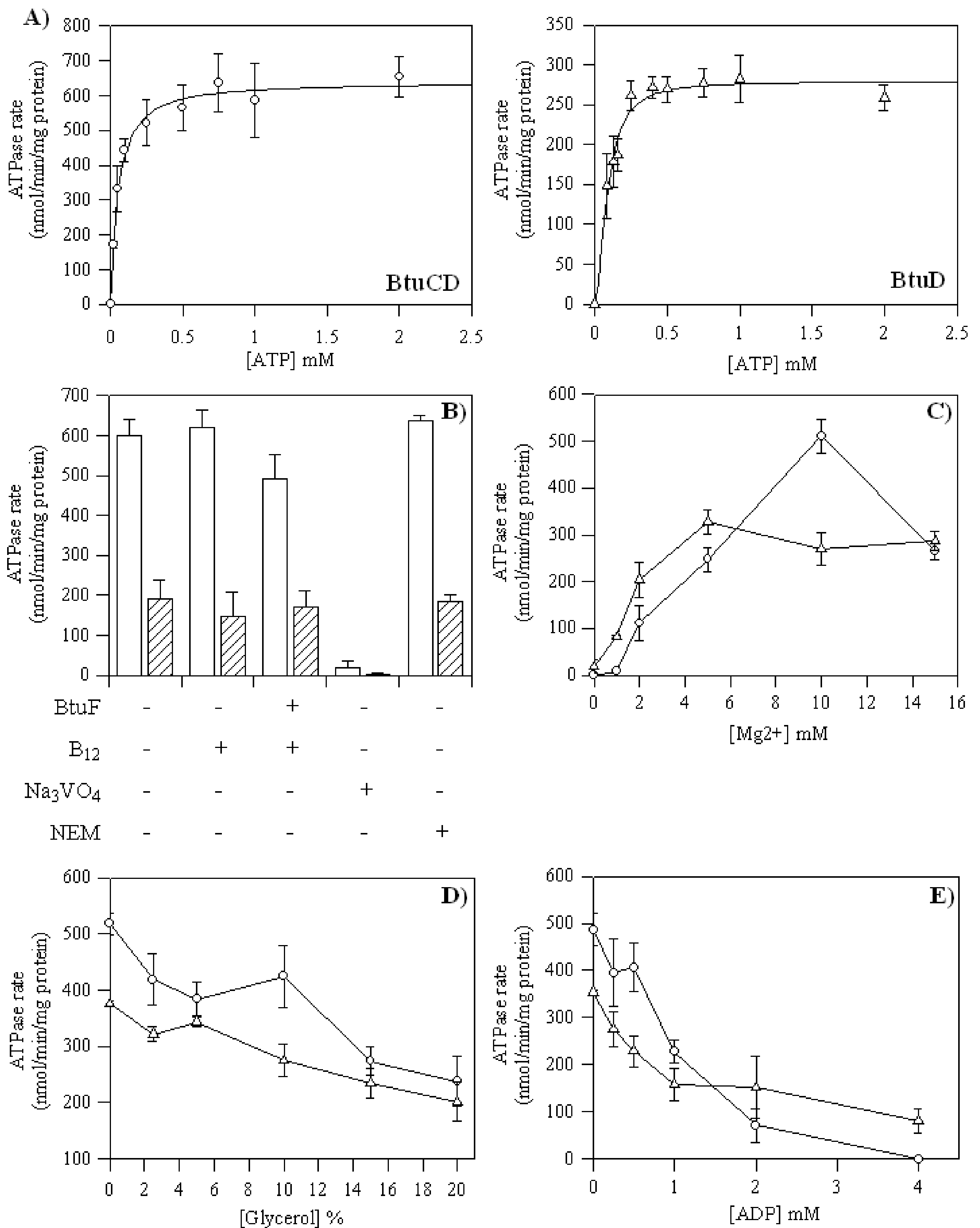

Figure S1. Characterising the ATPase activities of BtuCD purified in DDM and of purified BtuD under various conditions. (A) Basal rates of ATP hydrolysis of BtuCD (left, circles) and of BtuD (right, triangles) were measured as a function of concentration of ATP and in the presence of 10mM Mg^{2+}. Data was fit according to the Hill equation. All other ATPase assays were measured in the presence of 2mM ATP. (B) For these assays, vitamin B_{12} was used at a concentration of 50μM, BtuF at 10μM, sodium ortho-vanadate (Na_3VO_4) at 2mM and N-ethylmaleimide (NEM) at 10mM (BtuCD, empty bars and BtuD, stripped bars). Basal rates of ATP hydrolysis were also measured in the presence of varying concentrations of Mg^{2+} (C) glycerol (D) and ADP (E) (BtuCD, circles and BtuD, triangles).

In Vitro Folding and Assembly of the _Escherichia coli_ ATP-binding Cassette Transporter, BtuCD

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