Observations on the Calcium Dependence and Reversibility of Cobalamin Transport across the Outer Membrane of Escherichia coli

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The calcium dependence of cobalamin (Cbl) binding to the BtuB protein of Escherichia coli and the reversibility of its function in the transport of Cbl across the outer membrane have been examined. The results show that the two calcium-binding sites in BtuB that were identified previously by others are responsible for the calcium dependence of high affinity Cbl binding. The affinity of the pure BtuB protein for Cbl was ~1000-fold higher in the presence of saturating levels of calcium than in its absence. The affinities of BtuB for both Cbl and calcium were decreased by insertion of alanine residues at position 51 of the mature protein and were increased by several mutations and deletions in the TonB box. Experiments on the uptake of Cbl into the periplasmic space showed that this process is reversible and that the exit of Cbl back into the medium does not require the protonmotive force. Our interpretation of these results is that the role of the TonB-ExbB-ExbD complex, potentiated by the protonmotive force, is to reduce the affinity of the Cbl-binding site, thus increasing the rate of Cbl release into the periplasmic space. The evidence also indicates that access of the Cbl-binding site of BtuB to the periplasmic space does not require removal of the hatch domain from the barrel.

The BtuB protein in the outer membrane of Escherichia coli is the cobalamin (Cbl)3 carrier in an active transport process that pumps Cbl from the external medium into the periplasmic space. The mature protein consists of 594 amino acid residues folded into two major domains. The C-terminal 458 residues form a 22-stranded β-barrel with connecting loops that extend variously on either side of the membrane. The N-terminal 136 residues form a globular domain located within and potentially occluding the lumen of the barrel (1, 2). Similar structures are found in the outer membrane proteins FecA, FepA, and FhuA, which serve as the carriers for the ferric siderophores ferric dicitrate, ferric enterobactin, and ferric ferrichrome, respectively. The Cbl-binding site of BtuB consists primarily of interactions with residues on the external loops and nearby residues in the hatch (2). We know from a previous study that the rate of energy-dependent Cbl transport across the outer membrane is greater than the off-rate observed for high affinity Cbl binding by BtuB (3), indicating that the potentiation mechanism must involve an increased off-rate (lowered affinity) for Cbl when the binding site is exposed on the periplasmic side of the membrane. The energy source is the protonmotive force (pmf) of the inner membrane, which is coupled to Cbl transport through interactions with the TonB-ExbB-ExbD protein complex that is anchored in the inner membrane (4, 5). Numerous studies have indicated a direct interaction between the TonB protein and the TonB box (residues 6–12) of BtuB (6, 7). We have shown previously (8, 9) that high affinity Cbl binding (Kd ~ 1 nM) by BtuB is calcium-dependent and that calcium binding is dependent upon Cbl. The affinity of BtuB for Cbl is greatly reduced by suboptimal calcium concentrations. The structure for BtuB derived from x-ray studies shows two calcium-binding sites that are formed primarily from a group of 5 aspartate residues quite close to the Cbl-binding site (2). In this study, we show that mutagenesis of these residues results in impaired calcium and Cbl binding. We also show here that calcium and Cbl binding is modified by changes both in the TonB box and in other regions of the hatch, supporting our view that the intraprotein signaling that thus exists between the N terminus and the distant Cbl- and calcium-binding sites has an essential role in this transport process. We also present evidence that the exposure of the Cbl-binding site of BtuB to the periplasm does not require either TonB or the pmf. In addition, BtuB can catalyze the transport of Cbl from the periplasm to the exterior of the cell in the absence as well as in the presence of the pmf. Our results show that the exposure of the Cbl-binding site of BtuB to the periplasmic space does not require removal of the hatch from the lumen of the barrel.

**EXPERIMENTAL PROCEDURES**

_E. coli Strains and Plasmids_—The strains and plasmids used are listed in Table 1. The strain used for plasmid construction and maintenance was JM109 (Stratagene). Some of the other strains used in this study were derived from MC4100 (Δ[argF-lac]U169, araD, 139pS, L150, relA1, fbbB5301, deoC1, ptsF25, araC, lacZΔM15, galK, rpsL, leuB6, rfbD, trpC, thiA, and F′ lacU169 Δ(lacIQ)lacUV5).
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| Strain | Genotype | Source/Ref. |
|--------|----------|-------------|
| JM109  | thi, supE44, relA1, Δ(lac-pro) (F', traD36, proAB', lacIqZΔM15) | Stratagene |
| MC4100 | Δ(argF-lac)Δ1169, araD, 139py, L150, relA1, fbiB5301, deoC1, ptsE25, rbsR22, non-9, gyrA219 | Ref. 10 |
| RK5016 | MC4100 metE70, argH, btuB, recA; used to maintain WT and mutant btuB plasmids | Ref. 11 |
| RK8452 | MC4100 metE70ΔbtuB::Km; used to express chromosomal level btuB mutants | Ref. 7 |
| KBT103 | F', leu, pro, lysA, trp, purE, metE, str, lac, fhuA, btuC | Ref. 4 |
| C8111  |  | |

Plasmid | Genotype | Source/Ref. |
|--------|----------|-------------|
| pAG1   | WT btuB gene on pUC8 vector; used as backbone for all following mutant btuB plasmids | Ref. 11 |
| D179R  | Arg substituted for Asp at residue 179 | This study |
| D179E  | Glu substituted for Asp at residue 179 | This study |
| D193S  | Arg substituted for Asp at residue 193 | This study |
| D195S  | Arg substituted for Asp at residue 195 | This study |
| D230R  | Arg substituted for Asp at residue 230 | This study |
| D230E  | Glu substituted for Asp at residue 230 | This study |
| D241R  | Arg substituted for Asp at residue 241 | This study |
| D241Y  | Tyr substituted for Asp at residue 241 | This study |
| 1A     | Insertion of 1 Ala residue between residues 50 and 51 | This study |
| 2A     | Insertion of 2 Ala residues between residues 50 and 51 | This study |
| 3A     | Insertion of 3 Ala residues between residues 50 and 51 | This study |
| 4A     | Insertion of 4 Ala residues between residues 50 and 51 | This study |
| 5A     | Insertion of 5 Ala residues between residues 50 and 51 | This study |
| L8P    | Pro substituted for Leu at residue 8 | Ref. 11 |
| V10P   | Pro substituted for Val at residue 10 | Ref. 11 |
| △Box   | Deletion of residues 6–12 | This study |
| △1–11  | Deletion of residues 1–11 | This study |
| AlaBox  | Ala substituted for TonB box residues 6–11 | This study |
| 7,10Box | Ala substituted for residues 6, 8, 9, and 11 only | This study |
| V10G   | Gly substituted for Val at residue 10 | Ref. 11 |
| A12D   | Asp substituted for Ala at residue 12 | Ref. 11 |

rbsR22, non-9, gyrA219 (10). Strain RK5016 has the additional mutations metE70, argH, btuB, recA and was used as a host to assess the functionality of the various BtuB mutants present on plasmids. RK8452 is metE70ΔbtuB::Km and was used as a host to test the function of the BtuB mutants at chromosomal levels. Plasmid-bearing cells were grown in the presence of ampicillin (100 μg/ml).  

Construction of BtuB Mutants—All of the DNA manipulations followed standard protocols. The pAG1 plasmid carrying the wild-type btuB gene in pUC8 has been described previously (11). BtuB mutants were constructed using a two-step PCR method as described previously (12). All mutagenic primers incorporated a silent restriction site that was used to facilitate screening. PCR fragments containing the mutations were cloned back into pAG1 using restriction fragment digestion to confirm the presence of the mutated allele and loss of the Km cassette.  

Growth and Fractionation of Cells—The cells were grown to mid-log phase at 37 °C on the minimal medium described by Davis and Mingioli (14), supplemented with glucose (10 mg/ml) and, as required, with amino acids and antibiotics. For the preparation of cell envelopes, the washed cells were suspended in either 10 mM potassium phosphate (pH 7.1) or 10 mM potassium MOPS (pH 7.1) and broken by passage through a French pressure cell. Residual whole cells were removed by centrifugation at 5000 × g for 5 min, followed by 7000 × g for 7 min. Envelope particles were sedimented by centrifugation at 40,000 × g for 60 min and resuspended in an appropriate buffer at a concentration of ~4 mg/ml protein. Protein was determined by the Lowry method using fatty acid-free bovine serum albumin as the standard (24).  

Assay of Cobalamin Binding and Transport—The methods have been described previously and involved the use of 57Co-labeled cyanocobalamin (CN[57Co]Cbl), filtration through Millipore filters, and liquid scintillation counting (15). Uptake of Cbl in cells containing haploid BtuB is expressed as picomoles of Cbl/ml of cells with wild-type BtuB and grown in the absence of Cbl, contains 0.3–0.5 pmol of BtuB. Whole envelopes from such cells bind ~4–7 pmol of Cbl/mg of protein. The calculated theoretical specific activity for pure BtuB would be 15.0575 nmol of Cbl bound per mg of protein. CN[57Co]Cbl was prepared as described (7). The precise experimental conditions for each experiment are given in the figure legends. Specific free Ca2+ concentrations were generated with calcium EGTA buffers using the methods of Portzehl et al. (16).  

RESULTS  

Fig. 1 shows a representation of the structure of BtuB as determined by Chimento et al. (2), with the near side of the
β-barrel cut away and the domains that we have examined in this study highlighted.

Calcium Dependence

**Pure BtuB Protein**—We showed previously the interdependence and reciprocal relationship between the binding of Cbl and calcium by BtuB (8, 9). This is confirmed in Fig. 2 with a sample of the pure BtuB protein (a gift from David Cafiso, Chemistry Department, University of Virginia). In the presence of enough calcium to fully saturate the calcium-binding sites, the $K_D$ for Cbl was $5 \text{nM}$. However, in the absence of calcium, the $K_D$ for Cbl was $5 \text{µM}$.

**Calcium Cage Mutations**—The structure of BtuB derived from x-ray analysis shows two calcium-binding sites that are close to each other and to the Cbl-binding site (2). These calcium atoms are contained in a cage that consists primarily of 5 aspartate residues. Fig. 3 shows the effects on Cbl binding of changing these aspartates to other amino acid residues. The experimental material consisted of whole envelopes from cells containing plasmids bearing the mutant BtuB species. Polyacrylamide gel electrophoresis showed that the expression of BtuB was essentially the same in all of the strains. The concentration of Cbl used was enough to give at least 80% saturation of high affinity wild-type Cbl-binding sites. Calcium EGTA buffers were used to generate three different free calcium concentrations: 3 nM, which is too low for appreciable Cbl binding by wild-type BtuB, and 3 and 300 μM, both of which would fully saturate the wild-type calcium-binding sites. Single replacements of the aspartates with arginines at positions 179, 193, 195, and 230 almost completely eliminated Cbl binding at all three calcium concentrations. Arginine at position 241 showed less of an effect on Cbl binding and was consistent with an estimated $K_D$ for calcium binding of that strain of $5 \text{nM}$, compared with the wild-type value of $30 \text{nM}$. Substitution of glutamates for the aspartates had less of an effect on Cbl binding, with amounts bound of $10–30\%$ of the wild-type values.
Changing the aspartates to serines completely eliminated Cbl binding at positions 195 and 230, but ~20–40% activity was retained at positions 179 and 193. Our results indicate that of the two calcium atoms bound, that designated calcium 1 by Chimento et al. (2) may be more important for Cbl binding than calcium 2. Asp179 is required only for the calcium 1 site, and its change to arginine completely eliminated high affinity Cbl binding. In contrast, Asp241 is required only for the calcium 2 site, and its change to arginine had a much lesser effect on Cbl binding.

Fig. 4 shows the rates of transport of Cbl by cells that contained single copies of the wild-type or mutant btuB gene. In this experiment, each mutant contained arginine instead of aspartate at the specified position. They all showed measurable rates of Cbl transport, but the rates varied from a low of ~4% of the wild-type value for D195R to a high of ~40% for cells containing the D241R mutation.

The results shown in Figs. 3 and 4 confirm that the calcium-binding sites shown in the x-ray crystal structure are the ones that our previous functional studies had shown to be required for the high affinity binding of Cbl by BtuB (8, 9).

Insertions at Position 50—We showed previously (9) that a BtuB species that lacked the first 11 N-terminal residues and contained an aspartate-proline dipeptide between what had been residues 50 and 51 of the intact mature BtuB had a much reduced affinity for calcium. However, its affinity for Cbl was normal when the calcium concentration was increased to give full saturation of the reduced affinity calcium-binding sites. We also deduced that a second BtuB species that lacked the first 49 N-terminal residues showed no calcium-dependent, high affinity Cbl binding but still had the low affinity binding of Cbl that is characteristic of wild-type BtuB in the absence of calcium. We examined the effects on Cbl binding by the insertion of 1–5 alamines between residues 50 and 51. The results from cell envelope preparations in which BtuB expression was greatly amplified are shown in Fig. 5. Cbl binding was measured at Cbl concentrations of 21 nM and 18 μM in the presence of either 3 or 300 μM free calcium and either 21 nM or 18 μM CNOCbl. The results are expressed as picomoles of CNCbl bound per mg of protein.
on high affinity Cbl binding. We looked further at nine TonB box mutations and found that some have profound effects upon the calcium dependence of high affinity Cbl binding. All except A12D lacked Cbl transport. The experimental material consisted of whole cell envelopes from strains containing BtuB on a plasmid. Enough Cbl was contained in the reaction mixtures to ensure saturation of normal high affinity binding. Measurements were made over a range of free Ca\(^{2+}\) concentrations from 1 nM to 300 μM. The major part of the results from eight strains is shown in Fig. 6, where Cbl binding is expressed as a percentage of the maximal values obtained by each. In seven of these strains, the maximal amounts of Cbl bound ranged from 885 to 1212 pmol/mg of protein. The wild-type envelopes had a specific activity of 1086 pmol of Cbl bound per mg of protein. Envelopes from the ΔBox mutant contained less BtuB, as determined by polyacrylamide gel electrophoresis, and had a correspondingly lower specific activity of 496 pmol of Cbl/mg of protein. Envelopes from the ΔBox mutant contained less BtuB, as determined by polyacrylamide gel electrophoresis, and had a correspondingly lower specific activity of 496 pmol of Cbl bound per mg of protein. In all cases, there was a close correlation between the amount of BtuB expressed and the maximal observed amount of Cbl binding. All of these mutants showed what we have concluded to be increased affinities for calcium. These increases were comparatively modest for the point mutations L8P, V10G, and A12D. The affinities for calcium of the other mutants, V10P, 7,10Box, AlaBox, and ΔBox, plus two others (V9G and Δ1−11), for which the data are not shown, were all apparently very high, with better than 80% of maximal Cbl binding at the lowest calcium concentration used. Our lack of data at free calcium concentrations below 1 nM means that we cannot be certain whether these strains really have very high affinities for calcium or whether high affinity Cbl binding in these mutants has actually become independent of a calcium requirement. Cobalamin binding by the Δ1−11 mutant had >80% of maximal Cbl binding at 1 nM free calcium, whereas a previously described mutant (9) that also lacked the first 11 amino acid residues but, in addition, contained an aspartyl-prolyl dipeptide between residues 50 and 51 required 10 μM free calcium for half-maximal Cbl binding. We have also shown (11) that whole cells and envelopes from these TonB box mutants have slower off-rates for bound Cbl, indicating higher affinities for Cbl compared with the wild type. These results emphasize how sensitive Cbl and calcium binding is to structural changes in the hatch domain of the BtuB protein.

**Release of Bound Cbl from Cells and Envelopes**

Fig. 7 shows the rates of release of bound CN\(^{57}\)CoCbl from whole cells and from envelope particles upon the addition of a large excess of unlabeled CNCbl. Both preparations were from the same lot of cells that contained ~200 times the haploid

![Graph showing calcium dependence of CN\(^{57}\)CoCbl binding by BtuB species containing mutations in the TonB box.](image1)

**Fig. 6. Calcium dependence of CN\(^{57}\)CoCbl binding by BtuB species containing mutations in the TonB box.** The methodology was essentially the same as that described in the legend to Fig. 3. Whole envelope particles containing amplified BtuB were incubated in calcium EGTA/MOPS buffers. The reaction mixtures contained 67 nM CN\(^{57}\)CoCbl. The results are expressed as a percentage of the maximal binding found for each envelope preparation. Except for the ΔBox mutation, the maximal binding of each of the mutants was within 20% of that of the wild type, which was 1086 pmol of Cbl bound per mg of protein. The maximal binding of the ΔBox mutant was ~50% of that of the wild type, consistent with the lower expression of this BtuB species.

![Graph showing rates of release of bound CN\(^{57}\)CoCbl from whole cells and envelope particles containing amplified wild-type BtuB.](image2)

**Fig. 7. Rates of release of bound CN\(^{57}\)CoCbl from whole cells and envelope particles containing amplified wild-type BtuB.** The 15-ml reaction mixtures at pH 6.6 contained 90 mM potassium MOPS, 10 mM 2,4-dinitrophenol, 15.7 nM CN\(^{57}\)CoCbl, and either 5.7 μg of envelope protein or ~1.6 × 10⁶ whole cells/ml. Incubation was carried out at 37 °C, and 1-ml samples were removed at timed intervals. After 25 min, unlabeled CNCbl was added to a final concentration of 2.5 μM, and sampling was continued for an additional 15 min. The results show the amount of labeled Cbl remaining bound to the cells or envelopes, expressed as a percentage of the maximum bound before the addition of unlabeled CNCbl. The 100% value for binding by the whole cells was 62.7 pmol of Cbl/ml of cells at A\(_{660}\) = 0.6, and that for the envelope particles was 1745 pmol/mg of protein. WT Env., wild-type envelopes.
amount of wild-type BtuB. The envelope particles were prepared by cell breakage with a French pressure cell and differential centrifugation and were not exposed to any detergent or to EGTA or EDTA. The reaction mixtures and incubation conditions were identical in each case and included 1% fatty acid-free bovine serum albumin to reduce any binding of the non-intrinsic factor to the filters. The results are expressed as picomoles of Cbl/ml of cells at $A_{600} = 0.6$.

**Reversibility of Outer Membrane Cbl Transport**

We showed previously (17) that btuC mutants are incapable of Cbl transport across the inner membrane of *E. coli* and accumulate Cbl to high concentrations in the periplasmic space. This permitted us to study transport of Cbl across the outer membrane in isolation and showed that this was an active transport process that was potentiated by interactions requiring the pmf and the TonB protein. The transport of labeled Cbl quickly reached a plateau, indicating equality between the exit rate and the in rate, and the label was rapidly released back into the medium upon the addition of a large excess of unlabeled Cbl. Here, we examined the exit of Cbl in more detail. The results shown in Fig. 8 indicate that the pmf is not essential for BtuB-dependent Cbl transport from the periplasm to the external medium and argue against any mechanism for this transport process that requires the TonB- and pmf-dependent removal of the hatch from the barrel to permit access of the Cbl-binding site to the periplasm. The experimental cells were btuC and btuC/ mutants. Cells that lack a functional ATP synthase are unable to generate the pmf when the electron transport chain is blocked by cyanide. In two of the reaction mixtures shown in Fig. 8, cyanide was added at the beginning of the incubation, but inhibition of Cbl transport was observed only in the *atp* strain, confirming the dependence of this transport system upon the pmf. In the other reaction mixtures, the uptake of CN$^{57}$CoCbl was allowed to proceed for 25 min, at which point a variety of reagents were added, and the retention of $^{57}$Co-labeled Cbl by the cells was followed. The addition of a large excess of unlabeled Cbl resulted in a rapid loss of label from both strains, indicating the essential reversibility of this transport process. The addition of cyanide, which would eliminate the pmf only in the *btuC/atp* double mutant, resulted in the loss of label only from that strain, whereas the addition of dinitrophenol, which would abolish the pmf in both strains, released the label from both. Finally, when the labeled Cbl outside the cell was removed by the addition of the mammalian Cbl-binding protein non-intrinsic factor, the label was also released rapidly from the cells. It should be noted that the rate of release of Cbl from the cells was somewhat more rapid in the presence of the pmf (addition of either unlabeled Cbl or the non-intrinsic factor) than in its absence. In any case, the results show that Cbl can flow down its concentration gradient from a high concentration in the periplasm to a low external concentration.

**DISCUSSION**

The purpose of this study was to gain further insight into the mechanisms by which the TonB-ExbB-ExbD complex in the inner membrane of *E. coli* and the pmf potentiate the BtuB-dependent active transport of cobalamin across the outer membrane. From a previous study of ours (12), as well as a study on the analogous TonB-dependent transport of ferric siderophores (18), it is evident that there is a direct interaction between the C-terminal region of TonB and the TonB box part of the outer membrane transporters. The N-terminal region of TonB forms a complex with ExbB and ExbD that is embedded in the inner membrane and must provide a channel for the movement of protons from the periplasm into the cytoplasm. This movement presumably causes some sort of conformational change in the C-terminal region of TonB that stimulates the release of the transportable substrate from its outer membrane carrier into the periplasm. TonB is able to bind to the TonB box region of BtuB in the absence of energization by the pmf, but the catalyzed release of Cbl into the periplasm requires the pmf. The structures of BtuB and the ferric siderophore carriers FhuA, FepA, and FecA are basically the same and consist of a β-barrel embedded in the outer membrane, the potential channel of which seems to be occluded by an N-terminal region of ~150 amino acid residues that is commonly called the hatch. In BtuB, residues 6–12 form the TonB box. Fig. 1 shows a cutaway representation of the structure of the BtuB protein as determined Chimetto et al. (2), with some of the parts dis-

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**FIGURE 8. Reversibility of outer membrane CN$^{57}$CoCbl transport.** The experimental material consisted of cells that contained haploid levels of wild-type BtuB and nonfunctional btuC. In one series, the cells were also mutant in *atp*, which prevented the cells from generating the pmf when cyanide was present. The reaction mixtures contained 100 mM potassium MOPS, 1% glucose, 10 mM CN$^{57}$CoCbl, and $-3 \times 10^8$ cells/ml. Incubation was carried out at 37°C, and 1-ml samples were removed at timed intervals. In one series with each cell strain ( ), 2 mM potassium cyanide was added at the beginning. After 25 min of incubation (indicated by the arrows) in the other reaction mixtures, other additions were made, and the amount of labeled Cbl remaining with the cells was followed for an additional 15 min. These other additions were 2 mM KCN ( ), 2 μM unlabeled CN$^{57}$CoCbl ( ), 2 mM dinitrophenol (DNP; ), and the non-intrinsic factor (NIF; *) containing 30 pmol of Cbl binding activity. The reaction mixture to which the non-intrinsic factor was added also included 1% fatty acid-free bovine serum albumin to reduce any binding of the non-intrinsic factor to the filters. The results are expressed as picomoles of Cbl/ml of cells at $A_{600} = 0.6$.  

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cussed in this study highlighted. Further x-ray diffraction studies on crystals containing both BtuB and the C-terminal 93 residues of TonB showed that the unenergized interaction of these two proteins results in the conversion of the TonB box into a β-strand that aligns itself parallel to, and in contact with, the three-stranded β-sheet of TonB (19).

Superficially, the structures of BtuB and the other TonB-dependent, outer membrane transporters look like porins that have their aqueous channels occluded by hatch domains. This has led to the suggestion that they are selective, “ligand-gated porins” and that their mechanism of action involves substrate binding, followed by the TonB/pmf-potentiated removal of the hatch to expose the ligand-binding site to the periplasmic space (20). We object to the designation “ligand-gated porin” because such a mechanism is not capable of active transport, i.e. the movement of a substrate against its concentration gradient, and Cbl is clearly actively transported across the outer membrane. Similarly, we also dislike the designation “TonB-gated transporters” (21) because it implies that TonB is required for access of the bound ligand to the periplasmic space, whereas in the case of Cbl transport, such access is not TonB-dependent. Rather, the function of TonB is to increase the rate of dissociation of bound Cbl. Our view is that the BtuB protein on its own is probably capable of facilitated diffusion of Cbl across the outer membrane. However, to capture enough Cbl from the very low concentrations found naturally, it needs a high affinity Cbl-binding site. Although such high affinity binding is successful in scavenging subnanomolar levels of Cbl from the exterior, the dissociation rate is too slow to provide the 25–500 Cbl molecules that may be required in the cytoplasm of each cell for continued growth (22). Accordingly, some way is needed to increase the rate of dissociation of Cbl from the BtuB protein. This is accomplished by interaction with energized TonB. It must involve some conformational change in the hatch domain. It is even possible that this could include the partial or complete removal of the hatch from the barrel of BtuB, but it should be stressed that such movement is not required for exposure of bound Cbl to the periplasm. It should be noted that our work is confined to Cbl transport, and it remains possible that TonB-dependent conformational changes in the hatch domain are required to provide access of the ferric siderophore-binding sites to the periplasmic space.

Our proposed mechanism for Cbl transport across the outer membrane consists first of calcium-dependent, high affinity binding of Cbl to BtuB on the external surface of the outer membrane. This is followed by fluctuations in BtuB such that the occupied Cbl-binding site is sometimes exposed to the exterior and sometimes to the periplasm. The interaction of the TonB box of BtuB with the energized TonB protein transmits a conformational change through the hatch to the Cbl/calcium-binding sites and reduces the affinity of BtuB for both Cbl and calcium. The marked increase in the rate of dissociation of Cbl from its binding site stimulates the release of Cbl into the periplasm.

The evidence for calcium-dependent, high affinity binding of Cbl by BtuB has been well established previously (8, 9) and is shown above with the pure protein. Our earlier assumption that TonB and the pmf were not required for access of the Cbl-binding site to the periplasm was challenged by structural studies showing the apparent occlusion of the β-barrel by the hatch domain. This prompted the present study on access from the periplasm, which has confirmed our earlier suggestions. Fig. 7 shows that when an excess of unlabeled Cbl is added to preparations that have bound labeled Cbl, the rate of release of the labeled Cbl is faster from envelope particles, which have both sides of the outer membrane exposed to the reaction medium, than from whole cells. In both cases, the reaction conditions precluded involvement from either TonB or the pmf. In the whole cells, the unlabeled Cbl can exchange only with label when the binding site is toward the medium, whereas with the envelope preparations, the unlabeled Cbl has access to both sides of the outer membrane. It should be noted, even so, that the rate of Cbl release from the envelopes is slower than the observed rate of overall Cbl transport from the medium into the cytoplasm under optimal transport conditions, indicating a faster off-rate for Cbl toward the periplasm when the system is fully energized. More conclusive evidence that exposure of the Cbl-binding site on BtuB to the periplasm does not require the pmf is shown in Fig. 8. In btuC mutants, Cbl transport across the inner membrane is absent, and Cbl transport across the outer membrane can be studied in isolation. In such cells, in the absence of fully functional TonB and the pmf, Cbl accumulates in the periplasm until a plateau is reached when the rates of intake and exit become equal. The rates of Cbl exit from the periplasm into the medium can be measured by the addition of unlabeled Cbl after the plateau of labeled Cbl transport has been reached. In this counterflow experiment, the TonB/pmf energization is present. However, in the absence of addition of unlabeled Cbl, the accumulated periplasmic labeled Cbl can also be released by abolishing the pmf by the addition of dinitrophenol and, in the case of btuC/atp double mutants, also by cyanide. Thus, Cbl is able to flow down its concentration gradient to the outside of the cell, indicating that the BtuB protein on its own is capable of facilitated diffusion. This also means that, in the absence of the pmf, the Cbl-binding site is accessible to periplasmic Cbl and rules out any mechanism of Cbl transport that would require the removal of the hatch domain as a necessary step in the energization process. Consistent with the possibility of a capacity for facilitated diffusion are our observations that metE mutants that are also tonB will grow on minimal media containing 50 nM Cbl, whereas metE/btuB double mutants require 5 μM Cbl.

In any case, a facilitated diffusion mechanism is not adequate for growth in media containing subnanomolar Cbl, and a mechanism is needed to increase the off-rate of Cbl toward the periplasmic space. Our proposal now and previously is that this is what the ExbB/ExbD/pmf system does. It decreases the affinity of BtuB for Cbl and thus increases its off-rate. The interdependence of calcium and Cbl binding means that this interaction could be exerted primarily upon either Cbl or calcium binding. The effect would be the same: increased off-rates for both calcium and Cbl. Fig. 2 shows that the pure BtuB protein has a 1000-fold greater affinity for Cbl in the presence of saturating calcium than in its absence, indicating a high potential for an increased off-rate. We have also shown (Figs. 5 and 6) how exquisitely sensitive calcium
and Cbl binding is to structural changes in the hatch region, including the region at position 50 as well as the TonB box. Evidence has also been provided that the calcium-binding sites that were identified by x-ray structural analysis are the ones upon which high affinity Cbl binding is dependent. The report of some sequence homology between the ExbB and ExbD proteins and the proton-translocating proteins MotA and MotB of the flagellar motor (23) prompts us to support the view that the energized TonB protein undergoes some rotary motion and transmits some torque to the hatch, providing enough conformational change to decrease the affinity of BtuB for Cbl and calcium.

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