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Coupling of calcium and substrate binding through loop alignment in the outer membrane transporter BtuB

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Abstract

In Gram-negative bacteria, TonB-dependent outer membrane transporters (TBDTs) bind large, scarce organometallic substrates with high affinity preceding active transport. The cobalamin transporter BtuB requires the additional binding of two Ca^{2+} ions before substrate binding can occur, but the underlying molecular mechanism is unknown. Using the crystallographic structures available for different bound states of BtuB, we have carried out extended molecular dynamics simulations of multiple functional states of BtuB to address the role of Ca^{2+} in substrate recruitment. We find that Ca^{2+} binding both stabilizes and repositions key extracellular loops of BtuB, optimizing interactions with the substrate. Interestingly, replacement by Mg^{2+} abolishes this effect, in accordance with experiments. Using a set of new force field parameters developed for cyanocobalamin, we also simulated the substrate-bound form of BtuB, where we observed interactions not seen in the crystal structure between the substrate and loops previously found to be important for binding and transport. Based on our results, we suggest that the large size of cobalamin compared to other TBDT substrates explains the requirement of Ca^{2+} binding for high affinity substrate recruitment in BtuB.

Introduction

In order to import large yet essential nutrients (> 600 Daltons) such as iron-complexes and cobalamins, Gram-negative bacteria must actively transport them across both the outer membrane (OM) and the cytoplasmic membrane (CM)¹. At the CM, these nutrients use ATP-driven ABC transporters to cross the membrane^{2,3}. However, due to the high permeability of the OM to small molecules, chemical energy cannot be generated or stored in the periplasmic space; therefore, active transport at the OM has to rely on energy generated at the CM. To circumvent this limitation, one pathway bacteria have evolved is known as the TonB system. TonB interacts with proteins in the CM, where the proton-motive force is used to generate energy, and delivers that energy to TonB-dependent transporters (TBDTs) in the OM^{4,5}. These transporters share a common structure, possessing 22-stranded β -barrels connected to a large, N-terminal “luminal domain” folded inside the barrel. Various substrates bind on the

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extracellular side to the luminal domain, interacting simultaneously with the extracellular loops of the β -barrel, preceding transport. These loops, 11 in total (see Fig. 1), have been implicated in multiple functions, including the binding of substrate as well as lethal phages and colicins^{6–12}. They vary in length within a single TBDT and particularly between different TBDTs, inversely proportional to the size of the substrate, suggesting they might also function to occlude the binding site after substrate binding, thus preventing it from leaving during transport¹³.

Once the substrate is bound, interaction between TonB and the TBDT triggers its transport, although the mechanism is still largely unknown. A short N-terminal sequence in the TBDT, known as the Ton-box, serves as the point of interaction with TonB^{14–18}. The structures of TonB in complex with BtuB as well as in complex with FhuA, revealed that the Ton-box forms a β -strand which then joins the β -sheet in the C-terminal domain of TonB^{19,20}. Recent evidence suggests a mechanical mode of interaction in which force applied to TonB is transmitted to the luminal domain of the TBDT, causing unfolding and/or additional structural deformation by which a substrate pathway can form^{5,13,21}. In agreement with this model, experiments have demonstrated that residues in the luminal domain become accessible to fluorophores in the periplasm only during transport^{22,23}, and that TonB is simultaneously bound to both the CM and OM²⁴; however, alternative models of substrate path formation cannot yet be ruled out^{25–27}.

One of the best structurally characterized TBDTs is the cobalamin (Cbl, e.g., vitamin B₁₂) transporter BtuB. Crystallographic structures of BtuB have been determined in the apo, Ca²⁺-bound, substrate loaded, colicin-bound, and TonB-bound states^{8,9,19,28}. These structures have revealed how all the components (i.e., substrate, Ca²⁺ ions, and TonB) come together preceding transport; however, they still leave a number of questions unanswered. For example, Ca²⁺ binding is known to increase BtuB's affinity for Cbl 1000-fold, while it does not appear to be necessary for substrate binding in any other TBDT^{27,29}. In the Ca²⁺-bound structure of BtuB, the presence of Ca²⁺ ordered all or part of three extracellular loops not resolved in the apo structure (loops 3/4, 5/6, and 7/8, shown in Fig. 1) although how this ordering influences substrate binding is not clear⁹. Furthermore, Mg²⁺ can inhibit substrate binding to BtuB, suggesting that Mg²⁺ competes with Ca²⁺ for specific ion binding sites²⁹. The structure of the Ca²⁺/cyanocobalamin (CN-Cbl)-bound BtuB exhibited only a few differences compared to the Ca²⁺-bound form. Loops 3/4, 5/6, and 7/8 become fully resolved in the Ca²⁺/CN-Cbl-bound structure, although loop 9/10 is no longer seen⁹.

In order to determine the role of the extracellular loops of BtuB in Cbl recruitment and binding, we have performed an extensive set (over 250 ns) of molecular dynamics (MD) simulations of nearly all resolved structures of BtuB in a lipid/water environment. MD simulations have been applied to the study of many outer membrane proteins, including the TBDTs BtuB and FhuA^{21,30–35}. To include the substrate in our simulations, we first needed to parametrize CN-Cbl, missing in previous simulations²¹, for the CHARMM force field (see Methods). The subsequent simulations illustrate the dynamical differences between the extracellular loops in the apo, Ca²⁺-bound, and Mg²⁺-bound forms of BtuB. Furthermore, simulation of the Ca²⁺/CN-Cbl-bound state reveals multiple interactions between CN-Cbl and BtuB, including some not present in the original crystal structure. Simulations of two apo structures of BtuB obtained under different crystallization conditions reveals the fast, spontaneous binding of Ca²⁺ ions to the extracellular loops in both. Based on our results, we conclude that Ca²⁺ binding serves to both position the extracellular loops closer to the substrate binding site as well as stabilize them, permitting BtuB to bind Cbl with high affinity.

Results

The role of Ca²⁺ binding in BtuB

Prior to substrate binding, BtuB binds Ca²⁺, which is known to increase the affinity of BtuB for Cbl 1000-fold, through an unknown mechanism^{27,29}. According to the Ca²⁺-bound and Ca²⁺/CN-Cbl-bound crystal structures⁹, two Ca²⁺ ions are ligated between extracellular loops 3/4 and 5/6 primarily by a number of aspartic acid residues. To investigate how Ca²⁺ affects the structure and dynamics of these loops and the binding of Cbl, we simulated both the apo and the Ca²⁺-bound states of BtuB for 30 ns each, comparing the resulting structural and dynamical differences.

As a measure of loop stability, we calculated both the root mean-square deviation (RMSD) and the root mean-square fluctuations (RMSF) over the course of both simulations. As shown in Fig. 2A, the RMSD for the extracellular loops in the apo structure is significantly higher compared to that for the loops in the Ca²⁺-bound structure, providing further evidence that the binding of Ca²⁺ stabilizes these loops. The RMSF per residue (Fig. 2B) presents a more detailed picture, giving information on individual residues instead of the loops as a whole. For loops 3/4 and 5/6, the difference at peak RMSF is negligible, but for those residues closer to the Ca²⁺ binding site, the difference is statistically significant (defined as greater than one standard deviation from the mean RMSF for the apo structure; see Supplementary Fig. S1F). We also observe an increase in the stability of substrate binding loop 1 (SB-1, residues 57-65 in the luminal domain), in the Ca²⁺-bound structure. Given that residues in loop 3/4 near the Ca²⁺ binding site interact with SB-1, the stabilizing effect of Ca²⁺ on loop 3/4 may then also stabilize SB-1. Interestingly, it has been reported that the insertion of an aspartate-proline dipeptide or 1-5 alanines between residues 50 and 51 decreases both Ca²⁺ and Cbl affinity^{27,36}. This effect can be explained as the reverse of what we observe in our simulations; whereas we see a stabilizing effect of Ca²⁺ on SB-1, in the experiments, residue insertions near SB-1 likely destabilize the Ca²⁺ binding site.

The question still remains what purpose Ca²⁺ binding serves. It has been suggested that Ca²⁺ binding could restrain the loops and thus expose the Cbl binding site, increasing its binding affinity⁹. However, while we note a decrease in the loops' fluctuations, they still have the potential to occlude the Cbl binding site. In fact, we find that binding of Ca²⁺ shifts the average position of the loops closer to the Cbl binding site, up to 3.0 Å, 8.5 Å, and 3.4 Å for loops 3/4, 5/6, and 7/8, respectively (see Fig. 2C). Since all three loops contribute to CN-Cbl binding in the Ca²⁺/CN-Cbl-bound structure (5 out of 20 residues interacting with Cbl are found in these loops), bringing them closer to the binding site would position them for a closer interaction with the substrate, and, thus, could increase the affinity for Cbl⁹. The observed shift is in agreement with EPR data on the distance between certain residues in opposing loops (e.g., Thr188 in loop 3/4 and Gly399 in loop 13/14), which show a 2-3 Å decrease upon binding of Ca²⁺ (see comparison in Supplementary Table S1 and Supplementary Fig. S2)³⁷. Thus Ca²⁺ binding can serve both to reposition the extracellular loops for optimal Cbl binding and then to further stabilize the binding site.

Effects of cyanocobalamin binding on BtuB

Binding of Cbl to BtuB occurs with very high affinity (sub-nanomolar) once BtuB is in its Ca²⁺-loaded state^{27,29}. In the crystal structure, CN-Cbl interacts with 20 residues, both in the luminal domain and in extracellular loops 3/4, 5/6, 7/8 17/18, 19/20, and 21/22, divided approximately equally between hydrogen bonds and purely steric van der Waals contacts³⁸. To examine these substrate-protein interactions, we simulated the Ca²⁺/CN-Cbl-bound BtuB for 30 ns, using the parameters we developed for CN-Cbl (see Methods).

We measured the RMSD of the extracellular loops as well as the RMSF for the entire protein. The RMSD of the loops in the Ca^{2+} /CN-Cbl-bound state is comparable to that for the Ca^{2+} -bound one (see Fig. 3B). The RMSF is also quite similar to that for the Ca^{2+} -bound state, however with a few notable exceptions (see Fig. 3C). Extracellular loops 3/4 and 5/6 are significantly more stable due to their interactions with CN-Cbl; loops 7/8 and 19/20, which have few interactions with CN-Cbl, are instead less stable, and shift positions slightly (see also Supplementary Fig. S1C,D). The majority of hydrogen bonds between BtuB and CN-Cbl were the same as those observed in the crystal structure. However, an additional hydrogen bond between the side chain of Arg243 in loop 5/6 and the A-ring propionamide (the naming being defined in Chimento *et al.*³⁸) formed, at the expense of one with the backbone of Leu63. The carbonyl of Gly92 in SB-3 formed a stable hydrogen bond with the hydroxide of CN-Cbl's ribose moiety as well.

We also observed interactions between loops 13/14 and 15/16 and CN-Cbl. These loops do not interact with CN-Cbl in the crystal structure, but experiments have indicated that they might be important for both substrate binding and transport in BtuB and in FepA and for transport only in FhuA^{7,9–11}. Strands 13, 14, and their intervening loop form the “ β -cantilever” motif, so-named due to its positioning inside the barrel, separated from adjacent β -strands, thus positioning it closer to the Cbl binding site than otherwise possible¹³. Hydrogen bonds between the C-ring propionamide of CN-Cbl and Tyr453 in loop 15/16 as well as Asn397 and Gln400 in loop 13/14 formed transiently over the course of the simulation (minimum hydrogen-bonding distances of 2.6 Å, 2.9 Å, and 2.8 Å, respectively); one example is shown in Fig. 3A. Loop 15/16 also moved toward CN-Cbl by 3 Å by the end of the 30-ns simulation. This movement is less than the 7–8 Å shift seen in the structure of BtuB with TonB when compared to the Ca^{2+} /CN-Cbl-bound structure, although there, crystal contacts with an adjacent TonB may have influenced the loop's position¹⁹. The two different loop positions are also indicated in Fig. 3A.

The effect of Mg^{2+} in the Ca^{2+} binding site

Although the aspartate cage in BtuB is well suited for binding divalent ions, high affinity Cbl binding is dependent specifically on Ca^{2+} ²⁹. The presence of Mg^{2+} in solution even inhibits high affinity binding at non-saturating concentrations of Ca^{2+} , suggesting that Mg^{2+} ions can bind to BtuB, but do not confer the same increase in Cbl affinity as Ca^{2+} . The inhibitory effect of Mg^{2+} has been observed for other proteins with Ca^{2+} binding sites as well, including the C2 domains in eukaryotic signaling molecules and the tomato bushy stunt virus^{39,40}; the reverse effect, Ca^{2+} inhibiting a Mg^{2+} -dependent enzyme, has also been observed⁴¹. Additionally, the apo structure of BtuB was obtained in a high [Mg^{2+}] solution, yet loops 3/4, 5/6, and 7/8 were not resolved⁹. Mg^{2+} has a smaller radius than Ca^{2+} (0.65 Å vs. 0.99 Å) and as a result, has a shorter optimal coordination distance (2.05 Å vs. 2.4 Å)⁴². Additionally, coordination of Ca^{2+} ions by carboxyl groups can be monodentate or bidentate, while coordination of Mg^{2+} ions is typically monodentate⁴².

In order to determine how the differences between Ca^{2+} and Mg^{2+} lead to distinct functional behaviors in BtuB, we simulated the Ca^{2+} -bound crystal structure of BtuB with the Ca^{2+} ions replaced by Mg^{2+} . We compared the RMSD of the C_α atoms of the extracellular loops of Ca^{2+} -bound and Mg^{2+} -bound BtuB, finding the loops in the Mg^{2+} -bound state to exhibit much larger deviations over time than in the Ca^{2+} -bound state (see Fig. 4C). In fact, the RMSD for the loops in the Mg^{2+} -bound state has the same magnitude as that observed for the apo state, suggesting that the Mg^{2+} ions are unable to contain the loops' motions in the same way as Ca^{2+} ions. We also calculated the average structure of BtuB with Mg^{2+} bound, finding that the position of the loops is much closer to that in the apo state than in the Ca^{2+} -bound state (see Supplementary Fig. S3).

While the loops in the Mg^{2+} -bound BtuB resemble those in the apo state, the Mg^{2+} ions do remain bound to the protein for the entire 30 ns of the simulation. To further distinguish between the binding of Mg^{2+} and Ca^{2+} , we examined which residues coordinated the ions and with what coordination distances (Tables 1 and 2). Average coordination distances are approximately 0.3-0.5 Å shorter for Mg^{2+} compared to Ca^{2+} , as expected from the ions' radii. The primary ligands for both Ca-1 and Mg-1 are the four aspartate residues, 179, 193, 195, and 230. The carbonyl of Gln191 also contributes to Ca-1 binding (average distance, $d_{\text{avg}}=3.98$ Å), but moves away from Mg-1 ($d_{\text{avg}}=4.82$ Å). Additionally, while coordination of Ca-1 by aspartates is largely bidentate, Asp193 and Asp195 switch to monodentate for Mg-1 (judged by the decrease in coordination distance for only one of the two carboxyl oxygens). The difference between Ca-2 and Mg-2 is similar; coordination by Asp230 is bidentate for Ca-2 but monodentate for Mg-2. Thus, the shorter coordination distance combined with changes in the ligands coordinating Mg^{2+} compared to Ca^{2+} prevent adequate stabilization and positioning of the extracellular loops for Cbl binding.

Spontaneous binding of Ca^{2+} and Mg^{2+} to the apo structures of BtuB

To determine how Ca^{2+} binds to BtuB, we simulated the apo state of BtuB with Ca^{2+} ions present in bulk solution at an effective concentration of 71 mM, i.e., much higher than that required for binding *in vivo* ($K_D = 30$ nM), but within an order of magnitude of physiological extracellular Ca^{2+} concentration²⁹. Binding of Ca^{2+} ions to the expected binding site between loops 3/4 and 5/6 occurred spontaneously in the simulation within nanoseconds. The Ca^{2+} ion designated Ca-1 by Chimento *et al.*⁹ binds first to Asp179 at $t = 5$ ns. A second Ca^{2+} ion, Ca-2, binds first to Asp193 at $t = 12$ ns and then also Asp195 at $t = 13$ ns. Additionally, Asp230 contacts Ca-1 at $t = 35$ ns, helping to bring the ion much closer to its final location. The protein- Ca^{2+} interactions are strengthened over the course of the 50-ns simulation, resulting in Ca-1 being 4 Å from its crystallographic position and Ca-2 fluctuating between 6 and 9 Å away (see Fig. 5A). Interestingly, the order of binding of the Ca^{2+} ions is consistent with mutagenesis studies which suggest that Ca-1 is more important for Cbl binding than Ca-2²⁷. We also calculated the RMSD of extracellular loops 3/4, 5/6, and 7/8 in reference to the crystal structure of Ca^{2+} -bound BtuB⁹ (Fig. 5B). While initially increasing, the RMSD peaked at 5.7 Å and then decreased over the latter half of the simulation to 2.6 Å, indicating that the apo structure, after binding of two Ca^{2+} ions, is approaching the Ca^{2+} -bound structure.

Simulation of the apo state of BtuB with Ca^{2+} ions in solution was repeated in order to ascertain the reproducibility of the results of the first simulation discussed above. The ions' initial positions were randomized before starting. In this simulation, only one Ca^{2+} ion was observed to bind between loops 3/4 and 5/6, contacting OD1 and OD2 of Asp230 as well as the backbone carbonyl of Asp193 and Gln191 at $t = 5.5$ ns. As in the first simulation, this binding site is the same as that for Ca-1 in the crystallographic structure⁹. Although Ca^{2+} ions twice visited the region around the Ca-2 binding site during the 30-ns simulation, complete binding of an ion to this site was not achieved, an outcome which can be attributed to the short time scale of the simulation and the stochastic nature of the binding event.

Binding of Mg^{2+} ions from bulk solution to the apo state of BtuB was also observed in a separate 20-ns simulation in which all Ca^{2+} ions in solution were replaced by Mg^{2+} ions. After 6 ns, one Mg^{2+} ion became bound to Asp230 and Asp195, the two residues which ligate both Ca^{2+} ions in the crystallographic structure (see Fig. 4A). A second Mg^{2+} ion also interacts with Asp179, part of the binding site for Ca-1, from $t = 7$ to $t = 12$ ns. This interaction is, however, weak, with the ion-oxygen distance fluctuating between 4 Å to 13 Å before the ion completely leaves the binding site. A comparison of the ion-protein interaction energies reveals that the bound Mg^{2+} ion does not interact as strongly with BtuB as do Ca^{2+} ions in both spontaneous binding simulations; whereas the Mg^{2+} ion interacts with energy between -200 and -250 kcal/

mol, the Ca^{2+} ions all reach energies of -350 to -500 kcal/mol (see Supplementary Fig. S4). Therefore, while Mg^{2+} ions can bind spontaneously to the same binding site as Ca^{2+} , they do not do so in the same order or with the same strength.

An additional structure of the apo state of BtuB has been solved using different crystallization conditions. The majority of structures of BtuB are based on crystals in which the protein is surrounded by detergent micelles, referred to as the *in surfo* method^{9,19}. Alternatively, in the *in meso* method, the protein rests in a lipid bilayer, thereby attempting to more closely replicate the native state²⁸. Differences between the BtuB structures produced by the two methods include the resolution, or lack thereof, of certain regions. For example, the five N-terminal residues as well as loop 7/8 missing from the *in surfo* structure are resolved in the *in meso* structure, while SB-1 and loop 21/22 are present in the *in surfo* structure but not in the *in meso* one. Further differences include the positions of loops 9/10, 13/14, 15/16, and 19/20.

For comparison to the *in surfo* BtuB structure, we simulated the *in meso* structure for 30 ns. Similar to the *in surfo* structure, we observe two Ca^{2+} ions bind to loops 3/4 and 5/6 in the *in meso* BtuB structure. Ca-1 is 8.5 Å from its crystallographic position in the Ca^{2+} -bound structure of BtuB while Ca-2 is 13.5 Å away, both moving closer over time. These distances are comparable to those for the simulation of the *in surfo* structure with Ca^{2+} ions present where, after 30 ns, Ca-1 and Ca-2 were both 8 Å away. Thus, with the exception of two extracellular loops whose positions may be crystallographic artifacts, the two structures do not display remarkably different behaviors. More details of the *in meso* simulation are provided in the Supplementary Data.

Discussion

By carrying out extended MD simulations of various bound states of BtuB, we have characterized the initial steps in the Cbl transport cycle. We have examined the role of Ca^{2+} in Cbl binding, finding Ca^{2+} ions to bind spontaneously to extracellular loops 3/4 and 5/6, stabilizing them and causing them to move closer to the Cbl binding site. Simulations of extracellular proteins, e.g., cadherin and other extracellular matrix proteins, have also demonstrated that the binding of Ca^{2+} ions plays a structural role by stabilizing and positioning key regions^{43–45}. Additionally, we see a stabilizing effect of Ca^{2+} binding on SB-1, suggesting Ca^{2+} binding serves to position and stabilize multiple elements that contribute to the Cbl binding site. Destabilizing SB-1 may also affect the Ca^{2+} binding site, as suggested by the observation that amino acid insertions near SB-1 can diminish Ca^{2+} binding^{27,36}; further insertions targeting SB-1 directly are necessary to test this suggestion. In contrast to the stabilizing effect of Ca^{2+} , simulations of Mg^{2+} in the Ca^{2+} binding site demonstrated that while the Mg^{2+} ions remain bound, they significantly destabilize the binding site, explaining why experimentally, the presence of Mg^{2+} inhibits Cbl binding²⁹.

We also examined the effect of Cbl binding, made possible by our development of simulation parameters for CN-Cbl in the CHARMM force field. When bound to BtuB, CN-Cbl exhibits a stabilizing effect on a number of extracellular loops of BtuB, as well as interactions with residues in loops 13/14 and 15/16 not present in the crystal structure. Deletion of each of these two loops is known to abrogate substrate binding and transport in FepA and BtuB, and transport only in FhuA, although it is unclear whether this is due to the loss of key interactions with the substrate or destabilization of the transporter's structure^{7,10,11}. The role of loops 13/14 and 15/16 can now be tested by mutating the specific residues we observed to interact with CN-Cbl and then measuring the effect on its binding.

Based on these results, we can elaborate further on the transport cycle in TBDTs. One of the first steps, binding of Ca^{2+} ions, is only known to be necessary for BtuB, where we observe it

to position loops closer to the Cbl binding site on the extracellular side, and to stabilize them in this position^{9,37}. Since Ca^{2+} ions are likely always present at the outer membrane, this step does not seem to serve a regulatory role specific to Cbl transport³⁸. Instead, we suspect that because Cbl is the largest substrate among the known TBDTs, it requires a larger number of interactions with the transporter than other substrates in order to achieve high affinity binding; the simultaneous arrangement of such interactions is made possible indirectly through the binding of Ca^{2+} ions. In the next step, the substrate binds to the transporter, interacting with certain extracellular and luminal protein loops. These interactions, through an allosteric mechanism, dislodge the Ton-box on the periplasmic side of the TBDT^{46,47}. After binding of TonB to the Ton-box, energy generated at the inner membrane is transduced to the transporter. Although the details of the energy transduction step are still unknown, recent experiments indicate that TonB remains anchored to the CM during the process²⁴. Disulfide crosslinking and residue accessibility experiments demonstrate that the luminal domain undergoes some unknown conformational change during transport, either within the barrel, wholly or partially^{48,49}, or possibly being expelled from the barrel²². Previous simulations suggest a mechanical interaction in which the application of force partially unfolds the luminal domain is feasible²¹. However, it remains to be determined what is the specific balance between unfolding, removal as a single unit, and conformational change of the luminal domain within the barrel during transport^{5,22,50}.

Methods

Modeling

DMPE lipid bilayer—The outer membrane of Gram-negative bacteria is composed primarily of a combination of lipopolysaccharides (LPS) in the outer leaflet and phosphatidylethanolamine (PE) lipids on the inner leaflet^{51,52}. To approximate the outer membrane, we developed and used dimeristoyl phosphatidylethanolamine (DMPE) lipids. Previous simulations of outer membrane proteins have utilized various lipids including POPE^{21,51}, POPC³⁴, and DMPC^{30,53,54}, for example. DMPE lipids were chosen for the current study because they have only 14 carbons in each tail (compared to 16 and 18 for POPE) and thus better match the hydrophobic thickness of BtuB⁵⁵. A CHARMM topology file for DMPE is provided in the Supplementary Data.

Cyanocobalamin—Modeling of CN-Cbl for the CHARMM force field started from previously developed and tested AMBER parameters⁵⁶. Since the potential energy functions in CHARMM and AMBER are nearly identical, transfer of bonded parameters, including equilibrium values and force constants for bonds, angles, and dihedrals, was straightforward for those atom types not already present in the CHARMM force field. For the charges in the AMBER force field, the electron density for CN-Cbl was calculated using semi-empirical methods and the resulting Mulliken charges were taken⁵⁶. Although the accuracy of these charges has been questioned⁵⁷, recent simulations have demonstrated they are functional in the CHARMM force field⁵⁸. However, to avoid any potential discrepancies between the two different approaches, we chose to adopt CHARMM charges where possible from other compounds, and to calculate *ab initio* charges for the remaining atoms.

CN-Cbl can be broken down into a few separate chemical moieties, namely a corrin ring, associated methyl groups and acetamide side chains, a dimethylbenzimidazole (DMB), and a linker containing phosphate and ribose connecting the DMB to the corrin ring. To simplify calculations, *ab initio* charges were determined for only the corrin ring, along with the cobalt-bound cyano group and the DMB's cobalt-coordinating nitrogen, using the quantum chemistry package Gaussian 03⁵⁹. The reduced system contained 51 atoms. The geometry was optimized and charges were calculated using density functional theory at the B3LYP/6-31+G* level. The

normal procedure for developing CHARMM charges requires one to scale Mulliken charges resulting from the QM calculations in order to best reproduce the *ab initio* interaction of each atom with a TIP3P water molecule⁶⁰. However, because the proper placement of such water molecules is no longer apparent for such a large compound, charges were instead determined from the electron density using Natural Population Analysis (NPA), which is known to be significantly more accurate than Mulliken charges and also was used in the development of CN-Cbl parameters for the GROMOS force field^{57,61}. Charges on identical atom types were averaged and assigned, although variation was already small; these charges are given in Fig. 6A for all heavy atoms. Full topology and parameter sets are provided in the Supplementary Data.

The parameters and charges were tested by simulating CN-Cbl in a water box for 1 ns. Fig. 6B shows 250 frames from the simulation (1 every 4 ps) overlaying the initial structure. A large flexibility is seen for the side chains and the linker, while the corrin ring remains stable, as expected. The cobalt-nitrogen distance for the axial nitrogen was 1.998 Å on average, in excellent agreement with the experimental value of 2.017 Å⁵⁶.

Systems constructed—Multiple systems were built based on the available BtuB crystallographic structures. The apo state (PDB code 1NQE), the Ca²⁺-loaded state (1NQG), and the Ca²⁺/CN-Cbl-bound state (1NQH) were all simulated⁹. Additionally, we simulated the *in meso* structure of the apo state (2GUF)²⁸. Missing extracellular loops in some of the structures were modeled based upon the TonB-bound BtuB structure which was complete¹⁹. Specifically, loops 3/4, 5/6, and 7/8 in the apo structure; loops 5/6 and 7/8 in the Ca²⁺-bound structure; loop 9/10 in the Ca²⁺/CN-Cbl-bound structure; and loops 3/4, 5/6, 21/22, and SB-1 in the *in meso* structure were all modeled.

Crystallographic ions and water molecules inside the barrel or near the loops were retained, along with CN-Cbl when present. Protonation states of titratable residues were assigned based on pKa calculations using propKa and an assumed pH of 7^{62,63}. The protein was placed in a DMPE lipid bilayer and then solvated. In nearly all simulations, Ca²⁺ and Cl[−] ions were added with a net concentration of 100 mM. For one simulation of the apo state (represented in Fig. 2), Na⁺ was used instead of Ca²⁺. Each system contained approximately 85,000 atoms.

Molecular dynamics

We used NAMD 2.6⁶⁴ for all molecular dynamics simulations as well as the CHARMM27 force field with the CMAP correction^{60,65}. A multiple time-stepping algorithm was employed in which bonded interactions were calculated every 1 fs, short range non-bonded interactions every 2 fs, and long range interactions every 4 fs. Long range electrostatic interactions were calculated using the particle-mesh Ewald (PME) method with a grid density of more than 1/Å³.

Simulations were run at a constant temperature of 310K maintained using Langevin dynamics applied to all heavy atoms with a damping constant of 1 ps^{−1}. A pressure of 1 atm was enforced along the *z* direction (normal to the membrane) with a Nosé-Hoover-Langevin piston; the membrane area was only allowed to fluctuate for the first 2 ns of equilibration, after which it was fixed (NP_{*z*}TA ensemble). Periodic boundary conditions were used for all simulations.

Equilibration of each system was carried out in multiple stages. First, all atoms except the lipid tails were restrained for 250 ps of simulation, permitting the tails to “melt”. Next, protein backbone atoms were restrained for an additional 1.75 ns. Finally, all atoms were freed for the remainder of the simulation. We estimate the resulting area per lipid for our DMPE bilayer is 57-60 Å².

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Membrane-plane and extracellular views of the Ca^{2+} /CN-Cbl-bound structure of BtuB. The barrel and luminal domains are colored in grey, the two Ca^{2+} ions are in blue, and CN-Cbl is shown in a stick representation colored by atom type. The eleven extracellular loops are drawn using a thicker representation and labeled using different colors to distinguish them from their neighbors.

Figure 2.

Differences between simulations of the Ca²⁺-bound and the apo structures of BtuB. (A) Root mean-square deviation (RMSD) of C_α atoms of BtuB's extracellular loops vs. time for the Ca²⁺-bound structure of BtuB (black) and the apo structure of BtuB (red), both in comparison to the CN-Cbl-bound structure of BtuB. (B) Root mean-square fluctuation (RMSF) vs. residue number for the two structures, colored as in A. (C) Comparison of the average structures, shown from the extracellular side, calculated from the last 20 ns out of the 30-ns simulations. The apo structure is shown in blue and the Ca²⁺-bound structure, with corresponding Ca²⁺ ions, in yellow.

Figure 3.

Simulation of the Ca²⁺/CN-Cbl-bound structure of BtuB. (A) Unique interactions observed during the simulation. The two loops involved, loops 13/14 and 15/16, are colored as in Fig. 1; the specific residues along with CN-Cbl are seen in a stick representation, colored by atom name. A snapshot of the simulation is shown in which Tyr453 hydrogen bonds to CN-Cbl. The crystallographic position of loop 15/16, farther away from CN-Cbl, is also indicated in blue. (B) RMSD of the extracellular loops vs. time and (C) RMSF vs. residue number for the Ca²⁺/CN-Cbl-bound structure of BtuB (red) and the Ca²⁺-bound structure (black).

Figure 4. Comparison of Ca²⁺-bound and Mg²⁺-bound BtuB. (A) Ca²⁺ coordination after 30 ns. Residues interacting with the Ca²⁺ ions (yellow) are shown as sticks, colored by atom name. Gln191 is partially transparent so as not to obscure Asp193 behind it. (B) Mg²⁺ coordination after 30 ns. (C) RMSD of the extracellular loops vs. time for the simulation of the Mg²⁺-bound structure (red) compared to the simulation of the Ca²⁺-bound BtuB (black).

Figure 5.

Binding of Ca^{2+} to the apo structure of BtuB. (A) Stereo view of the Ca^{2+} binding site after 50 ns of simulation. The Ca^{2+} -binding residues are shown in a stick representation and the Ca^{2+} ions are drawn as red spheres. The positions of the ions in the crystal structure of the Ca^{2+} -bound BtuB are indicated as blue spheres. (B) RMSD vs. time for loops 3/4, 5/6, and 7/8, compared to their positions in the crystal structure of the Ca^{2+} -bound BtuB.

Figure 6.

Parametrization of CN-Cbl. (A) Reduced model used for quantum chemical calculations in a ball and stick representation. The atoms are colored by type, with cobalt in green, and the final partial charges assigned to each heavy atom is given. (B) Simulation of CN-Cbl in a water box. The initial conformation is indicated as a thick stick representation colored by atom type. Snapshots of CN-Cbl taken every 4 ps from the 1-ns trajectory are overlaid as thin lines.

Table 1Ca/Mg-1 coordination^a.

Atom name	occ. time (Ca)	occ. time (Mg)	avg. dist. (Ca)	avg. dist. (Mg)
Asp179 OD1	93.58	98.84	3.14	3.04
Asp179 OD2	98.39	100.00	2.32	1.88
Asp193 OD1	91.32	100.00	3.30	3.29
Asp193 OD2	100.00	100.00	2.14	1.85
Asp195 OD1	99.26	100.00	3.44	3.54
Asp195 OD2	100.00	100.00	2.13	1.85
Asp230 OD1	13.15	99.98	4.16	3.61
Asp230 OD2	100.00	100.00	2.14	1.84
Gln191 O	54.88	1.61	3.98	4.82
Asp193 O	5.58	29.82	4.86	4.19
Asp179 O	2.92	1.71	6.11	5.87

^a Ca-1 and Mg-1 coordinating atoms are indicated by residue number and atom name. Occupancy (occ.) time is the fraction of time that each atom was within 4 Å of the Ca²⁺ or Mg²⁺ ion. The final two columns indicate the average distance (avg. dist.) between each atom and the corresponding ion over the course of the entire 30 ns of the simulation.

Table 2Ca/Mg-2 coordination^a.

Atom name	occ. time (Ca)	occ. time (Mg)	avg. dist. (Ca)	avg. dist. (Mg)
Asp193 OD1	100.00	100.00	2.15	1.84
Asp193 OD2	66.84	99.92	3.51	3.65
Asp195 OD1	100.00	100.00	2.13	1.85
Asp195 OD2	87.30	100.00	3.74	3.47
Asp230 OD1	100.00	100.00	2.17	1.85
Asp230 OD2	99.97	100.00	2.67	3.34
Tyr229 O	15.79	36.95	4.28	4.22
Gln191 OE1	1.35	2.21	6.35	7.82

^a Ca-2 and Mg-2 coordinating atoms, along with their occupancy time and average distance from the ions, are given. See Table 1 for more details.