Nucleotide-Dependent Dimerization of the C-Terminal Domain of the ABC Transporter CvaB in Colicin V Secretion

Xiangxue Guo,1 Robert W. Harrison, 1,2 and Phang C. Tai1∗
Department of Biology1 and Department of Computer Sciences,2 Georgia State University, Atlanta, Georgia 30303

Received 22 December 2005/Accepted 19 January 2006

The cytoplasmic membrane proteins CvaB and CvaA and the outer membrane protein TolC constitute the bacteriocin colicin V secretion system in Escherichia coli. CvaB functions as an ATP-binding cassette transporter, and its C-terminal domain (CTD) contains typical motifs for the nucleotide-binding and Walker A and B sites and the ABC signature motif. To study the role of the CvaB CTD in the secretion of colicin V, a truncated construct of this domain was made and overexpressed. Different forms of the CvaB CTD were found during purification and identified as monomer, dimer, and oligomer forms by gel filtration and protein cross-linking. Nucleotide binding was shown to be critical for CvaB CTD dimerization. Oligomers could be converted to dimers by nucleotide triphosphate-Mg, and nucleotide release from dimers resulted in transient formation of monomers, followed by oligomerization and aggregation. Site-directed mutagenesis showed that the ABC signature motif was involved in the nucleotide-dependent dimerization. The spatial proximity of the Walker A site and the signature motif was shown by disulfide cross-linking a mixture of the A530C and L630C mutant proteins, while the A530C or L630C mutant protein did not dimerize on its own. Taken together, these results indicate that the CvaB CTD formed a nucleotide-dependent head-to-tail dimer.

CvaB is an ATP-binding cassette (ABC) transporter from Escherichia coli that is responsible for the export of protein toxin colicin V (10). ABC transporters are ubiquitous in eukaryotic and prokaryotic cells. More than 80 ABC proteins, about 5% of the genome, were identified in E. coli K-12 (15). Some human genetic disorders, like cystic fibrosis and Wegener’s granulomatosis, are due to mutations in ABC transporters (2), and there are at least 48 ABC transporter genes in Homo sapiens (6). Therefore, it is important to understand the mechanism and structure of ABC transporters.

ABC transporters consist of at least two domains, a nucleotide-binding domain (NBD) and a transmembrane domain (TMD). The functional transporter consists of at least a dimer of the NBD (18). The NBDs show significant sequence similarity and structural homology, while the TMDs do not (38). The lack of homology in the TMDs reflects the wide range of compounds for which export and import are controlled by ABC transporters. ABC transporters control the export or import of compounds such as cytotoxins, antibiotics, antifungal agents, herbicides, and anticancer drugs (14). The structural homology of the NBDs reflects the underlying common mechanism where hydrolysis of a nucleotide triphosphate provides the energy for substrate transport across the cell membrane (17).

The crystal structures of ABC NBDs in both eukaryotes and prokaryotes have been determined, including HlyB (36, 47), MalK (5) from E. coli, MJ0796 from Methanococcus jannaschii (45), HisP from Salmonella enterica serovar Typhimurium (22), GicV from Sulfolobus solfataricus (42), LmrA from Lactococcus lactis (PDB accession code 1MV5; provided by Y.-R. Yuan et al.), TAP1 from Homo sapiens (9), and the cystic fibrosis transmembrane conductance regulator from Mus musculus (28). Two whole ABC transporters have been crystallized, i.e., the lipid transporter MsbA (3, 4, 33) and the vitamin B12 importer BtuCD in E. coli (30).

The NBD structures show significant structural similarity. The consensus structure is an alpha/beta fold (25). One of the most striking features of the NBD structure is the highly conserved Walker A and B sites, as well as the ABC signature motif and Q loop, form parts of the nucleotide-binding site and are critical for the function of the ABC transporter (26, 29, 37). However, these motifs are not adjacent to each other in the monomer structure. Clearly, the monomer cannot be the active ABC molecule, and therefore, the factors that control dimer formation are potentially important in understanding the function of the ABC transporters. The canonically accepted model for the role of the NBD in ABC transporters is a chemical pump. The Rad50 crystal structure (19) and the MJ0796 NBD structure (45) with nucleotide triphosphates or triphosphate analogs showed that the NBD dimers formed the nucleotide-binding site. This leads to the hypothesis that nucleotide binding is essential for the formation of dimers, which supplies the “power stroke” for ABC transporter pumps. It is necessary to construct a soluble NBD in order to test this hypothesis, and such a system can be constructed from the E. coli CvaB protein (23, 49).

CvaB is part of the dedicated type 1 exporter for export of the bacterial toxin colicin V. This exporter consists of the cytoplasmic membrane proteins CvaA and CvaB and outer membrane protein TolC (10). The CvaB-NBD is located at the C terminus and has nearly all of the conserved sites from ABC transporters, including the Walker A (G526ASGAGKT) and Walker B (I650LFMDE) sites (16), the Q loop (Q574), the H switch (H684), and the ABC signature motif (L630SGGQ). The most homologous NBD with a high-resolution crystal structure is the α-hemolysin exporter HlyB (36), where the sequence...
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>K-12 ompF44 lacIq169 (808 locZΔM15)</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>11</td>
</tr>
<tr>
<td>BL21</td>
<td>F' ompT hsdSd</td>
<td>40</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHK11</td>
<td>pBR322 with cavaABC and cvi</td>
<td>41</td>
</tr>
<tr>
<td>pTrcHisB</td>
<td>Expression vector in E. coli with trc promoter and N-terminal His6 tag</td>
<td>In vitro</td>
</tr>
<tr>
<td>pXG11</td>
<td>CvaB-CTD (amino acids 459–698) gene on pTrcHisB between BamHI and EcoRI sites</td>
<td>This study</td>
</tr>
<tr>
<td>pXG530</td>
<td>CvaB-CTD mutation Ala530 to Cys (A530C) at Walker A site</td>
<td>This study</td>
</tr>
<tr>
<td>pXG630</td>
<td>CvaB-CTD mutation Leu630 to Cys (L630C) in ABC signature motif</td>
<td>This study</td>
</tr>
<tr>
<td>pXG654</td>
<td>CvaB-CTD mutation Asp654 to His (D654H) at Walker B site</td>
<td>49</td>
</tr>
</tbody>
</table>

MATERIALS AND METHODS

Bacterial strains, plasmids, media, chemicals, and reagents. All of the E. coli strains used in this study are described in Table 1. Bacterial culture medium (10 g of tryptone, 5 g of NaCl, 40 mM potassium phosphate buffer [pH 7.0], 6 mM ammonium sulfate, 1.6 mM sodium citrate per liter) was used for both liquid and solid (with 1.5% agar) plates. The antibiotic ampicillin was used at a final concentration of 100 μg/ml. Liquid media, cultivated at 37°C until the optical density at 600 nm reached between 0.4 and 0.6, and induced by 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at various temperatures for 4 h. The induced cells were harvested, resuspended in affinity binding buffer (25 mM phosphate-buffered saline buffer [pH 8.0], 100 mM KC1, 200 mM NaCl, 7 mM β-mercaptoethanol, 20% glycerol), and passed through a French press (GE Healthcare) twice at 12,000 lb/in 2. The CvaB CTD was purified from cell supernatant through Ni-nitrilotriacetic acid (NTA) affinity chromatography (QIAGEN), Q-Sepharose FF ion-exchange chromatography, and Superose 6 gel filtration chromatography (Pharmacia). The molecular sizes of the different forms were identified by gel filtration chromatography on Superdex 75 HR 10/30 (Pharmacia) in buffer containing 20 mM HEPES (pH 7.6), 100 mM NaCl, 10% glycerol, and 1 mM dithiothreitol (DTT). The purified proteins were stored in aliquots at ~80°C in 100 mM 3-(cyclohexylaminol)-1-propanesulfonic acid (CAPS) buffer (pH 10.4)–20% glycerol–10 mM DTT. All of the plasmids were purchased from Sigma unless otherwise noted. The reagents were reagent grade and recommended by the manufacturers. All other chemicals were reagent grade and purchased from Sigma unless otherwise noted.

Plasmid construction, protein expression, and purification. The cavaABC gene was cloned into the expression vector pTrcHisB (Invitrogen) between the BamHI and EcoRI sites. The vector carried a DNA fragment encoding 31 extra amino acids, which included six histidines as an Ni2+-binding domain, as used previously in our laboratory (49). Recombinant DNA manipulations were performed essentially as previously described (35). For high-level expression, the constructed plasmids were freshly transformed into the host, E. coli DH5α. The overnight cell culture of the transformant was diluted 40 times into fresh TA medium, cultivated at 37°C until the optical density at 600 nm reached between 0.4 and 0.6, and induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at various temperatures for 4 h. The induced cells were harvested, resuspended in affinity binding buffer (25 mM phosphate-buffered saline buffer [pH 8.0], 100 mM KC1, 200 mM NaCl, 7 mM β-mercaptoethanol, 20% glycerol), and passed through a French press (GE Healthcare) twice at 12,000 lb/in 2. The CvaB CTD was purified from cell supernatant through Ni-nitrilotriacetic acid (NTA) affinity chromatography (QIAGEN), Q-Sepharose FF ion-exchange chromatography, and Superose 6 gel filtration chromatography (Pharmacia). The molecular sizes of the different forms were identified by gel filtration chromatography on Superdex 75 HR 10/30 (Pharmacia) in buffer containing 20 mM HEPES (pH 7.6), 100 mM NaCl, 10% glycerol, and 1 mM dithiothreitol (DTT). The purified proteins were stored in aliquots at ~80°C in 100 mM 3-(cyclohexylaminol)-1-propanesulfonic acid (CAPS) buffer (pH 10.4)–20% glycerol–10 mM DTT.

Site-directed mutagenesis. Plasmid DNA isolation, digestion, transformation, and other routine DNA manipulations were done as previously described (35). Mutagenesis was performed by overlap extension PCR (21) with pHK11 as the cava gene template. cavaB CTD forward and reversed primers 5'-CCGGGA TCCAATGAGTCTGCACAAATGTAATATATATATACTCT (incorporated BamHI and EcoRI restriction sites are underlined), respectively, and mutant 5′ primers A530C (5’-GCTTCGGTGTTGGCAGAAA ACC) and L630C (5’-GAAGTTGTTCTGGCAGG) were used. PCR conditions were 95°C for 5 min, 28 cycles of denaturation at 95°C for 45 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and then an extra extension for 10 min by PCR enzyme [Pfu (Pharmacia)]. The parent plasmid and newly (PCR) synthesized DNA fragments were digested separately by BamHI and EcoRI. They were then ligated and transformed into E. coli DH5α. DNA sequencing was performed with an ABI 377 Sequencer (Applied Biosystems) to verify the mutations and the rest of the DNA sequence.

Gel electrophoresis and Western blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10%) was performed by the method of Laemmli (27). Proteins were transferred to polyvinylidene difluoride membranes (Applied Biosystems) and treated for Western blotting (35) with a polyclonal antibody for the CvaB CTD (49). Alkaline phosphatase-conjugated goat antirabbit IgG (Bio-Rad) was used as the secondary antibody.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) cross-linking. The protocol used for EDC cross-linking was that recommended by the manufacturer (Pierce). Protein samples at 1.0 μg/ml in reaction buffer [100 mM 2-(N-morpholino)ethanesulfonic acid (MES; pH 5.5), 5 mM GTP, 5 mM MgCl2, 5% glycerol, 1 mM DTT] were preincubated on ice for 5 min and at 25°C for 1 min before a final concentration of 4 mM EDC was added. Cross-linked samples were taken at various times, and the reaction was stopped by adding 1/10 volume of 2 M Tris-HCl (pH 6.8). The cross-linked protein samples were run on SDS-PAGE and identified by Western blotting with CvaB antibodies (49).

Dimerization and dissociation assays. To test the effect of nucleotide binding on protein dimerization, the purified oligomer fraction of the CvaB CTD (30 μg/ml) was incubated at 4°C with 10 mM GTP-Mg2+ for various times in 20 mM HEPES buffer (pH 7.6) with 5% glycerol and 1 mM DTT. To determine the effect of nucleotide-magnesium release on the dissociation of protein, the nucleotide-bound CvaB CTD dimer (30 μg/ml) was incubated at 20°C with 15 mM EDTA for various times in 10 mM CAPS buffer (pH 10.4) with 5% glycerol and 1 mM DTT. Finally, each sample was subjected to analytical gel filtrationchromatography (Superdex 75 PC 3.2; Amersham) at a flow rate of 0.2 ml/min at 4°C.

Disulfide cross-linking. The disulfide cross-linking procedures used were previously described (34). After freshly removing the DTT from protein stock through gel filtration chromatography, 48-μl protein samples at 2.5 μg/ml in 20 mM HEPES buffer (pH 7.6) were mixed with 12 μl of 15 mM CuSO4, 45 mM 1,10-phenanthroline, 50 mM sodium phosphate (pH 7.3), and 10% glycerol. The samples were incubated at 37°C for 10 min or as indicated. The reactions were stopped by addition of SDS sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS) containing 50 mM EDTA without reducing agent. The reaction mixtures were subjected to SDS-PAGE (10%) and Western blotting (49).

RESULTS

Both dimers and oligomers of the CvaB CTD were observed. Previous studies in our laboratory (49) utilized constructs expressing the CTD of CvaB (residues 439 to 698) with either a His6 tag or the gene for glutathione S-transferase fused to the N terminus. However, this construct formed inclusion bodies easily. In this study, we constructed several shorter versions of the CTD with 190, 201, 210, 220, 230, and 240 residues. The expression vector pTrcHisB has been used widely for its convenience of purification through Ni2+ binding (49). IPTG induction was per-
formed at 27.5°C, which was found to be the optimal temperature for expression and prevention of the formation of excess inclusion bodies. When expressed in *E. coli* BL21 with 0.5 mM IPTG induction, the 240-residue CTD (residues 459 to 698) had better expression and remained more soluble than others in the cytoplasm. This protein was purified through Ni-NTA affinity, ion-exchange, and gel filtration chromatography to more than 99% purity. Two forms of the CvaB CTD were isolated; one eluted at 50 mM and the other eluted at 250 mM imidazole on Ni-NTA chromatography. The apparent molecular mass of the CvaB CTD monomers of both forms was 32 kDa on SDS-PAGE, and both forms were confirmed by immunoblotting (Fig. 1A). The 50 mM imidazole-eluted fraction was about 62 kDa and was identified as a dimer of the CvaB CTD in 20 mM HEPES buffer (pH 7.6) by analytical gel filtration chromatography (Fig. 1B). The *A*<sub>280</sub>/<A>260</sub> ratio of the purified dimer fraction was 1.35, indicating the existence of a nucleotide bound to the purified CvaB CTD dimer. Based on calculation, the molecular ratio of protein to nucleotide was close to 1:1, suggesting that two nucleotides were bound to each CvaB CTD dimer. On the other hand, the fraction of the CvaB CTD eluted by 250 mM imidazole was identified as oligomers that appeared at the void volume on the gel filtration column (Fig. 1C). The *A*<sub>280</sub>/<A>260</sub> ratio of the
oligomer fraction was about 1.68, implying that nearly no nu-
cleotide was bound. The bound nucleotides in the dimer form
were confirmed by thin-layer chromatography (TLC). Both the
nucleotide diphosphates (minor) and nucleotide triphosphates
(major) were found in the dimer but not in the oligomer (Fig.
1D). These results suggested that the bound nucleotide(s) was
strongly associated with the dimer and might be critical for the
formation of the CvaB CTD dimer.

Induction of dimers from oligomers by nucleotide binding.
To identify the effect of nucleotide binding on protein dimer-
ization, the purified nucleotide-free CvaB CTD oligomers
were incubated with different nucleotides. The relative dimer
and oligomer amounts were then detected by gel filtration
chromatography. Based on our previous finding that the CvaB
CTD bound GTP much better than ATP at low temperatures
(49), we used GTP-Mg, attempting to convert the CvaB CTD
from an oligomer to a dimer. As the time of GTP-Mg incuba-
tion on ice increased, the proportion of the oligomer decreased
while that of the dimer increased (Fig. 2A). These results
indicated that the nucleotide binding to the CvaB CTD in-
duced the oligomers into dimers. This induction was rapid;
about 80% of the dimer form appeared after 20 min.

To determine whether other nucleotides might induce the for-
formation of CvaB CTD dimers, different nucleotides were tested.
Almost no dimer was obtained with GMP. However, GDP, GTP,
and GMP-PNP displayed similar induction rates for the CvaB
CTD (Fig. 2B, left), indicating that the equilibrium of the mono-
mer and dimer was not directly dependent on the hydrolysis of
nucleotides from the triphosphate to the diphosphate. ATP and
GTP displayed similar induction rates for the CvaB CTD. How-
ever, CTP and TTP showed a much lower rate than that of ATP
or GTP (Fig. 2B, right). These results indicated that the purine
base of the nucleotide is important for binding to ABC NBDs.
This finding is consistent with our previous report that the radio-

![Graph A](https://example.com/graph_a.png)

![Graph B](https://example.com/graph_b.png)

![Graph C](https://example.com/graph_c.png)

**FIG. 2.** Formation of CvaB CTD dimers from oligomers induced by nucleotides. (A) GTP-Mg (10 mM) was incubated with CvaB CTD
oligomers in HEPES buffer on ice for various times; only data taken at 0 (dotted line), 10 (bold line), or 20 (thin line) min are shown (the oligomer
and dimer were located at 7.12 and 10.02 ml, respectively). (B) Effects of different nucleotides on dimer formation (the amount of dimer is
indicated as scanning data from Western blots; different sets of data were normalized). (C) Lack of dimer formation by the nucleotide
binding-defective D654H mutant protein.
active GTP-bound CvaB CTD could not be competed by 100 μM unlabeled CTP or UTP but was partially competed by ATP or ADP and highly competed by GTP or GDP (49).

To further demonstrate the importance of nucleotide binding for the function of the CvaB CTD, we determined the oligomer state by EDC cross-linking on the D654H defective mutant, which has a point mutation in the Walker B site of CvaB that severely impairs both nucleotide triphosphatase activities and nucleotide binding and completely abolished the secretion of colicin V (49). The wild-type CvaB CTD dimer could be cross-linked by EDC in a short time at room temperature (25°C), and more dimers were obtained with increased EDC cross-linking time (Fig. 2C, left). However, the nucleotide binding-defective D654H mutant did not form dimers; no dimer band was detected on EDC cross-linking (Fig. 2C, right). These results indicated that nucleotide binding was important for the CvaB CTD to be in the dimer form.

Monomers and oligomers induced by nucleotide removal from dimers. During these studies, it was found that oligomer forms appeared in the purified dimer fraction of the CvaB CTD upon storage at 4°C in HEPES buffer, and a low concentration of EDTA (5 mM) could greatly accelerate this oligomerization process. To further investigate the role of bound nucleotides on maintaining the dimer form of the CvaB CTD, EDTA was used to chelate the magnesium ion from the nucleotide-bound dimer, resulting in eventual nucleotide release from the CvaB CTD. To accelerate magnesium chelation, the temperature was set at 20°C and CAPS buffer (pH 10.4) was used, under which conditions the EDTA was fully deprotonated as the most efficient chelator (46). By analyzing the protein samples with increased time of EDTA chelation on gel filtration chromatography, it was found that the main fraction of the CvaB CTD shifted from dimers to monomers. Some intermediate forms were also observed in the first 2 h (Fig. 3A...
and B). After 4 h of EDTA incubation, the oligomer appeared; by 24 h of EDTA incubation, only the oligomer form could be detected and almost half of the protein was lost, presumably due to protein aggregation (Fig. 3A). In comparison with the CvaB CTD dimerization induced by GTP-magnesium (Fig. 3A), this oligomerization due to nucleotide release was relatively slow (Fig. 3C). Nevertheless, it is clear that the oligomer and dimer forms of the CvaB CTD are interconvertible under different conditions and that nucleotide binding is critical for maintaining the dimer form.

**Proximity of the Walker A site and the ABC signature motif shown by disulfide cross-linking.** Shown in some ABC transporters, the highly specific ABC signature motif is adjacent to the nucleotide-binding pocket (Walker A and B sites) and functions to couple the nucleotide binding and conformational changes in TMDs (31, 39). To test the relative locations of the Walker A (GASGA<sup>530</sup>GKT) site and the signature motif (L<sup>630</sup>SGGQ) in CvaB CTD dimers, A530C and L630C site-directed mutagenesis, based on experimental data obtained with the human P glycoprotein (31), was done and disulfide cross-linking by oxidative copper phenanthroline was performed. As a control, the wild-type CvaB CTD was treated by oxidation under the same conditions. No disulfide-cross-linked dimer band was obtained under the oxidative condition (Fig. 4A, lane 1), even though each had four cysteine residues. Without nucleotide, A530C or L630C mutant protein or a mixture of the two at a ratio of 1:1 showed only monomer bands on SDS-PAGE (lanes 2 to 4). However, with nucleotide (GTP) preincubation, the mixture of L530C and L630C mutant proteins showed both monomer and dimer bands (lane 7); the dimer band disappeared when the sample was treated with 100 mM DTT after disulfide cross-linking (lane 8). The time course of the mixture of A530C and L630C mutant proteins in disulfide cross-linking is shown in Fig. 4B. These results indicate that the nucleotide binding induced a dimer form in which the Walker A site and the signature motif from two monomers were spatially proximal.

The observation that the A503C or L630C mutant protein individually did not show the disulfide-cross-linked dimer band (lanes 5 and 6 in Fig. 4A) but the mutant mixture did (lane 7) suggested that neither the two Walker A sites nor the two ABC signature motifs were spatially close to each other in the CvaB CTD dimer. These data indicated that the Walker A site and the signature motif from two monomers were spatially proximal.

**Head-to-tail model of the nucleotide-dependent CvaB CTD dimer.** To describe the effect of nucleotide binding on CvaB CTD dimerization, homology models of both the monomer and dimer forms of CTD were made (12, 13) with the crystal structures of the HlyB NBD as the template (36, 47). In addition, crystal structures of nucleotide-bound MJ0796 (45) and MalK (5, 7) were compared. A sandwich-like homodimer model of the CvaB CTD was obtained (Fig. 5A). By analyzing the models, it was found that the Walker A site and the signature motif were located far apart in a CvaB CTD dimer. Shown in the dimer model, the selected residues (Ala530 and Leu630) were within a 10-Å separation with side chains directed toward each other to facilitate disulfide bond formation once they were replaced by cysteines. As the functional form, the CvaB CTD homodimer was very possibly maintained by the interactions between subunits in nucleotide-binding pockets. We concluded that the nucleotide-dependent dimers were formed in a head-to-tail arrangement (Fig. 5A).

**DISCUSSION**

In this study, the nucleotide-bound CvaB CTD was identified as a dimer, nucleotide binding was found to be critical for CTD dimerization, and nucleotide release from the dimer was found to result in the formation of monomers and oligomers.
Site-directed mutagenesis and disulfide cross-linking studies verified that nucleotide binding drives the formation of a head-to-tail dimer in which the Walker A site and the ABC signature motif from different subunits are spatially close to each other.

The monomer and dimer forms are in equilibrium in the ABC NBDs of HlyB (1) and OpuAA (20). In this study, equilibrium of the monomer, dimer, and oligomer forms was observed in the CvaB CTD in vitro and nucleotide binding was found to have a critical role in this equilibrium. The oligomeric state of the CvaB CTD was converted into dimers rapidly with the purine nucleotide diphosphates and triphosphates (Fig. 2A and B), while the nucleotide binding-defective mutant protein was not (Fig. 2C). With the removal of nucleotides, the dimer form of the CvaB CTD was converted transiently and rapidly into monomers (Fig. 3A and B). Both nucleotide diphosphates and triphosphates were found in the purified CvaB CTD dimer, with triphosphates being the dominant form (Fig. 1D). Furthermore, the CvaB CTD dimers can be induced by both diphosphate and triphosphate purine nucleotides (Fig. 2B).

Diphosphate nucleotides are widely used in crystallizing ABC NBDs (5, 9, 26). However, the nucleotide triphosphate has also been used to crystallize some ABC NBD dimers, such as MJ0796 (45), GlcV (43), Rad50 (19), and MalK (7). In this study, we found that CvaB CTD dimerization can be induced by both GTP and GDP, but not by GMP. Furthermore, different nucleotide triphosphates, such as ATP and GTP, can promote the formation of the CvaB CTD dimer at similar rates (Fig. 2B). The nucleotides with pyrimidine rings (CTP and UTP) had no effect on the oligomeric state of the CvaB CTD, which confirmed our previous result for the competitive binding of nucleotides (49). In vitro, the monomers oligomerized slowly and all were converted into oligomers and aggregates eventually (Fig. 3A and C). However, such oligomerization probably does not occur in vivo for several reasons. First, in whole CvaB, the CTD is merged with TMDs that integrate into the cytoplasmic membrane with the CTD in the cytoplasm. When the CvaB dimer is dissociated, the hydrophobic CvaB CTD monomers might partially interact with the membrane so
that oligomers cannot form. Second, in cells under physiological conditions, there is a high concentration of ADP or ATP and GDP or GTP in vivo, where nucleotides can promote the formation of the CvaB CTD dimer.

In most ABC transporters, nucleotide binding and hydrolysis by NBDS result in conformational changes in TMDs which open the cytoplasmic membrane channel for the secretion of substrates (3, 4, 32, 33). In addition to the ABC transporter CvaB, the ColV secretion system contains the outer membrane tunnel protein ToIC and the accessory protein CvaA. To describe the role of nucleotide binding and hydrolysis in ColV secretion, three states of the CvaB CTD (monomer, inactive dimer, and active dimer) are proposed here (Fig. 5C). With no bound nucleotide, the CvaB CTD exists as monomers (Fig. 5C, part I); once a nucleotide is bound, the CvaB CTD will be in a dimer form. Considering that a high concentration of nucleotides exists in vivo and different nucleotides (GTP, GDP, ATP, and ADP) can induce CvaB CTD dimerization, the CTD monomers may be just a transient state. The nucleotide triphosphate was found to dominate in the CTD dimer, suggesting low-level nucleotide triphosphate hydrolysis in vitro. However, ColV precursor processing requires energy from nucleotide binding and hydrolysis. Thus, we propose that there are two states of CvaB dimer, i.e., inactive and active forms. In an inactive CvaB dimer (Fig. 5C, part II), even bound with nucleotides, the cytoplasmic CvaB CTD channel is closed. Only when the substrate (ColV) binds to the CvaB TMD and in the presence of CvaA and ToIC, the ATP- or GTP-bound CvaB dimer becomes active (Fig. 5C, part III) and the channel is open for export of the substrate ColV.

Nucleotide hydrolysis provides energy for substrate secretion. Two proposals have been described for the roles of nucleotide hydrolysis in ABC-involved exporters. First, nucleotide hydrolysis directly promotes ABC channel opening for secretion. Secondly, nucleotide hydrolysis occurs after substrate secretion for initiation of the next cycle. In the first proposal, as previously described (8, 16, 24), the CvaB dimer is in an inactive state without substrate binding (Fig. 5C, part II). Binding of the substrate ColV stimulates nucleotide triphosphate binding and consequent hydrolysis to a CvaB dimer (Fig. 5C, part III), which provides energy for conformational changes in CvaB TMDs and for opening of the ABC channel for substrate export. However, several recent studies indicated that it is the binding, rather than the hydrolysis, of nucleotide triphosphate that provides the power stroke for ABC-involved export (32, 44). Thus, in the second proposal, the binding of nucleotide diphosphates and triphosphates marks the lower-energy and high-energy states of the CvaB dimer, respectively. When the nucleotide diphosphate is bound, the CTD dimer is in an inactive state and the CvaB cytoplasmic channel is closed (Fig. 5C, part II). Substrate (ColV) binding promotes the replacement of nucleotide diphosphates with nucleotide triphosphates on CTDs. Nucleotide triphosphate binding to CTDs results in a high-energy active form of CvaB dimer (Fig. 5C, part III) for substrate export. After secretion, the bound nucleotide triphosphate is hydrolyzed, the CvaB active dimer changes back into an inactive low-energy status, and the ABC channel becomes closed. Regardless of when nucleotide hydrolysis takes place, only the active form of the channel composed of CvaA, CvaB, and ToIC allows secretion of the bound substrate ColV.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health research grants GM53766 (P.C.T.) and GM6762 (R.W.H.), the Georgia Cancer Coalition (R.W.H.), a Molecular Basis of Disease Program fellowship (N.G.), and Research Enhancement Program funds from Georgia State University. The facilities were supported by funds obtained through the Georgia Research Alliance.

REFERENCES


Downloaded from http://jb.asm.org/ on September 8, 2017 by guest


