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Molecular Basis for Differential Nucleotide Binding of the NBD of ABC-transporter CvaB[†]

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Abstract

The cytoplasmic membrane protein CvaB, involved in colicin V secretion in *Escherichia coli*, belongs to the ABC transporter family in which ATP hydrolysis is typically the driving force for substrate transport. However, our previous studies indicated that the nucleotide-binding domain of CvaB could also bind and hydrolyze GTP, and indeed highly preferred GTP over ATP at low temperatures. In this study, we have examined the molecular basis of this preference. Sequence alignment and homology modeling of the CvaB nucleotide binding domain predicted that the aromatic stacking region of CvaB (Y⁵⁰¹DSQ-loop) had a role in the differential binding of nucleotides, and the Ser503 and Gln504 provided potential hydrogen-bonds to GTP but not to ATP. Site-directed mutagenesis of the Y⁵⁰¹DSQ-loop: mutations S503A, Q504L, and double mutation S503A/Q504L, was made to test the predicted hydrogen-bonds with GTP. The double mutation S503A/Q504L increased the affinity for ATP by 6-fold, whereas the affinity for GTP was reduced slightly: the ATP/GTP-binding ratio increased about 10-fold. The temperature-effect assays on nucleotide binding and hydrolysis further indicated that the double-mutant protein had largely eliminated the difference to substrate ATP and GTP and behaved more closely to the NBD of typical ABC transporter HlyB. Therefore, we conclude that the Ser⁵⁰³ and Gln⁵⁰⁴ in aromatic stacking region of CvaB block the ATP binding and are important for the GTP-binding preference.

Keywords

ABC transporter; CvaB; Molecular modeling; GTP-preference; Aromatic stacking; Nucleotide-binding domain; UV-cross linking

Colicin V (ColV)¹, an anti-bacterial toxin produced by *Enterobacteriaceae*, kills sensitive target cells by disrupting their cell membrane potential (1). In the Gram-negative bacterium

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Escherichia coli, ColV is secreted by a dedicated type-I exporter system across both the cytoplasmic and outer membranes directly into the environment outside the cells. At least three protein components participate in the ColV export system: two plasmid-born proteins CvaA and CvaB in the cytoplasmic membrane and a host chromosomal gene product TolC in the outer membrane (2,3).

The cytoplasmic membrane protein CvaB plays pivotal roles in the ColV export system. CvaB belongs to the family of ATP-binding cassette transporters (4,5), like the α -hemolysin exporter HlyB (6), the Cystic Fibrosis trans-membrane conductance regulator (7), and the multidrug resistance P-glycoprotein (8). The N-terminal proteolytic domain of CvaB processes the leader region of ColV precursor at the double-glycine site (9–11), the trans-membrane domains in the middle of CvaB form the cytoplasmic channel (3,9,12), and the C-terminal nucleotide-binding domain supplies energy for ColV secretion. Although the sequence of the proteolytic N-terminal domain of CvaB is unique among ABC transporters (11), the C-terminal nucleotide binding domain (NBD) retains a high level of homology with other family members (4,5,13). Several highly conserved ABC-NBD motifs are found in the CvaB sequence, such as the Walker A and B sites, the ABC Signature motif, Q-loop and H-switch (14–17). The Walker A and B sites are common to most ATPases involved in the binding and hydrolysis of nucleotides, while the ABC Signature motif is highly specific to ABC-NBDs. The Q-loop and H-switch are also specific to ABC transporters; they interact with the bound magnesium-nucleotide through non-covalent forces, such as ionic or hydrogen bond interactions (16). Three main aspects of nucleotide binding are noted by analysis of crystal structures of bacterial ABC-NBDs, such as HlyB (6), HisP (18), MJ0796 (19), MalK (20,21), and GlcV (22). First, the Walker A and B sites interact with oxygen atoms on the β -, and γ - phosphates of bound nucleotide, and orientate the nucleotide phosphates in the protein nucleotide-binding pocket (17). Secondly, the nucleotide-binding cofactor magnesium (Mg^{2+}) is important for connecting the nucleotide and protein through the ionic and hydrogen bond interactions involving oxygen atoms from β - and γ -phosphates of the nucleotide and from residues of the Walker A and B sites, and the Q-loop directly or via a water molecule (22). The magnesium stabilizes the nucleotide in the binding site, and participates directly in the nucleotide hydrolysis (17). Thirdly, amino acid residues from the ABC Signature motif are also involved in the binding of nucleotide as shown in the ABC-NBD dimer structures (19,20,23,24).

Alignment of the sequences of bacterial ABC transporters revealed that CvaB-NBD is phylogenetically the most similar to the HlyB-NBD. HlyB is a typical ATP-binding cassette protein on the cytoplasmic membrane that acts in the export of α -hemolysin in *E. coli* (6). Although the HlyB lacks the proteolytic domain at the N-terminus for processing substrate precursor that is present in CvaB (11), their C-terminal NBDs share a high sequence similarity. Recent studies indicated that the HlyB-NBD functions as a dimer providing energy for substrate transport (24,25), while the nucleotide-dependent formation of the CvaB-NBD dimer has also been reported (26). In an active ABC transporter, ATP-hydrolysis is widely assumed to supply energy to (14–16,27,28) for the translocation process. Many crystal structures of the ABC-NBD are stabilized by binding ADP or ATP analogs (6,29,30), and site-directed mutagenesis of the conserved binding sites will affect the binding and hydrolysis of nucleotide (31). Biochemical studies indicated that the bound ATP interacted extensively with the HlyB-NBD nucleotide-binding sites (6,24), induced the dimerization of the protein (26,32), and showed cooperation in the ATP hydrolysis (25). However, our previous results *in vitro* indicated that the CvaB-NBD had highly preferential binding of GTP at low temperatures, and the hydrolysis of ATP is cold-sensitive compared to that of GTP (33). In this study, we have determined the molecular basis of the nucleotide-binding preference of CvaB-NBD. Homology modeling of

¹The abbreviations used are: ABC, ATP-binding cassette; ColV, colicin V; NBD, nucleotide-binding domain; NTP, nucleotide triphosphate; PDB, protein data bank.

the CvaB-NBD dimer identified potential interacting residues for differential binding of nucleotides, and site-directed mutagenesis of those residues was used to test the binding and hydrolysis of nucleotides at different temperatures. The results showed that the aromatic loop (Y⁵⁰¹DSQ) is involved in the high GTP-binding preference of CvaB.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media and Reagents

E. coli strains ^{DH5} α (K12 *supE44* *AlacU169* (ϕ 80 *lacZ* Δ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*) (34) and BL21 (B *F⁻ ompT hsdS_B*) (35) were used as the hosts for plasmid transformation and protein expression, respectively. Plasmid pHK11 contains genes *cvaA*, *cvaB*, *cvaC* and *cvaI* (36). Plasmid pPSG122 (gift from Dr. Schmitt) expresses N-terminal His-tagged fusion protein HlyB-NBD (residues 467-707) under the control of an arabinose-inducible promoter in pBAD18 (6). The vector pTrcHisB is used for expressing CvaB-NBD (residues 459-698) (Invitrogen Corp., Carlsbad, CA).

Bacterial culture medium TA (10 gram of Tryptone and 5 gram of NaCl per liter with Buffer A: 40 mM potassium phosphate buffer (pH 7.0) with 7.6 mM ammonium sulfate and 1.6 mM sodium citrate) was used as both the liquid and solid plates (with 1.5% agar). The antibiotics ampicillin and chloramphenicol were used at a final concentration of 100 and 30 μ g/ml, respectively. Restriction enzymes, T4 DNA ligase, and EDTA-free protease inhibitor were obtained from Roche Applied Sciences Inc. (Indianapolis, IN) and were used essentially as recommended by the manufacturers. All other chemicals were reagent-grade and were purchased from Sigma Corp. (St. Louis, MO) unless otherwise noted.

Construction, Expression and Purification of CvaB-NBD

Recombinant DNA manipulations were performed essentially as described (37). Wild type and mutants of CvaB-NBD with the His6-tag on the N-terminus were expressed with the vector pTrcHisB. For high-level expression, the constructed plasmid was freshly transformed into the host *E. coli* BL21, and the overnight cell culture was diluted 40 times into fresh TA medium and cultivated at 37°C until the OD₆₀₀ was between 0.4 and 0.6, then the culture was induced by 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 27.5°C for 4 hours. CvaB-NBDs were purified from cell supernatant through Ni-NTA (Qiagen Inc., Valencia, CA) affinity, Q-Sepharose FF ion exchange, and Superose 6 FPLC (Pharmacia Corp., Peapack, NJ) gel filtration chromatography. The purified proteins were stored at -80°C in 100 mM 3-(cyclohexylamino) propanesulfonic acid (CAPS) buffer (pH 10.4) with 20% glycerol and 10 mM DTT.

Construction of CvaB-NBD models with nucleotides

The molecular model of CvaB-NBD was built by homology to the templates of the HlyB-NBD monomer (PDB entry 1MT0 (6)) and dimer (1XEF (24)) crystal structures, respectively. The molecular modeling program AMMP was used as described (38,39). The HlyB-NBD dimer structure provided the ATP and Mg as templates to generate the ATP and GTP-bound models of CvaB-NBD. The ligand structure (Mg and ADP) of TAP1-NBD (PDB entry 1JJ7)(29) were also considered when docking nucleotides onto CvaB-NBD. A discrete water model was used to treat solvation. The new atoms in the hydrated dimer models were generated by 20 cycles of the analytic procedure of AMMP (38) with molecular mechanics potentials. Finally, the models were optimized by 1000 steps of conjugate gradients minimization.

Site-directed Mutagenesis

Plasmid DNA isolation, digestion, transformation, and other routine DNA manipulations were as described (37). Mutagenesis was performed by overlap extension PCR with pHK11 as the template (40). DNA sequencing was performed to verify the mutations, using an ABI 377 Sequencer (Applied Biosystems, Foster City, CA) in the Biology Core Facilities, Georgia State University.

Gel Electrophoresis, Western Blotting, and Western blot

Standard SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (41). For Western blotting, electrophoresed proteins were transferred to polyvinylidene difluoride membranes (Applied Biosystems, Foster City, CA) and treated by established procedures for Western blotting (42). The polyclonal antibody for CvaB-NBD was made using the 12-KDa C-terminus of CvaB (33). Alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad Lab, Hercules, CA) were used as the secondary antibody.

ATP/GTP UV Cross-linking and Competitive Binding

UV cross-linking was performed as described (43). Samples (20 μ l) containing 2 μ g CvaB-CTD protein and 1 μ M α - 33 P-labeled ATP or GTP (3000 Ci/mmol from PerkinElmer Inc., Boston, MA) were pre-incubated for 5 minutes on ice in the binding buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$). Samples were irradiated in XL-1000 UV-crosslinker (Spectronics Inc., Westbury, NY) at 254 nm at 120 mJ/cm² twice for 15 minutes in an ice-water bath or twice for 10 minutes at other temperatures (16, 25, and 37 °C) as indicated. Cross-linked samples were directly mixed in SDS loading buffer, and separated by 12% SDS-PAGE. The gel was dried and the radioactive bands were quantified by a PhosphorImager (Fujifilm Medicals, Edison, NJ).

For the competitive binding assay, after the proteins were incubated with radioactive nucleotides, different concentrations of non-radioactive ATP or GTP were added to compete on ice for 10 minutes, and then UV-irradiation was performed as described (44). The competitive binding data were fitted to the linear regression by the Prism (GraphPad Software Inc., Ober-Olm, Germany) to obtain the best fit and the half of effective concentration, which were displayed as R^2 and EC_{50} , respectively.

ATPase/GTPase Activity Assay

The complete reaction mixture (20 μ l) contained 5 mM magnesium chloride, 100 mM potassium acetate, 40 mM Tris-acetate (pH 7.5), and 2 μ g ovalbumin, 1 mM DTT, and 0.5 μ g of CvaB NBD protein. [γ - 33 P]GTP or ATP (1 μ Ci; PerkinElmer Inc., Boston, MA) was added to samples incubated at various temperatures. The assay was carried out as described (33). Briefly, after 10-min incubation at the temperatures indicated, the reaction mixtures were mixed with 800 μ l of a 5% suspension of activated charcoal (Sigma Corp., St. Louis, MO) in 20 mM phosphoric acid, incubated on ice for 10 min, and then centrifuged for 10 min to pellet the charcoal and absorbed nucleotides. A 200 μ l aliquot of the supernatant fraction containing the liberated radioactive phosphate was analyzed by a Beckman scintillation counter. Sample data were normalized by subtracting the background count from the control without CvaB-NBD protein.

The temperature effect on NTPase activities was calculated according to the Arrhenius equation ($k = Ae^{-E_a/RT}$). Here, k is the reaction rate constant at different temperatures; R is equal to 0.00831 kJ/mol/K; E_a is the activation energy (kJ/mol); T is temperature (K); and A is the frequency factor as $\ln(k)$ intercept. The slopes ($-E_a/R$) of the $\ln(k)$ vs $1/T$ linear relationship displayed the activation energy of the reaction (E_a) at different temperatures.

RESULTS

Differential Binding of Nucleotides by HlyB and CvaB

Our previous studies have shown that the CvaB-NBD (C-terminal domain of CvaB, residues 459-698) binds GTP with much higher affinity than ATP at low temperatures (33). In this work, further analysis of this preference for binding GTP was made in comparison with that of the typical ABC transporter HlyB nucleotide-binding domain (HlyB-NBD). As reported previously (33), the GTP-binding level of CvaB-NBD was about 12.5 times higher than that for binding ATP shown on their nucleotide bound amount. Similar binding trends were observed when using radioactive ADP and GDP (data not shown). In contrast, the HlyB-NBD cross-linked nucleotides with a slight preference for ATP compared with GTP (Figure 1). The results suggested that CvaB may be unique among ABC transporters in the preference for binding GTP.

In order to determine the molecular basis of the nucleotide-binding preference, the sequences of CvaB and other ABC transporters were aligned. The sequences shared similar Walker A and B sites, ABC Signature motif, D-loop, Q-loop, and H-switch regions (Figure 2). High identity (42.9%) and similarity (61%) was especially evident for the NBDs of HlyB and CvaB. Analyzing ABC-NBD sequences and available crystal structures suggested which residues provided hydrogen bonds and hydrophobic interactions with the base ring of bound nucleotides. Conserved aromatic residues (Tyr or Trp) were identified recently to stack on the nucleotide base ring in the multidrug resistance protein (15,45). The corresponding aromatic stacking region of CvaB is the Y⁵⁰¹DSQ-loop (Figure 2). Since the ATP and GTP molecules differ only in their purine rings, the Y⁵⁰¹DSQ-loop region was proposed to be responsible for the differential nucleotide binding of CvaB-NBD.

Modeling CvaB-NBD Interactions with Nucleotides

To further investigate the interactions of CvaB-NBD with the bound nucleotides, molecular models were built of the structure of CvaB-NBD in complex with ATP or GTP. In the models, the Walker A & B sites interacted very similarly with the magnesium, β - and γ - phosphates of the two nucleotides ATP or GTP. The CvaB-NBD dimer model predicted that the Signature motif was also involved in the binding of β - and γ - phosphates of nucleotide, but showed no significant differences in the interaction with ATP or GTP (data not shown).

Analysis of the molecular models predicted a stacking interaction between the aromatic rings of the conserved Tyr⁵⁰¹ and the nucleotides GTP or ATP. This stacking interaction was a little weaker for ATP than for GTP considering the separation of the aromatic groups of 4.2 and 3.9 Å, respectively (Figure 3). Furthermore, the Ser⁵⁰³ and Gln⁵⁰⁴ of the Y⁵⁰¹DSQ-loop formed two hydrogen bonds to the guanine base of GTP, Ser⁵⁰³-O γ ...N²-GTP and Gln⁵⁰⁴-O ϵ ¹...N²-GTP (Figure 3, right), which are not possible for ATP due to the lack of an N² group on the purine ring. No equivalent interaction appears in the model of CvaB-NBD with bound ATP (Figure 3, left). These potential hydrogen bonds were predicted to increase the binding affinity for GTP compared to ATP. Therefore, the molecular models support our previous observation (33) that CvaB-NBD bound preferentially to GTP over ATP. Instead of the classical nucleotide-binding sites (Walker A and B and the Signature motif), the Y⁵⁰¹DSQ-loop was predicted to be important for the preferential binding of CvaB-NBD to GTP rather than ATP.

Lack of Effect on GTP-preference for Residues of the Walker A site, B Site, or the ABC Signature Motif

Site-directed mutagenesis experiments were designed to test the predictions of homology modeling. Comparing the NBD sequences of CvaB and HlyB, several variable residues were

found in the Walker A and B sites. Ala⁵²⁷, Ala⁵³⁰ and Thr⁵³³ (A site), and Phe⁶⁵² and Met⁶⁵³ (B site) of CvaB differed from the consensus sequence (Table 1).

Site-directed mutagenesis of those variable residues in the Walker A and B sites was performed to test the effect on differential binding of ATP or GTP to CvaB. Mutant proteins with the single-site substitutions (A527R, A530S, T533S, F652I and M653F) and their combinations were purified and tested by UV cross-linking to radioactive ATP or GTP. Only the double-mutation A527R/A530S on the Walker A site decreased the GTP/ATP-binding ratio by about 25%, and this decrease was partially recovered if this double mutation was combined with the double mutation F652I/M653F on the Walker B site (Table 1). Single mutations and other double- or triple-mutations did not show much difference in the ratio of GTP or ATP binding. These results showed that phylogenetically variable residues in the Walker A and B sites did not account for the high GTP-binding preference of CvaB-NBD, as predicted.

Site-directed mutagenesis of the ABC Signature motif of CvaB showed similar results for the UV cross-linking to ATP and GTP. While the G633D and K635A mutants showed similar ATP- and GTP-binding levels to those of wildtype, the mutant Q634H showed significantly reduced levels for binding of both GTP and ATP. However, the GTP/ATP-binding ratio was similar to that of wildtype (Table 1). These results demonstrated that, like the Walker A and B sites, the ABC Signature motif was not involved in differential binding of ATP and GTP.

ATP-binding Enhancement in the Double-Mutant of Y⁵⁰¹DSQ-loop

To test if the aromatic stacking region (residues 501-504) was indeed involved in the differential binding of nucleotides ATP and GTP as predicted, the single mutations S503A and Q504L and the double-mutant S503A/Q504L were made. The substitutions of Serine to Alanine and Glutamine to Leucine replaced polar side chains with hydrophobic side chains of approximately similar size. Purified wildtype and mutant proteins were tested by UV cross-linking to [α -³³P] ATP and [α -³³P] GTP, respectively. The autoradiogram results indicated that the single-mutation proteins S503A and Q504L had little effect on the ATP-/GTP-binding ratio. However, the double-mutant protein S503A/Q504L showed about 6-fold increase in binding affinity for ATP while that of GTP was reduced slightly, compared with those of the wildtype protein. The ATP/GTP-binding ratio of mutant S503A/Q504L increased from 0.08 to 0.8, about ten times more than that of the wildtype (Figure 4). Thus, the double-mutant protein S503A/Q504L largely eliminated the high preference of CvaB-NBD wildtype for GTP by increasing the affinity for ATP at low temperatures.

Reduced Preference for GTP of the Double-Mutant Protein S503A/Q504L

To further analyze the affinity for different nucleotides by the double-mutant protein S503A/Q504L, the binding of radioactive [α -³³P] ATP or GTP to CvaB-NBDs was competed by a series of concentrations of cold ATP or GTP. Their half effective concentrations (EC₅₀) were calculated as an estimate of the nucleotide-binding affinity. For the radioactive GTP-bound wildtype, the EC₅₀ of cold ATP was as high as 112.1 μ M while that of GTP was 47.8 μ M (Figure 5B), indicating the high GTP affinity for CvaB-NBD wildtype. Data for ATP-bound wildtype protein (Figure 5A) were not shown due to the low ATP-binding level, which resulted in inaccurate scanning.

However, the double-mutant protein S503A/Q504L showed different competition patterns for the ATP-bound and GTP-bound forms. Non-radioactive ATP and GTP displayed a similar low competitive level (Figure 5C) for the radioactive ATP-bound double-mutant protein. However, for the radioactive GTP-bound double-mutant protein, more than three-fold of non-radioactive ATP was needed compared with that of GTP (Figure 5D). It appeared that the double-mutant

protein S503A/Q504L still slightly preferred GTP over ATP, though the degree of preference was much lower than that of wildtype.

Temperature Effects of Double Mutant on Binding of Nucleotides and ATP/GTPase Activity

The temperature effects of the double-mutant on the binding of nucleotides were assayed by UV cross-linking. As reported previously (33), the UV cross-linking of GTP or ATP by CvaB-NBD decreased with the increase of temperatures, likely due to molecular motion. However, in contrast to the wildtype, the double-mutant protein displayed relatively similar binding levels for ATP and GTP at different temperatures (Figure 6).

At the same time, the ATP/GTPase activities of the double-mutant protein were further tested at different temperatures. As reported previously (33), the ATPase activity in the wildtype CvaB was cold sensitive: the ATPase activity became 8–10 times lower than that of GTPase at 10 °C. However, for the double-mutant protein S503A/Q504L, the ATPase activity at low temperatures increased to a level similar to that of the GTPase activity (Figure 7).

From those temperature-effect assays, it was concluded that the double-mutant protein changed the properties of the wildtype on both the nucleotide binding and hydrolysis, and behaved more closely to that of HlyB-NBD which is of a typical ABC transporter.

DISCUSSION

In bacterial cells, the physiological concentration of ATP is about ten times higher than that of GTP, and it is widely assumed that the ATP-hydrolysis supplies energy to an active ABC translocation process (14–16,27,28). However, our results showed the GTP-binding preference of CvaB-NBD at lower temperatures *in vitro*. This is different from most of NBDs of typical ABC-transporter, such as HlyB (25,27) and MalK (21), although the potential meaning of physiological function is not clear. In this study, we examined the molecular basis of the differential binding of CvaB-NBD to ATP and GTP at low temperatures. The approaches utilized were modeling of the CvaB-NBD bound to different nucleotides to search for potential interactions, and then, site-directed mutagenesis to verify those predictions. Mutations of the traditional nucleotide-binding motifs, such as the Walker A and B sites, and the ABC Signature motif, had little effect on the preference of CvaB-NBD for GTP over ATP (Table 1). On the other hand, the recently identified aromatic stacking region in the Y⁵⁰¹DSQ-loop of CvaB-NBD was predicted to provide two potential hydrogen bonds to GTP, but not to ATP (Figure 3). Amino acid substitutions were designed to remove the two potential hydrogen bonds. Thus, the hydrophilic side chains of Ser⁵⁰³ and Gln⁵⁰⁴ were replaced by the hydrophobic side chains of Alanine and Leucine, respectively, which cannot form hydrogen bonds. Site-directed mutagenesis results indicated that, unlike the wildtype CvaB-NBD, the double-mutant protein S503A/Q504L increased both the binding affinity for ATP (Figure 4 and 6) and the ATPase activities to almost same level as for GTP at low temperatures (Figure 7). We conclude that the Y⁵⁰¹DSQ-loop directly participated in the differential binding of nucleotides and the double mutant S503A/Q504L largely eliminated the high GTP preference of CvaB-NBD at low temperatures by increasing the binding affinity for ATP.

It is worth noting that the double mutant did not decrease the GTP-binding significantly (only about 30% of GTP-binding decrease was observed in Figure 4), which is not as a huge difference as we expected. Hence, we consider the Ser⁵⁰³ and Gln⁵⁰⁴ function to block ATP from binding to the NBD of CvaB, and the replacement of those two residues largely increase the ATP-binding at the lower temperatures. However, the GTP-binding of double mutant is quite intense, compared to NBDs of HlyB and other ABC transporters. Due to the complexity of enzyme-substrate interaction, we suspect that other factors, such as the angle between

aromatic rings of Tyr⁵⁰¹ and nucleotide, may also contribute to the GTP-binding preference of CvaB-NBD, which needs to be further identified.

The aromatic stacking effect has been observed in some crystal structures of ABC transporters, such as human TAP1 (29), MalK (21), and MJ0796 (46), and described by Locher (14), Schmitt (15), and Higgins (16). Site-directed mutagenesis and biochemical studies of the aromatic residue of HisP (47) and MRP1 (multidrug resistance protein) (45) showed the significance of the aromatic ring for the binding of nucleotide. In this study, we provide evidence for specific interactions between residues of the Y⁵⁰¹DSQ-loop and the base ring of different nucleotides using both homology modeling predictions and site-directed mutagenesis, and suggested the mechanism of the highly preferential binding of CvaB-NBD to GTP. Two hydrogen bonds were predicted between the side chains of Ser⁵⁰³ and Gln⁵⁰⁴ and the N² group of GTP, which cannot occur in ATP due to lack of the 2-amino group on the purine ring. The absence of these potential hydrogen bonds may produce little difference in stacking observed in the model between aromatic rings of Tyr⁵⁰¹ and the ATP.

The aromatic stacking loop has been found in many ABC transporters. However, only the aromatic residue (Y or F) is conserved (45,48,49), the other residues are very variable (Figure 2). Possibly, it is the unique sub-domain structure of CvaB YDSQ-loop that differentiates between the base rings of nucleotides ATP and GTP. The replacement of amino acids in the HlyB aromatic region (Y⁴⁷⁷KPD), substituting serine for proline and glutamine for aspartic acid, did not change the trends in ATP/GTP-binding of HlyB-NBD (data not shown). Recently, the dimeric crystal structure of HlyB-NBD was obtained in complex with nucleotide (24). Through docking with different nucleotides in the crystal structure, it was found that the corresponding aromatic stacking region of HlyB-NBD did not display any significant preference for nucleotides. Possibly the reason is that the Pro⁴⁷⁹ and Pro⁴⁸² made a sharp turn in the structure of the aromatic region (Y⁴⁷⁷KPDSPVIL), which might prevent the residues, such as Asp⁴⁸⁰ and Ser⁴⁸¹, from interacting with the GTP base. Thus, the HlyB-NBD was predicted to show similar affinity for both ATP and GTP, consistent with our experimental results, which identified a slight preference for binding ATP (Figure 1). The temperature effects of HlyB-NBD on nucleotide binding and hydrolysis were also characterized (Figure 6 and 7) in comparison with the effects on the wild type and double mutant of CvaB-NBD. The double mutant S503A/Q504L behaved similarly to HlyB-NBD, indicating that the double-mutation of the aromatic stacking region (Y⁵⁰¹DSQ-loop) had changed the GTP-preference of CvaB-NBD. We conclude that sequence and structural differences in the aromatic stacking region are important for the specific nucleotide preference of NBDs.

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Figure 1.

Different nucleotide-binding preferences of CvaB-NBD and HlyB-NBD at 0 °C. The protein amount was indicated by Commassie blue staining (below). The radioactive GTP-bound CvaB-NBD was diluted for 10 and 20 times for comparison with the ATP-bound form.

MsbA-NBD : DVEFRN--VTFTYTPGR-DVPA LRNINIKTPAGKTVALVGRSGSGKSTIASLITR
TAP1-NBD : LVQFQD--VSFAYPNRPDVLVLQGLTFTLRPGEVTA LVGPNNGSGKSTVAALLQN
HlyB-NBD : DITFRN--IRFRYKPD-SPVILDNINLSIKQGEVIGIVGRSGSGKSTLTCLKIQR
CvaB-NBD : PISLETNGLSYRYDSQ-SAPIFSALSLSVAPGESVAITGASGAGKTTLMKVLCG

-- Walker A

Aromatic stacking

MsbA-NBD : FYDIDEGEILMDGHD LREYTLASLRNOVALVSONVHLFNDTVANNIAYARTEQY
TAP1-NBD : LYQFTGGQLLLDGGKPLPQYEHRYLHRQVAAVGQEPQVFG RSLQENIAYGLTQKP
HlyB-NBD : FYIPENGQVLIDGHD LALADPNWLRROVGVVLQDNVLLNRSIIDNIS-LANPGM
CvaB-NBD : LFEFDSGRVLINGIDIRQIGINNYHRMIACVMODRDLFGSGSIRENIC-GFAEEM

-- Q-loop

MsbA-NBD : SREQIEEAARMAYAMDFINKMDNGLD TVIGENGVL LSGGQRQRIAIARALLRDS
TAP1-NBD : TMEEIITAAAVKSGAHSFISGLPQGYDTEVDEAGSQLSGGQRQAVALARALIRKP
HlyB-NBD : SVEKVIYAAKLAGAHDFISELREGYNTIVGEQAGLSSGGQRQRIAIARALVNNP
CvaB-NBD : DEEWMVECARASHIH DVMNMPMGVETLIGELGEG LSGGQKQRIFIARALYRKP

***** ABC Signature Motif

MsbA-NBD : PILILDEATSALDTESERAIQAA LDE LQK--NRISLVIAHRLSTIEKADEITVVV
TAP1-NBD : CVLILDDATSALDANSQLQVEQLLYESPERYSRSVLLITQHLSSLVEQADHILFL
HlyB-NBD : KILIFDEATSALDYESEHVIMRNMHKICK--GRTVIIIAHRLSTVKNNADRIIVV
CvaB-NBD : GILFMDATSALDSESEHFVNVAIKNMNI--TR--VIIA HRETTLRTVDRVISI

***--** Walker B D-loop ***--* H-switch

Figure 2.

Alignment of the NBD sequences of CvaB and other ABC transporters. All NBD protein sequences are from *E. coli* except the TAP1-NBD, which is an endoplasmic reticulum resident protein in *Homo sapiens*. (HlyB: transporter for α -hemolysin; TAP: transporter associated with antigen process; MsbA: transporter for lipid A.) (Note: (1) the grey and dark colors showed the conservation of amino residues of protein sequence; (2) those identical or nearly identical residues in each conserved site are marked by a star *.)

Figure 3.

Predicted interactions between the side-chains of Y⁵⁰¹DSQ-loop and the bases of bound nucleotide ATP (left) and GTP (right). The molecular model was illustrated with the molecular visualization software RasMol (<http://www.umass.edu/microbio/rasmol/>), the color red indicates oxygen, grey is carbon, and blue nitrogen atoms. Dashed lines indicate hydrogen bonds, and dotted lines are stacking interactions, with interatomic distances in Å. A distance cutoff of 3.2Å was used to identify hydrogen bonds.

Figure 4.

The double mutation in the Y⁵⁰¹DSQ-loop changes the preference of CvaB-NBD for binding GTP at 0°C. (A/G: the ratio of binding amount for ATP and GTP normalized by protein amounts).

Figure 5.

Competitive binding assay of CvaB-NBD wildtype and the mutant. Radio-labeled nucleotides (at 0°C) bound to CvaB-NBD or mutant protein were competed at 0°C by cold ATP and GTP, respectively. Concentrations of cold ATP or GTP were 0, 6.25, 12.5, 25, 50, 100, 250, 500 $\mu\text{mol/L}$ from left to right. All samples were exposed for 3 hrs in (A) ATP-bound WT, (B) GTP-bound WT, (C) ATP-bound S503A/Q504L, and (D) GTP-bound S503A/Q504L.

Figure 6. Temperature sensitivity of the binding affinity for ATP (◇) and GTP (■). Data used were average values of three repeats.

Figure 7.

Temperature sensitivity of hydrolysis activity for ATPase (◇) and GTPase (■). The temperature effects were calculated based on the Arrhenius equation, data used were average values of three repeats.

Table 1

Site directed mutagenesis on Walker A & B sites and the Signature motif of CvaB-NBD (G/A Ratio is the average value of nucleotide bound amount of three-time repeats.)

	Walker A	Walker B
CvaB	G- <u>A</u> ⁵²⁷ -S-G- <u>A</u> ⁵³⁰ -G-K- <u>T</u> ⁵³³ -T.....I-L- <u>F</u> ⁶⁵² - <u>M</u> ⁶⁵³ -D-E	
HlyB	G- <u>R</u> ⁵⁰³ -S-G- <u>S</u> ⁵⁰⁶ -G-K- <u>S</u> ⁵⁰⁹ -T.....I-L- <u>I</u> ⁶²⁸ - <u>F</u> ⁶²⁹ -D-E	
Consensus	G- <u>R</u> -S-G- <u>S</u> -G-K- <u>S</u> -T.....I-L- <u>I</u> - <u>L</u> -D-E	
Mutants	Location	G/A Ratio
Wildtype	Wildtype	12.5 ± 0.7
A527R	Walker A	10.2 ± 0.5
A527R-A530S	Walker A	8.9 ± 0.5
T533S	Walker A	13.0 ± 0.5
A527R-T533S	Walker A	12.7 ± 0.9
F652I	Walker B	11.9 ± 0.5
M653F	Walker B	12.9 ± 0.7
M653L	Walker B	12.1 ± 0.5
F652I-M653F	Walker B	11.4 ± 0.9
F652I-M653L	Walker B	11.7 ± 0.6
A527R-F652I-M653F	Walker A & B	10.0 ± 0.5
A527R-T530S-F652I-M653F	Walker A & B	9.4 ± 0.9
G633D	Signature motif	12.1 ± 0.5
Q634H	Signature motif	9.1 ± 0.3
K635A	Signature motif	13.5 ± 0.4