The Q-loop Disengages from the First Intracellular Loop during the Catalytic Cycle of the Multidrug ABC Transporter BmrA*

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The ATP-binding cassette is the most abundant family of transporters including many medically relevant members and gathers both importers and exporters involved in the transport of a wide variety of substrates. Although three high resolution three-dimensional structures have been obtained for a prototypic exporter, MsbA, two have been subjected to much criticism. Here, conformational changes of BmrA, a multidrug bacterial transporter structurally related to MsbA, have been studied. A three-dimensional model of BmrA, based on the “open” conformation of Escherichia coli MsbA, was probed by simultaneously introducing two cysteine residues, one in the first intracellular loop of the transmembrane domain and the other in the Q-loop of the nucleotide-binding domain (NBD). Intramolecular disulfide bonds could be created in the absence of any effectors, which prevented both drug transport and ATPase activity. Interestingly, addition of ATP/Mg plus vanadate strongly prevented this bond formation in a cysteine double mutant, whereas ATP/Mg alone was sufficient when the ATPase-inactive E504Q mutation was also introduced, in agreement with additional BmrA models where the ATP-binding sites are positioned at the NBD/NBD interface. Furthermore, cross-linking between the two cysteine residues could still be achieved in the presence of ATP/Mg plus vanadate when homobifunctional cross-linkers separated by more than 13 Å were added. Altogether, these results give support to the existence, in the resting state, of a monomeric conformation of BmrA similar to that found within the open MsbA dimer and show that a large motion is required between intracellular loop 1 and the nucleotide-binding domain for the proper functioning of a multidrug ATP-binding cassette transporter.

ATP-binding cassette (ABC) transporters form one of the largest protein families in all species and are involved in the cellular uptake or export of an extraordinary variety of substrates, including ions, sugars, lipids, amino acids, organic compounds, peptides, or even proteins. Several human ABC transporters are associated with genetic diseases such as cystic fibrosis and adrenoleucodystrophy, whereas others are responsible for multidrug resistance (MDR) phenotype of cancer cells, namely P-glycoprotein, MRP1, and BCRP (2, 3). In microorganisms as well, many medically relevant members are found, including MDR efflux pumps such as pfMDR1 in parasites, Pdr5p in yeast, and LmrA in bacteria (4). The core structure of ABC transporters is composed of four domains: two transmembrane domains (TMDs), quite divergent in sequence and topology, are involved in substrate translocation, and two nucleotide-binding domains (NBDs) energize the transporters through ATP binding and hydrolysis (5). These four domains are either found on the same polypeptide (full-length transporter) or on separate subunits, from two (half-transporter) to four (1). The NBDs are highly conserved in sequence and show a similar fold regardless of their origin and function (prokaryotic versus eukaryotic and importers versus exporters), suggesting that all ABC transporters share a similar overall mechanism for energy transduction (6). Four high resolution three-dimensional structures of complete ABC transporters are available: the lipid A exporter, MsbA, was crystalized either as an open (EcMsbA) or a closed (VcMsbA and StMsbA) dimer, and the vitamin B12 importer, BtuCD, shows a closed compact structure (7–10). Although the NBD structure in BtuCD agreed well with the tertiary structure of isolated NBDs, the one found in VcMsbA was only partly in agreement with other known structures, showing an unprecedented fold. This new fold was therefore regarded as a possible consequence of crystal packing constraints (6, 11). An even stronger skepticism greeted the EcMsbA structure because the partially resolved NBDs are oriented toward the outside of the dimer interface with an unusually limited TMD interface (6, 11–14). Thus, a complete rotation of the NBDs with respect to the TMDs would be required during the catalytic cycle to reconstitute the two ATP-binding sites at the NBDs interface in a conformation supported by many pieces of biochemical and structural evidence (15–19). The third MsbA structure very recently obtained, StMsbA, appears more consensual than the two previous ones and might reflect a post-hydrolytic conformational state (10).

We have previously shown that the sequence of BmrA (Bacillus multidrug resistance ATP), a new multidrug resistance bacterial half-ABC transporter (20), was akin to that of MsbA; on the other hand, EcMsbA was found to exhibit some drug efflux abilities (21). Here, several three-dimensional models of BmrA were built, using EcMsbA, VcMsbA, or StMsbA structure as template. In contrast to the different three models based on the closed conformation of MsbA (two based solely either on...
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StMsBA or VcMsBA, plus two chimeric ones based on VcMsBA for the TMD and either BtuD or MJ0796 for the dimeric association of the NBDs, the model based on the open conformation of EcMsBA suggested a close proximity between residues of the first intracellular loop, ICL1 (also known as IC1 (7)), that belongs to the TMDs and residues of the so-called Q-loop of NBDs. Introducing two cysteine residues simultaneously in these sequences allowed the formation of disulfide bonds, resulting in an inactive transporter for both drug efflux and ATPase activity, whereas addition of DTT restored both activities. Furthermore, addition of ATP/Mg plus vanadate prevented the disulfide bond formation in a BmrA double mutant, whereas ATP/Mg alone was sufficient when the additional E504Q mutation that abolishes ATP hydrolysis was simultaneously introduced. In the vanadate-trapped state, the cysteine residues introduced in wild-type BmrA could still be cross-linked upon addition of homobifunctional reagents of sufficient lengths. Our results clearly show that during the catalytic cycle of BmrA the NBDs must move away from the TMDs, thereby preventing the disulfide bond formation; this conformational motion between the two domains is likely to play a key role in the catalytic cycle of ABC multidrug transporters.

EXPERIMENTAL PROCEDURES

Unless otherwise stated, all chemicals and restriction enzymes were purchased from Sigma and Promega, respectively.

Site-directed Mutagenesis—pET23b(+)C436S was used as a template for site-directed mutagenesis to introduce a single cysteine using Kunkel’s method as previously described (22). To screen for positive clones, oligonucleotides with the following sequences were designed to introduce the desired mutations and to simultaneously introduce or remove a restriction site without modifying the protein sequence: N118C, ACCGGCTGACTGTGTTCCTCGAAGACCATGTATCGGAAATAA, A119C, GACTGTTTCTCCGGAACATTGATCGGAAAT, and T123C, CGTTACACCGGCTGACACATTTCCTCGAGACTATTGTAT removing an AccIII restriction site; V127C, ACCATGTATGTTGATCAACCGGCTGACGTGT adding a BsrGI (New England Biolabs) restriction site; and C436S containing a single cysteine codon were digested by 5 units of NheI and StuI. Fragments were purified by 0.7% agarose gel electrophoresis.

Preparation of BmrA-enriched Membranes—E. coli membranes vesicles containing over-expressed BmrA were diluted at 5 µg/µl (final volume of 50 µl) in a buffer containing 50 mM Hepes, pH 7.0, 50 mM NaCl, 5 mM MgCl₂. When indicated, membrane vesicles were incubated in the presence of effectors for 20 min at room temperature. Cysteine oxidation was promoted by addition of 200 µM CuSO₄, and samples were incubated for either 1 h on ice or 30 min at 30°C. For the different methanethiosulfonate cross-linkers (Toronto Research Chemicals, Toronto, Canada), membrane vesicles were incubated on ice for 5 min with 200 µM methanethiosulfonate compound. When indicated, free sulfhydryl groups were modified by addition of 10 mM N-ethylmaleimide for 30 min at 25°C. 15 µg of protein were resolved by SDS-PAGE (10%) using a 10% acrylamide gel. After staining with Coomassie Blue R250.

Molecular Modeling—The three-dimensional model of BmrA based on EcMsBA was built by using Geno3D, a comparative molecular modeling program for proteins (25). Protein structures of TAP1 (PDB accession code 1J7F-A; the closest homologue whose structure found in the PDB has been published) and EcMsBA (PDB accession code 1SOQ-A) were taken as templates for molecular modeling of the open monomeric conformation of BmrA. On the basis of sequence identity after sequence alignment with BmrA, distance restraints and dihedral angles were calculated on the template structures. These measurements were performed for all common atoms revealed by alignment of BmrA with the templates. The approach used in Geno3D allows reconstruction of the protein three-dimensional model by fragments. For that, it is necessary that sequence alignment between query protein and templates show partial overlapping sequences. In these segments, the generated restraints resulted from both templates simultaneously. The CNS 1.1 program (26) was used to generate the model by a distance geometry approach similar to that used in modeling from NMR experiments. Each structure was regularized by simulated annealing (2000 steps) and energy minimization (2000 steps). Ten models were built, all exhibiting closely similar features, and were superimposed with the ANTHEPROT three-dimensional package by minimizing the root mean square deviation between α carbons (27). Mirror images were eliminated on the basis of energy calculation. The model retained was that with the lowest energy (−19334 kcal/mol) and regular chemical features, and its quality was assessed with the PROCHECK tools (28) (91% of residues are located in the favorable region of the Ramachandran plot). The visualization of the molecule was performed using PyMol software (Delano Scientific LLC).

Two “closed” BmrA models based solely on the VcMsBA template (PDB accession code 1PF4) were built using either the same program as
for the open BmrA model or, alternatively, the MODELLER program (version 7.5) (29), which allows building models faster than the previous method. Both models gave essentially similar parameters when assessing their quality, and similar distance values were obtained between the two residues replaced by cysteines (only the values obtained from the model made by the MODELLER program are shown in TABLE ONE). Another closed BmrA model was built with the same program based on homology with the recently resolved structure of MsbA from Salmonella typhimurium (PDB accession code 1Z2R). The missing residues in the structures of VcMsbA and StMsbA have been left out in the models of BmrA. Ten models were generated with the MODELLER program from both VcMsbA and StMsbA, and the models with the lowest objective functions were kept. They will hereafter be referred to as ModVc and ModSt.

To build the closed BmrA models with an NBD/NBD interface based on either BtuCD (VcMsbA/BtuCD) or MJ0796 (VcMsbA/MJ0796) NBDs dimers, the NBD of BmrA was first modeled using the NBD of TAP1 as a template. The sequences of BmrA (from Glu-324) and TAP1 were aligned with CLUSTALW (alignment score 532) on the NPS@ web server (npsa-pbil.ibcp.fr), and again 10 models were generated with MODELLER from this alignment. The model with the lowest objective function (1431.7) was retained. Then the NBD model of BmrA was fitted sequentially to each of the two NBDs of the vitamin B12 transporter BtuCD (PDB accession code 1L7V) and of the archaeal ABC transporter MJ0796 (PDB accession code 1L2T), both being crystallized in a closed conformation with a correct positioning of the NBD/NBD interface. The difference between the two structures lies in the fact that BtuCD was crystallized in the absence of ATP (replaced by cyclo-tetra-vanadate) in a “nonenergized” state, whereas the NBDs from MJ0796 were obtained in the presence of ATP/Na⁺. The fit was done by least square superpositioning of the four β strands corresponding to the conserved core β sheet of the NBDs, namely residues Val-369 to Val-373, Ile-417 to Val-420, Ile-498 to Leu-501, and Thr-529 to Ile-533 in BmrA. Backbone atoms only were selected for the fits resulting in root mean square deviations of 1.84 and 1.81 Å for the fit to BtuCD and MJ0796, respectively. (Similar root mean squares were obtained for the fits of the two NBDs.) In the next step, the two NBDs now forming a correct interface were docked as a rigid body onto the TMDs built by homology from Vibrio cholerae (ModVc). This was done by least square superpositioning of the three β strands comprising residues Gly-418 to Ser-421, Ile-498 to Leu-501, and Thr-529 to Ile-533 in BmrA. Backbone atoms only were selected for the fits resulting in root mean square deviations of 3.9 and 3.7 Å for the anchoring of the NBDs based on BtuCD (ModBtu) and on MJ0796 (ModMJ), respectively. Finally, the four models of complete BmrA dimers in the closed state, including residues Lys-9 to Gln-562 (the loop Phe-202 to Leu-236 was left out in the models of BmrA).
energies were as follows: ModVc, \( E_{\text{tot}} = -25280 \) and \( E_{\text{harm}} = 216 \); ModSt, \( E_{\text{tot}} = -25950 \) and \( E_{\text{harm}} = 164 \); ModBtu, \( E_{\text{tot}} = -21481 \) and \( E_{\text{harm}} = 1188 \); and ModMJ, \( E_{\text{tot}} = -25283 \) and \( E_{\text{harm}} = 818 \). The model built from BtuCD appears to be the least stable and shows the largest restraint energy term, whereas the model made from \( S. typhimurium \) is the most stable. Interestingly, the chimeric model built from Vc/MsbA and MJ0796 is more stable by 600 kcal/mol (if we subtract the restraint term) than the model made by direct homology from VcMsbA alone.

**RESULTS**

The fairly good homology found between BmrA and MsbA (20) allowed us to build BmrA models based on either the open or the closed conformation of MsbA. In the open BmrA model, the first intracellular loop of TMD was found very close to the Q-loop of NBD (Figs. 1A and 2A). To experimentally test this model, double mutants were created where two cysteine residues were simultaneously introduced into a fully

| Residues | S-S bond formation | Distances between the residues (Å) |
|----------|--------------------|-----------------------------------|
|          |                    | Open BmrA (EcMsbA) | Closed BmrA (VcMsbA) | Closed BmrA (VcMsbA/BtuD) | Closed BmrA (VcMsbA/MJ0796) | Closed BmrA (StMsbA) |
| Ser-424  | Asn-118            | +                   | 3.5                  | 18.4                  | 11.6                  | 13.8                  | 9.1                  |
| Ala-119  | +                   | 4.9                  | 22.1                  | 17.3                  | 18.7                  | 20.9                  | 13.7                  |
| Thr-123  | −                   | 7.1                  | 28.7                  | 18.0                  | 20.9                  | 15.3                  |                      |
| Met-427  | Val-124            | −                   | 8.2                  | 22.6                  | 13.8                  | 20.3                  | 18.1                  |
| Val-127  | −                   | 4.1                  | 17.8                  | 11.3                  | 17.6                  | 14.9                  |                      |
| Ser-428  | Thr-123            | ++                  | 3.8                  | 21.75                 | 12.0                  | 19.1                  | 15.6                  |
| Val-124  | +                   | 4.2                  | 28.3                  | 18.0                  | 25.4                  | 20.7                  |                      |
| Val-127  | −                   | 9                   | 24.0                  | 13.7                  | 21.9                  | 15.0                  |                      |

(96.5, 96.7, 95.9, and 96.7% for ModVc, ModSt, ModBtu, and ModMJ, respectively).
The functional cysteine-less BmrA mutant (24). The two residues mutated at the same time by a cysteine residue (Fig. 1, B and C) were separated by a distance in the open model (TABLE ONE). The double mutants thus created were S424C/N118C, S424C/A119C, S424C/T123C, M427C/V124C, M427C/V127C, S428C/T123C, S428C/V124C, and S428C/V127C. Interestingly, with the exception of M427C/V127C, all the double mutants predicted to be separated by less than 5 Å in the model, namely S424C/N118C, S424C/A119C, and S428C/V124C, were indeed able to form a disulfide bond in the presence of slightly oxidizing conditions. The fourth one, S428C/T123C, separated by 3.8 Å in the model, was even able to form a disulfide bond spontaneously without the addition of cupric ion as a catalyst. By contrast, the three double mutants whose residues were predicted to be separated by more than 7 Å in the open BmrA model were totally unable to form a disulfide bond (TABLE ONE).

Incubation of the S428C/T123C double mutant in a buffer deprived of oxygen almost totally prevented the S–S bond formation, whereas incubation with increasing concentrations of cupric ion progressively transformed the reduced cysteine residues into a disulfide bond (Fig. 3A). A further addition of dithiothreitol fully restored the cysteine residues to the reduced form. The ability to create a disulfide bond in several double mutants indicates that the two cysteine residues must be separated by only a few Å in the three-dimensional structure of BmrA. This validates our BmrA model and, consequently, suggests that the template used to create our model, i.e. the conformation of the monomer of MsbA within the EcMsbA open dimer, is valid as well. Indeed, three additional BmrA models were built, one based solely on the closed structure of VcMsbA and two chimeric ones based on VcMsbA for the transmembrane domain and on the three-dimensional structure of either BtuD or MJ0796 for the two NBDs. These two latter models allow positioning of the two ATP-binding sites at the NBDs interface, a feature generally assumed to reflect a conformation competent for the transport (6, 31). According to each of these three closed models, a comparison of the distances between the two residues chosen to be replaced jointly by two cysteine residues clearly ruled out the possibility that disulfide bonds would have been formed in either additional model (Fig. 2, B–D and TABLE ONE). Furthermore, using a fourth BmrA model based on the latest MsbA closed structure released (StMsbA) also revealed that the distances between the two cysteine residues would be incompatible with S–S bond formations (Fig. 2E and TABLE ONE).

The formation of disulfide bonds had strong consequences on the functioning of the transporter. It abolished the Hoechst transport activity, whereas restoring the reduced forms of BmrA by a subsequent addi-
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A large body of evidence, including modification in tryptic digestion pattern (32, 33), quenching of the Trp intrinsic fluorescence (34), and deuterium/hydrogen exchange profiles (35), has shown that global conformational changes are associated with the catalytic mechanism of ABC transporters. Accordingly, large domain movements have been detected in the low resolution three-dimensional structures of P-glycoprotein multidrug exporter (36, 37). On the other hand, based on three-dimensional structures of several NBDs either empty or loaded with different nucleotides (i.e. ATP or ADP), the Q-loop was proposed to be one of the most mobile elements of this domain (38, 39), and this was further supported by molecular dynamics studies of HisP (40, 41). Here, the physical displacement of the Q-loop of the NBD relative to ICL1 is directly demonstrated; furthermore, it is shown that this motion is mandatory for the proper functioning of wild-type BmrA. Indeed, ATP/Mg is thought to induce the dimerization of the two NBDs in a conformation where the two ATP-binding sites are shared between the NBDs (18, 36, 37), while addition of vanadate freezes the transporter in a subsequent transition state conformation (16). In the latter conformation, a reduction in the S–S bond formation was detected in the wild-type BmrA, indicating that the Q-loop has disengaged from ICL1. This result was exacerbated when the additional E504Q mutation was present in the double mutant; moreover, it was even observed in the absence of vanadate. This glutamate residue has been proposed to be the catalytic base of ABC transporters (18, 43, 44), and accordingly this mutation was shown to totally abolish ATP hydrolysis in BmrA (22). In fact, mutation of this Glu-504 in several residues, including the E504Q one, was also shown to induce a vanadate-insensitive tight trapping of ATP/Mg, most

FIGURE 5. The cross-linking of the two cysteine residues in the double mutant is still allowed, in the presence of ATP/Mg plus vanadate, by some homobifunctional reagents. A, 200 μm methanethiol sulfonate cross-linking reagent of different lengths was added to an ice-chilled suspension of E. coli membrane vesicles containing the overexpressed S428C/T123C double mutant either preincubated or not with 0.5 mM Vi plus 5 mM ATP/Mg at 25 °C for 30 min. B, free SH groups were derivatized by 10 μm N-ethylmaleimide, and samples were analyzed by SDS-PAGE without reducing agent. The white arrowhead indicates the position of BmrA with no intramolecular cross-link; the black arrowhead shows the position of BmrA containing an intramolecular cross-link. Panel A was adapted from Ref. 58.

FIGURE 6. Putative scheme to explain the disulfide bond formation, or prevention, in the BmrA double mutants. This model is based on Chang's model (9), with one ATP molecule shown in color at the interface of the NBDs.
likely at the NBDs interface of BmrA (22). This interpretation was consistent with the effects produced by equivalent mutations in other ABC transporters, i.e., a drastically altered ATP hydrolysis and a tight trapping of nucleotides (18, 42, 45–47). Therefore, it is reasonable to assume that ATP/Mg binding induces the dimerization of the two NBDs in BmrA, thereby moving the NBD away from ICL1 and thus preventing the disulfide bond formation. ICL1 would therefore act as a central pivot with the NBD moving around this TMD subdomain, conceivably by a hinge-like motion (48) that might involve as well a swiveling motion (9). In this way, the interaction between ICL1 and the Q-loop would be maintained throughout the catalytic cycle. This would be consistent with the three-dimensional structure of BtuCD, where the Q-loop is shown to mediate most of the NBD interactions with the TMD, and more precisely with the L-loop in this domain (also known as the “EAA” loop (49)) in a tightly packed conformation with the two ATP-binding sites located at the NBDs interface (8). The L-loop, which has been proposed to be functionally equivalent to ICL1 of MsbA (50) and to the N-terminal domain of the DrrB subunit of the daunorubicin/doxorubicin transporter (51), plays a key role in the function of ABC importers, and its interaction with the Q-loop is mediated by nucleotides (49, 52). This assumption also agrees, on the one hand, with the molecular dynamics studies of HisP and, on the other hand, with the conformations of the NBD loaded with different nucleotides (i.e., ATP or ADP), both showing that the Q-loop is one of the most mobile elements of this domain (38, 40).

As emphasized previously, one cannot accurately determine the displacement of the NBD from ICL1 induced by ATP-Mg binding (see Fig. 5). However, the stably trapped BmrA conformation thus generated either by the presence of vanadate or by the additional E504Q mutation is consistent with the two chimeric models based on either BtuD or MJ0796 three-dimensional structures, where the two ATP-binding sites are positioned at the NBDs interface (18). According to these two models and whatever the double mutants considered, the estimated distances between the two cysteine residues in an ATP/Mg bound conformation of BmrA would be at least 11.3 Å, a distance too long to allow a disulfide bond formation. On the other hand, according to the BmrA model solely based on the closed VcMsbA, no distances shorter than 18.4 Å would be expected between two cysteine residues. Recently, a new structure of MsbA was published that probably corresponds to a post-hydrolytic step (ADP + vanadate present in one of the NBDs) (10). Using this new structure as a template to model BmrA gave essentially the same results for the distances between the newly introduced cysteine residues as those presented in the table for the hybrid models: the shorter distance, 9.1 Å, was obtained for the double mutant S424C/N118C, whereas all other double mutants gave distances above 13.6 Å. Moreover, this new model also explains the prevention of disulfide bond formation observed for the double mutant in the presence of ATP/Mg plus vanadate (see Fig. 4; transition state akin to the new MsbA structure related to MJ0796 with two ATP/Mg plus vanadate (see Fig. 4; transition state akin to the new MsbA structure)), thereby lowering the likelihood of forming a disulfide bond between ICL1 and NBD. The great flexibility and possibly the rotation of NBDs relative to TMDs could be a peculiar feature of half-ABC transporters because both domains are only tethered through a long connecting loop that precedes the NBD. This loop appears quite flexible in MsbA (9), and it might act as a loose “leash” to maintain the NBD in close proximity to the TMDs without restraining its motion. Throughout the different conformations of the half-ABC transporter, contacts between NBDs and TMDs might be secured by ICL1 that would thus play a central role in interconnecting these two domains, as evidenced from either mutational (54–56) or structural studies (7, 9). By contrast, in full-length transporters such as Pgp, or even in ABC importers where the different domains are borne on separate polypeptides, the NBDs motion might be more restricted (57) and, for instance, swing back and forth in a tweezers-like motion (19) without any swiveling motion.

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