Substrate Binding Stabilizes a Pre-translocation Intermediate in the ATP-binding Cassette Transport Protein MsbA*

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Background: During substrate transport, ATP-binding cassette exporters switch between an inward-facing and an outward-facing state in a nucleotide-dependent fashion.

Results: Substrate binding to bacterial MsbA initiates dimerization of nucleotide-binding domains without opening the membrane domains at their external side.

Conclusion: Substrate binding to MsbA stabilizes an “inward-facing closed” pre-translocation state that binds ATP.

Significance: Our observations suggest a fundamental mechanism by which substrates stimulate ATP hydrolysis.

ATP-binding cassette (ABC)3 exporters are found in all living organisms and are responsible for the active efflux from the cell of a wide range of molecules such as ions, drugs, lipids, peptides, and proteins. Their dysfunction has been linked to many disease conditions, including multidrug resistance, cystic fibrosis, Stargardt disease, age-related macular degeneration, adrenoleukodystrophy, Tangier disease, Dubin-Johnson syndrome, and progressive familial intrahepatic cholestasis. All ABC exporters share a conserved dimeric architecture in which each monomer is composed of a nucleotide-binding domain (NBD) and a cognate membrane domain (MD) (1). For multidrug ABC transporters, the structural conservation between the mammalian full-transporter ABCB1 and bacterial half-transporter homologs LmrA, Sav1866, and MsbA translates into functional similarities, including overlapping substrate specificities (2–5).

MsbA functions as a homodimer and has a recognized role in mediating lipid A export in Escherichia coli (6). Two MsbA orthologs were recently crystallized in distinct but complementary conformations as follows: nucleotide-free inward-facing (E. coli MsbA) and AMP-PNP (a nonhydrolyzable ATP-analog)-bound outward-facing (Salmonella typhimurium MsbA); inward and outward refer to cytoplasmic and periplasmic side of the plasma membrane, respectively (7). ADP\(\cdot\)V, (hydrolysis intermediate)-trapped MsbA was also observed in the outward-facing conformation, similar to the AMP-PNP-bound structure (7). Biochemical verifications of these conformations include electron paramagnetic resonance (EPR) studies on MsbA (8–10) and LmrA (11), hydrogen/deuterium exchange-mass spectrometry on BmrA (12), inter-molecular cysteine cross-linking on MsbA in membrane vesicles (13, 14), and most recently, by luminescence resonance energy transfer on MsbA (15).

These studies provide an overall view of the dynamic movements associated with an ATP hydrolysis (ATPase) cycle that is in agreement with an alternating access model of transport (1, 7, 16). Briefly, this model suggests that an ABC exporter alternates between an inward-facing and outward-facing conformation to present the substrate-binding pocket in the MDs to the inside and outside of the cell, respectively. These movements are guided by the binding and hydrolysis of ATP that cause alternating dimerization and dissociation, respectively, of the two NBDs (1). Although compelling, the current model does not address a fundamental property of ABC exporters, namely the substrate-dependent stimulation of the ATPase activity (17–20). Our understanding of this phenomenon requires insight into the conformation adopted by an ABC exporter upon substrate binding, and its relation to the cycle of ATP binding and hydrolysis at the NBDs.

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3 The abbreviations used are: ABC, ATP-binding cassette; MD, membrane domain; NBD, nucleotide-binding domain; TM, transmembrane helix; CuPhe, copper phenanthroline; TNP-ATP, 2’3’-O-(2,4,6-trinitropheryl)adenosine-5’-triphosphate tetra(triethylammonium); AMP-PNP, adenosine 5’-(β,γ-imido)triphosphate; V, sodium orthovanadate; NEM, N-ethylmaleimide; ISOV, inside-out membrane vesicle; a.u., arbitrary unit.
At present, detailed structural information regarding substrate binding by ABC transporters is limited. Mouse ABCB1a was recently crystallized with cyclic peptide inhibitors (21). Although the peptide-bound ABCB1a structures identify a substrate-binding surface (1, 21, 22), these structures appear identical to the drug/nucleotide-free inward-facing ABCB1a and MsbA conformations. The suggestion that the protein structure is unaltered when progressing from a substrate/nucleotide-free apo-state to a substrate-bound state is inconsistent with previously published biochemical data, nearly all of which point to a measurable conformational change (19, 23–27). Furthermore, it is unclear how an inward-facing conformation with disengaged NBDs could accelerate ATP hydrolysis.

We recently found that the disruption of molecular contacts within a structurally conserved element, termed the tetrahelix bundle, inhibits the formation of a stable, ATP-bound, outward-facing state of E. coli MsbA (14). In this previous study, we used two cysteine reporters in MsbA-cl (cysteine-less) to measure nucleotide-dependent conformational changes, E208C (reports NBD dimerization) and A281C (reports the separation of two groups of TMs (TM1 and -2, TM3, -4', -5', and -6' separate from TM1' and -2' and TM3, -4, -5, and -6)) during “wing” formation in the MsbA dimer.

Here, we have conducted cysteine cross-linking, Förster/fluorescence resonance energy transfer (FRET), and cysteine accessibility studies on these reporters to biochemically study substrate binding-induced conformational changes in MsbA. We have further performed substrate-binding assays and substrate-stimulated ATP binding and ATPase measurements to assign a role for substrate binding in the current alternating access model for substrate transport.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Lactococcus lactis strain NZ9000 ΔlmrA ΔlmrCD, which is devoid of the major endogenous multidrug transporters LmrA and LmrCD, was used as a host for pNZ8048-derived plasmids; E. coli strain XL1 Blue was a host for the pGem-5Zf(+) (Promega) cloning vector. _L. lactis_ and _E. coli_ cells were grown in M17 (Oxoid) and LB (Formedium) medium, respectively, as described previously (28).

**Site-directed Mutagenesis**—Construction of N-terminal His₆-tagged cysteine-less MsbA (MsbA-cl), E208C MsbA-cl, and A281C MsbA-cl has been reported (13). pGEM-A281C MsbA-cl was used as template to generate the E208C/A281C MsbA-cl double mutant. The gene was subcloned as an NcoI-Sacl fragment into pNZ8048 downstream of the nisin A-inducible promoter, yielding pHN-E208C/A281C MsbA-cl. The mutated _msba_ gene was sequenced to confirm that only the intended mutations were introduced.

**Inside-out Membrane Vesicles**—The expression of MsbA proteins in lacticoccal cells, and the preparation of inside-out membrane vesicles (ISOVs) in 100 mM K-HEPES buffer, pH 7.0, were performed as reported previously (28).

**Cysteine Cross-linking**—Cysteine cross-linking on the E208C, A281C, and E208C/A281C mutants was performed in ISOVs as reported previously (13). For each cross-linking reaction, ISOVs were diluted to 5 μg of protein/μl in 100 mM K-HEPES buffer, pH 7.0, containing 5 mM MgSO₄ in a reaction volume of 90 μl in microcentrifuge tubes. To reduce background signals due to cross-links formed before nucleotide addition, 0.5 mM dithiothreitol (DTT) was added, and the samples were incubated at 20 °C for 2 min. Nucleotides (2 mM ADP, 2 mM AMP-PNP, or 2 mM ATP plus 2 mM sodium orthovanadate, referred to as ADP-V₅) or substrates (Hoechst 33342, verapamil, lipid A) or an equal volume of solvent was added following incubation at 20 °C for 5 min. The cross-linking reactions were initiated by the addition of 0.5 mM copper phenanthroline solution, which was added from a 50 mM stock made by mixing CuSO₄ and 1,10-phenanthroline in a ratio 1:4 (w/w) in ultrapure H₂O. The tube lids were pierced, and the reaction was allowed to occur for 5 min in a 30 °C shaker incubator (model 3032, GFL Gesellschaft für Laborteknik mbH, Germany) at 200 rpm. The reactions were stopped by the addition of excess (10 mM) of the thiol alkylator N-ethylmaleimide (NEM) (added from a freshly prepared stock of 100 mM in ultrapure H₂O) and incubation at 20 °C for 1–2 min. Next, 10–15 μg of ISOVs from the cross-linking reactions were mixed with 5× SDS sample loading buffer devoid of DTT and separated on an SDS-PAGE without any incubation. Band intensities on Western blots were compared by densitometry analyses using ImageJ software version 1.43 (National Institutes of Health).

**FRET**—The purification of His-tagged MsbA, as described previously (28), was modified as follows. For each experiment, after the overnight binding step the Ni²⁺ affinity resin was washed once with 3 ml of buffer B and suspended in 3 ml of buffer B. Then 3.3 μM Atto 590 + 3.3 μM Atto 665 (Atto-tec, Germany; prepared as 20 mM stocks in DMSO) were added, and after incubation on ice for 5 min, 1 μl of copper phenanthroline (prepared as above) was added, and the sample was incubated on a rotating wheel for 1 h at 4 °C. Subsequent wash and elution steps were identical to the above-mentioned. 7–10 μg of the labeled protein per measurement and ligands, 2 mM ATP plus 2 mM sodium orthovanadate or 2 mM ADP or 40 μg/ml lipid A or equal volume of DMSO, were used in a 2-ml reaction set up in SOG cuvettes. The LS 55B luminescence spectrometer (PerkinElmer Life Sciences) settings were as follows: excitation at 594 nm, slit width 5 nm, emission 600–800 nm, slit width 10 nm, scan speed 500 nm/min, stirrer “low.” All measurements reported here have been made after 10 min of stirring.

**Cysteine Cross-linking and Accessibility**—For cysteine cross-linking combined with Atto590 labeling, ISOVs were diluted to 7 μg of total membrane protein/μl in 100 mM K-HEPES buffer, pH 7.0, containing 5 mM MgSO₄ in a reaction volume of 90 μl in microcentrifuge tubes. Nucleotides (2 mM AMP-PNP) or substrates (Hoechst 33342, verapamil, or lipid A or an equal volume of DMSO) were added following incubation at 20 °C for 5 min. 20 μM Atto590 was then added (from a 2 mM stock in DMSO), and after incubation at room temperature for 5 min, cross-linking reactions were initiated by the addition of 0.5 mM copper phenanthroline solution (prepared as above). After piercing their lids, the tubes were kept in a 30 °C shaker (200 rpm) incubator for 15 min. For E208C, prior to the addition of nucleotides, the ISOVs were incubated for 3 min with 0.5 mM DTT. After incubation with nucleotides for 5 min, 0.5 μM copper phenanthroline was added; the tube lids were pierced, and samples were placed in a 30 °C shaker (200 rpm) incubator for 5
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min. Next, 20 μM Atto590 was added, and the reaction was further continued in a 30 °C shaker (200 rpm) incubator for 10 min. All reactions were stopped by the addition of 10 mM NEM, followed by incubation at 20 °C for 1–2 min. Subsequently, 25 μg of ISOVs from the cross-linking reactions were mixed with 3–5× SDS sample loading buffer devoid of DTT and separated on an SDS-PAGE without any incubation. Gels were first viewed under UV light to detect the Atto590-labeled protein bands and subsequently Coomassie Brilliant Blue-stained to visualize all protein bands. Monomer intensities from UV images and dimer intensities from Coomassie Brilliant Blue-stained gel scans were subjected to densitometry analyses using the ImageJ software, version 1.43 (National Institutes of Health).

Ethidium Transport—Ethidium transport in intact cells was measured by fluorimetry as described (28).

Hoechst 33342 Binding—Binding of Hoechst 33342 to purified MsbA in solution was detected fluorimetrically (28). MsbA cysteine single/double mutants or MsbA-cl was purified as described before (28) with the following modifications. After the overnight binding step, the resin was washed once with buffer B and resuspended in 3 ml of buffer B. 16 μM CuPhen was added, and the suspension was incubated on the rotating wheel for 1 h at 4 °C, before proceeding with the washes and elution as described above. 10–12 μg of purified pre-cross-linked protein was diluted in 2 ml of assay buffer (100 mM KPi, pH 7.0, 5 mM MgSO4), and an equal volume of the elution buffer diluted in the assay buffer was used as a negative control. Hoechst 33342 was added as follows: 2×10 μl of 5 μM Hoechst 33342, 2×10 μl of 25 μM Hoechst 33342, followed by 10–μl additions of 50 μM Hoechst 33342 until a plateau in the fluorescence intensity was obtained. All steps of Hoechst 33342 were separated by 30 s.

Fluorescence was monitored on an LS 55B luminescence spectrometer (PerkinElmer Life Sciences) using excitation and emission wavelengths of 407/610 Å and 407/630 Å for this pair of dyes was deemed suitable with or without 50, 100, or 200 μM verapamil. TNP-ATP (Molecular Probes, Invitrogen) was added as follows: 2×5 μl of 100 μM TNP-ATP, followed by 5-μl additions of 500 μM TNP-ATP until a plateau in fluorescence emission was obtained. All increments were separated by 30 s. Equal volume of elution buffer was used as negative control. Fluorescence was monitored in an LS 55B luminescence spectrometer (PerkinElmer Life Sciences) using excitation and emission wavelengths of 407 and 535 nm, and slit widths of 10 and 5 nm, respectively. Fluorescence intensities of the elution buffer control were subtracted.

TNP-ATP Binding—WT MsbA was purified as described (28), and 40 μg of protein was diluted in a 2-ml reaction buffer (50 mM KP, pH 7.0, 10% glycerol, 0.01% n-dodecyl-β-D-maltoside) with or without 50, 100, or 200 μM verapamil. TNP-ATP was added as follows: 2×5 μl of 100 μM TNP-ATP, followed by 5-μl additions of 500 μM TNP-ATP until a plateau in fluorescence emission was obtained. All increments were separated by 30 s. Equal volume of elution buffer was used as negative control. Fluorescence was monitored in an LS 55B luminescence spectrometer (PerkinElmer Life Sciences) using excitation and emission wavelengths of 407 and 535 nm, and slit widths of 10 and 15 nm, respectively.

Substrate-stimulated ATPase Activity—ATPase activity of purified MsbA was measured using the malachite green colorimetric method (13). Briefly, for each data point, 1–5 μg of protein was added to 100 mM K-HEPES buffer, pH 7.0, with 5 mM MgSO4, containing either 100 μg/ml lipid A (Sigma, diposphoryl from E. coli F583 (Rough strain lipopolysaccharide (Rd) mutant); prepared as a 1 mg/ml stock in anhydrous DMSO) or equal volume DMSO (anhydrous, Sigma) for 5 min on ice. For incubations with verapamil, 10 μM of the compound was added instead of lipid A. Then the mixture was added to a range of ATP concentrations, 0–8 mM (high grade ATP from Sigma), in a total volume of 60 μl at 4 °C. The assay was then incubated for 5 min at 30 °C, after which A600 was determined following the addition of malachite green-ammonium molybdate, freshly activated with 0.1% Triton X-100.

Curve-fitting and Statistical Analysis—Graphs were plotted using Origin Pro 8 (OriginLab). Equilibrium Hoechst 33342 binding, TNP-ATP binding, and ATPase kinetics data were fit using the built-in hyperbola function (y = P1+x/P2 + x). P1 and P2 obtained for the resulting fits were designated Pmax (maximum binding)/Vmax (maximum rate of hydrolysis) and Kd (dissociation constant)/Km (ATP hydrolysis affinity constant), respectively. All nonlinear fits were subjected to the built-in fit statistics, including reduced χ2 and adjusted R2 to determine the fit status. Data from FRET, Hoechst 33342 binding, and ATPase kinetics were statistically analyzed using the Student’s t tests, in which p < 0.05 was taken as significant (*), and p < 0.04 as highly significant (**).

RESULTS

Substrate Binding to MsbA Causes NBD Dimerization—We previously described the use of inter-molecular cysteine cross-linking between E208C and E208C’ in the cysteine-less MsbA (MsbA-cl) homodimer in ISOVs to dissect the conformational changes associated with the ATPase cycle in the absence of added transport substrates (13, 14, 16). Here, we used this assay to test the effect of typical MsbA substrates, including Hoechst 33342 and verapamil and the physiological substrate lipid A (2, 3, 5, 6, 26, 28), in the absence of added nucleotides. Upon the exposure of the E208C mutant to these substrates at room temperature prior to performing oxidative cross-linking, significant concentration-dependent increases in the proportions of cross-linked dimers were observed (Fig. 1, a–c). As the proportion of E208C cross-links reports the conformational movements associated with the dimerization of the NBDs (13), our results suggest that substrate binding to MsbA causes a closure of the NBDs.

To test the effect of substrate on conformational changes in the MsbA dimer by an alternative technique, we applied a FRET-based method to purified protein. In this method, the Ni2+ affinity resin-bound, His6-tagged E208C/E208C’ MsbA-cl homodimer was labeled through an oxidative cross-linking reaction with a FRET pair of thiol-reactive dyes (donor (D), Atto590, and acceptor (A), Atto665; termed E208C-Atto590-Atto665). The predicted Förster distance (R0, the distance at which FRET is 50% efficient) of ~70 Å for this pair of dyes was deemed suitable for our purpose, because the nucleotide-free, inward-facing crystal structure of E. coli MsbA predicts a distance of ~55 Å between Glu-208 and Glu-208’ (7). The washed and eluted probe-labeled protein samples were analyzed by SDS-PAGE. As per the manufacturer, and as confirmed in our experiments, D (but not A) absorbs strongly in the UV region (260–280 nm) and is fluorescent at 610–630 nm (Fig. 2, a and b). The observation that the UV fluorescence of D, when reacted with the E208C mutant in the presence of equimolar amounts of A, was reduced to half that in the absence of A, led us to conclude that D and A were equally reactive with an incorporation at an
approximate ratio of 1:1 when applied together at equal concentrations (Fig. 2, b and c). Incubation of E208C labeled with D or A or D and A (termed E208C-D, E208C-A, and E208C-DA, respectively) with the oxidizing agent CuPhen did not yield any increase in cysteine cross-linking or A labeling efficiency (Fig. 2, a and b). This indicated that the oxidation reaction was complete, i.e. no free cysteine residues and unreacted probe molecules remained in the eluted protein samples. Upon exciting E208C-D, E208C-A, or E208C-DA at the excitation maximum for D (594 nm) and following the emitted fluorescence over 600–800 nm, we found a strong signal at ~620 nm for E208C-D but not for E208C-A (Fig. 2, d and e). The fluorescence intensity for E208C-DA at ~620 nm was reduced to about 0.5–0.75 times that of E208C-D, although an additional signal at ~680 nm was observed that corresponded to the emission region of A (Fig. 2, d and e). These results point to the transfer of fluorescence energy from D to A in E208C-DA. Our control experiments revealed that the FRET signal at 680 nm could only be obtained with E208C-DA, as opposed to background signals for the elution buffer, the dyes D and A free in the elution buffer solution, or MsbA-cl incubated with D and A (Fig. 2, d and e). Taken together, the findings suggest that, although labeling of the E208C/E208C’ MsbA dimer with D and A will yield dimers populations that contain AA (25%), DD (25%) or DA or AD (50%), FRET signal is only obtained for the latter populations containing both A and D.

To validate the use of FRET for detection of conformational changes as a complement to cysteine cross-linking at position Glu-208 in MsbA, FRET signals for E208C-DA were tested in the presence of ADP, ADP, and ADP (Fig. 3). These were found to be in excellent agreement with the cysteine cross-linking results reported for E208C (13). Namely, ADP-bound trapped E208C-DA yielded a significantly higher FRET intensity compared with the no-nucleotide control, in significant contrast to ADP binding (p < 0.04, Fig. 3), suggesting that purified MsbA, similar to MsbA in ISOVs, is in the outward-facing conformation when trapped with ADP, although it is in the inward-facing state with ADP alone.

When 40 µg/ml lipid A was added to E208C-DA instead of the substrates, and in the absence of added nucleotides, we also observed a significant increase in the FRET signal compared with the DMSO (solvent) control or the ADP-bound condition (p < 0.04 for both comparisons, Fig. 3). This observation confirmed our conclusions from substrate-responsive E208C cross-linking (Fig. 1, a–c), which point to substrate binding-dependent NBD dimerization. Furthermore, we also conducted
control experiments where MsbA-cl mixed with DA was incubated with lipid A or where E208C-A was incubated with ADP-V, ADP, or lipid A, but all these spectra were near-background (Fig. 2f), suggesting that the inclusion of nucleotides or lipid A did not produce any major background interferences in the region where FRET was observed for E208C-DA, i.e. 680 nm. The only fluorescent signals contributed by lipid A were found to be near ~600 nm (Fig. 2f). Taken together, our cross-linking and FRET data on E208C suggest that substrate binding to the MsbA dimer brings the NBDs into close proximity.

**Substrate Binding to MsbA Does Not Cause MD Separation**—NBD closure has previously been reported for the steps of ATP hydrolysis.
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FIGURE 4. Substrate binding by MsbA does not cause MD separation. a and b, cysteine cross-linking-Atto590 labeling with A281C, which is located at the extracellular side of the MDs. We previously used this reaction to detect nucleotide-dependent conformational changes in the MsbA dimer in ISOVs (14). Here, we did not observe any marked changes in the proportion of dimers (D, dimers visualized on Coomassie Blue-stained SDS-polyacrylamide gels) or Atto590-labeled monomers (M, monomers visualized through in-gel UV fluorescence from Atto590) when increasing concentrations of the substrates verapamil, Hoechst 33342, or lipid A, or the solvent controls were included in the reaction instead of nucleotides. In contrast, incubations with AMP-PNP served as a robust positive control for the detection of MD separation in the outward-facing state, and gave clear alterations in dimer and monomer band intensities. c, densitometry on dimer (left) and monomer (right) signals is presented as -fold change in intensity relative to the no substrate/nucleotide control, which was taken as 1 in each experiment (**, p < 0.04 AMP-PNP versus verapamil/Hoechst 33342/lipid A; n = 3, mean ± S.E.).

binding and ATP hydrolysis in the catalytic cycle of MsbA (7, 8, 10–16). However, ATP binding and ATP hydrolysis were also reported to cause MD separation in MsbA and homologs (8, 9, 11, 12, 29). To investigate the effects of substrate binding on the conformation of MsbA at the MDs, we used our previously described cysteine reporter at the extracellular end of the MDs, A281C in MsbA-cl.

We have successfully used A281C at the extracellular end of TM6 in MsbA-cl to report nucleotide binding-dependent conformational changes in the MDs by measuring its accessibility to the thiol-reactive probe Atto590 in conjunction with cysteine cross-linking (14). In this assay, an increase in cysteine cross-linked dimers suggests MD closure/inward-facing MsbA, whereas an increase in Atto590-labeled monomers, detected through the UV fluorescence of Atto590 (explained under “Substrate Binding to MsbA Causes NBD Dimerization”), reports MD separation/outward-facing MsbA.

When we performed this cysteine cross-linking Atto590-labeling assay using A281C with substrates in the absence of added nucleotides, we observed no major changes in cysteine cross-linked dimers or Atto590-labeled monomers (Fig. 4, a–c). We used the incubation with AMP-PNP as a control in this experiment, where the inward- to outward-facing conformational shift was successfully detected (14). Our results suggest that as opposed to the dimerization near the NBDs caused by substrate binding (Figs. 1 and 3), the MDs do not undergo a detectable conformational change at the same substrate concentrations (Fig. 4). This observation distinguishes the substrate binding-led conformation from the nucleotide-dependent conformations reported previously, e.g. AMP-PNP/ADP-V, binding gives NBD dimerization and concomitant MD separation (8–14). We further confirmed that the disparity in substrate-responsive cross-linking observed between E208C and A281C was not due to the use of Atto590, by reproducing some of the substrate-dependent data by crossing-over methods, i.e. using cysteine cross-linking accessibility for E208C (Fig. 5, a and b) and using cysteine cross-linking alone for A281C (Fig. 5, c and d).

Taken together, our data from the studies on the two reporters, E208C and A281C, suggest that the substrate-bound MsbA dimer exists in an inward-facing closed conformation, in which both NBDs and MDs are in close proximity.

Hoechst 33342 Binding to MsbA Is Preferred in Inward-facing Closed Conformation—To further study the inward-facing closed state, we introduced both the cysteine mutations in MsbA-cl to create the double mutant E208C/A281C, which was expressed at a similar level as MsbA-cl (Fig. 6a) and which, in the absence of cysteine cross-linking, was equally active in ethidium efflux activity as MsbA-cl (Fig. 6b).

A281C-A281C’ cross-links in MsbA-cl are highly intense in the inward-facing conformation of MsbA under nucleotide-free or substrate-bound conditions (Fig. 5), compared with the nucleotide-bound conditions. In contrast, we previously found that E208C-E208C’ cross-links are less intense in the nucleotide-free inward-facing conformation (13, 14). Thus, we predicted that in the absence of added nucleotides, the double mutant E208C/A281C could possibly exist as two kinds of inward-facing species, either as A281C-A281C’ single cross-linked (inward-facing “open”) or E208C-E208C’ + A281C-A281C’ double cross-linked (inward-facing “closed”). We per-
formed equilibrium Hoechst 33342 binding on purified and pre-cross-linked single cysteine mutants, E208C and A281C, and double cysteine mutant E208C/A281C, alongside MsbA-cl. The results revealed that the dissociation constants ($K_d$) for substrate binding to E208C and E208C/A281C MsbA-cl were $1.7\times$ and $2.0\times$-fold lower, respectively, than for A281C ($p < 0.01$ for both comparisons), with MsbA-cl itself lying in between (Table 1). Because both pre-cross-linked protein samples E208C and E208C/A281C have comparatively higher proportions of the inward-facing closed species compared with pre-cross-linked A281C or noncross-linked MsbA-cl and E208A MsbA-cl proteins (14), these data suggest that the
inward-facing closed conformation of MsbA is the preferred high affinity substrate-binding state. These data also show that the double E208C/A281C mutant was functionally and conformationally different from the single A281C mutant, thus distinguishing the inward-facing closed conformation from the inward-facing open conformation. It is important to note here that the overall Cys-Cys cross-linking efficiencies in purified proteins were found to be much lower than for proteins embedded in ISVs (e.g. purified A281C cross-linking efficiency was ~31% versus >75% in ISVs; purified E208C cross-linking efficiency was ~22% versus >40% in ISVs, based on densitometry of monomer and dimer signals on Western blot). This could be one of the reasons for the modest changes in $K_d$ that were observed in these experiments. Another reason might be interferences from the detergent itself, which may also bind as a substrate (30, 31), reducing the substrate binding on the binding of ATP to MsbA in a fluorescent TNP-ATP-based assay (14). Significant hydrolysis of TNP-ATP in this assay was prevented by the exclusion of Mg$^{2+}$ (solvent control), whereas the apparent affinity for ATP in the hydrolysis reaction was unaltered ($K_m = 1.24 \pm 0.05$ $\mu$M ATP in presence of lipid A versus 1.31 $\pm$ 0.17 $\mu$M ATP in presence of DMSO (solvent control)), whereas the apparent affinity for ATP in the hydrolysis reaction was unaltered ($K_m = 1.24 \pm 0.05$ $\mu$M ATP in presence of lipid A versus 1.31 $\pm$ 0.17 $\mu$M ATP in presence of DMSO (solvent control)), whereas the apparent affinity for ATP in the hydrolysis reaction was unaltered ($K_m = 1.24 \pm 0.05$ $\mu$M ATP in presence of lipid A versus 1.31 $\pm$ 0.17 $\mu$M ATP in presence of DMSO (solvent control)), whereas the apparent affinity for ATP in the hydrolysis reaction was unaltered ($K_m = 1.24 \pm 0.05$ $\mu$M ATP in presence of lipid A versus 1.31 $\pm$ 0.17 $\mu$M ATP in presence of DMSO (solvent control)), whereas the apparent affinity for ATP in the hydrolysis reaction was unaltered ($K_m = 1.24 \pm 0.05$ $\mu$M ATP in presence of lipid A versus 1.31 $\pm$ 0.17 $\mu$M ATP in presence of DMSO (solvent control))).

**DISCUSSION**

The current alternating access model for substrate transport does not yet explain a key observation made for many ABC exporters that transported substrates stimulate the rate of ATP hydrolysis. To investigate this further, we assessed the conformational changes at the NBDs and MDs following substrate binding by the prototypical multidrug/lipid A ABC exporter MsbA. In addition, we studied the effects of substrate binding to MsbA on the kinetics of ATP binding and hydrolysis.

We show that substrate binding by MsbA stimulates the maximum rate of ATP hydrolysis by facilitating the dimerization of NBDs in an overall conformation of the MsbA dimer that is markedly distinct from the previously described nucleotide-free, inward-facing open and nucleotide-bound, outward-facing conformations of ABC exporters. This new state of MsbA might share features with the inward-facing closed conformation depicted by an x-ray crystal structure of Vibrio cholerae MsbA, in which both the MDs and NBDs are in closer proximity (Fig. 8) (7). The physiological relevance of this inward-facing closed conformation was not clarified, but it was proposed to act as an intermediate between the inward-facing open state and outward-facing state (7). This suggestion is supported by our finding that substrate binding to MsbA enhances the maximum rate of ATP hydrolysis without changing the binding affinity for the nucleotide. Inward-facing closed, pre-translocation conformations that are stabilized by substrate binding were also recently observed for the ABC importer MalFEGK$_2$ (35) and the sodium-coupled hydantoin transporter Mhp1 (36).

The increased proximity of the reporter residues E208C and E208C' in the MsbA-cl dimer that is associated with the substrate binding-induced NBD closure (this work) was also observed in response to ATP binding (14). This earlier study on _E. coli_ MsbA focused on the role of the tetrahelix bundle that is formed by the cytoplasmic extensions of TMs 3 and 4 and that contains Glu-208 and Glu-208'. Disruption of critical inter-

### TABLE 1

| MsbA protein         | $K_d$ a | $B_{max}$ a |
|----------------------|---------|-------------|
| MsbA-cl              | 0.65 $\mu$M | 33.5 $\pm$ 4.0 |
| MsbA-cl E208C        | 0.47 $\pm$ 0.06 b | 34.7 $\pm$ 2.7 |
| MsbA-cl A281C        | 0.79 $\pm$ 0.03 c  | 31.9 $\pm$ 7.8 |
| MsbA-cl E208C/A281C  | 0.39 $\pm$ 0.04 c  | 34.3 $\pm$ 2.5 |

Data represent mean $\pm$ S.E. obtained in three independent experiments with different batches of purified proteins.

a The difference between $K_d$ values is statistically significant with a $p$ value of 0.01.

b The difference between $K_d$ values is statistically significant with a $p$ value of 0.004.

c The difference between $K_d$ values is statistically significant with a $p$ value of 0.0009.

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**Coupling between Substrate Binding and ATP Hydrolysis**

To investigate the mechanism by which substrate binding stimulates ATP hydrolysis, we studied the effect of substrate binding on the binding of ATP to MsbA in a fluorescent TNP-ATP-based assay (14). Significant hydrolysis of TNP-ATP in this assay was prevented by the exclusion of Mg$^{2+}$ from the assay buffer. Despite adding increasing amounts of verapamil in the TNP-ATP binding assay, we were unable to find any detectable change in the TNP-ATP binding properties of WT MsbA (Fig. 7a). The dissociation constant ($K_d$) for TNP-ATP binding by WT MsbA was 0.36 $\pm$ 0.02 $\mu$M TNP-ATP in the absence of verapamil as follows: $50 \mu$M verapamil, 0.33 $\pm$ 0.03 $\mu$M TNP-ATP; $100 \mu$M verapamil, 0.35 $\pm$ 0.01 $\mu$M TNP-ATP; $200 \mu$M verapamil, 0.31 $\pm$ 0.01 $\mu$M TNP-ATP), and the maximum TNP-ATP binding ($B_{max}$) was 119.3 $\pm$ 6.1 a.u. in the absence of verapamil; $50 \mu$M verapamil, 116.2 $\pm$ 5.6 a.u.; $100 \mu$M verapamil, 128.0 $\pm$ 8.0 a.u.; $200 \mu$M verapamil, 125.7 $\pm$ 3.4 a.u. (Fig. 7a).

The effects of lipid A on TNP-ATP binding could not be tested due to the direct quenching of TNP fluorescence by lipid A.

We further assessed the effect of substrate binding on the kinetics of ATP hydrolysis by MsbA. To study this, we added a fixed concentration of lipid A or an equal volume of DMSO
monomer interactions by alanine substitution mutations in the tetrahelix bundle did not significantly affect the binding affinity for nucleotides or Hoechst 33342 (14). However, this disruption strongly inhibited the maximum rate of ATPase activity due to impaired formation of the outward-facing state (14). Taken together, these published findings and our current observations suggest that substrate binding to MsbA stabilizes an intermediate state (an inward-facing closed conformation) that precedes the outward-facing conformation.

A TP binding to this intermediate state switches MsbA into the outward-facing conformation through the formation of stabilizing tetrahelix bundle interactions (Fig. 8). ATPase activity is then required to resolve the outward-facing conformation back to inward-facing.

In conclusion, substrate binding to MsbA stimulates progression of the catalytic cycle by promoting the formation of an inward-facing closed, pre-translocation state that binds ATP.

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