Published in final edited form as:

J Mol Biol. 2009 October 30; 393(3): 586–597. doi:10.1016/j.jmb.2009.08.050.

Conformational Cycle of the ABC transporter MsbA in Liposomes. Detailed Analysis using Double Electron-Electron Resonance Spectroscopy

Ping Zou, Marco Bortolus¹, and Hassane S. Mchaourab^{*}

Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, 2215 Garland Avenue, Nashville, TN 37232, USA

Abstract

Driven by the energy of ATP binding and hydrolysis, ATP binding cassette (ABC) transporters alternate between inward- and outward-facing conformations allowing vectorial movement of substrates. Conflicting models have been proposed to describe the conformational motion underlying this switch in access of the transport pathway. One model, based on three crystal structures of the lipid flippase MsbA, envisions a large amplitude motion that disengages the nucleotide binding domains and repacks the transmembrane helices. To test this model and place the crystal structures in a mechanistic context, we use spin labeling and Double Electron Electron Resonance (DEER) spectroscopy to define the nature and amplitude of MsbA conformational change during ATP hydrolysis cycle. For this purpose, spin labels were introduced at sites selected to provide a distinctive pattern of distance changes unique to the crystallographic transformation. Distance changes in liposomes, induced by the transition from nucleotide-free MsbA to the highest energy intermediate, fit a simple pattern whereby residues on the cytoplasmic side undergo 20–30 Å closing motion while a 7–10 Å opening motion is observed on the extracellular side. The transmembrane helices undergo relative movement to create the outward opening consistent with that implied by the crystal structures. DEER distance distributions reveal asymmetric backbone flexibility on the two sides of the transporter that correlates with asymmetric opening of the substrate binding chamber. Together with extensive accessibility analysis, our results suggest that these structures capture features of the motion that couples ATP energy expenditure to work providing a framework for the mechanism of substrate transport.

Keywords

ABC transporter; MsbA; double electron-electron resonance (DEER); site-directed spin labeling; electron paramagnetic resonance

Active transporters transduce various forms of energy into the mechanical work of substrate translocation. For the class of ATP binding cassettes (ABC) transporters, ATP energy drives protein conformational motions to carry molecules ranging from small ions to large polypeptides across membranes^{1,2}. The spectrum of these movements, their amplitudes and

^{*}Corresponding author. Mailing address for Hassane S. Mchaourab: 741 Light Hall, 2215 Garland Ave., Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN 37232, USA, hassane.mchaourab@vanderbilt.edu.

¹Current address: Dept. Scienze Chimiche, Università degli studi di Padova, Padova, Italy.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

substrate specificity remain an area of intense interest³. High resolution crystal structures of ABC importers and exporters have defined the molecular architectures of ABC transporters in multiple conformations^{4–10}. A functional unit of an ABC transporter consists of two ABCs or nucleotide binding domains (NBD) that bind and hydrolyze ATP each linked to a transmembrane domain (TMD) that provides a transport pathway across the bilayer. The signal of ATP binding and hydrolysis is transmitted through coupling loops and helices that contact the two domains³.

Initial expectations of a conserved transport mechanism across the two ABC transporter classes have been tempered by fundamental differences in their architecture and packing. The twisting and extensive intertwining of helices observed in ABC exporter structures^{5,10} deviate significantly from the side by side packing of the four modules characteristic of ABC importers. Indeed, the crystallographic snapshots of ABC transporters have inspired divergent models for the molecular mechanism of transport. Common to these models is the concept of alternating access¹¹ wherein the substrate transport pathway is alternately exposed to the two sides of the membrane. While evidence supports an inward- to outward- facing transitions during the transport cycle, the nature and magnitude of the underlying motion are controversial^{3,5}. Specifically, the questions are focused on the separation between the two NBDs during the cycle, the extent of the opening in the transmembrane domains and the nature of transmembrane helix motion mediating substrate translocation. The prevalent model of transport by importers invokes limited conformational rearrangements in the transmembrane domain consisting mostly of helix rotation driven by ATP-induced repacking at the NBD interface⁶. While this model is grounded in a series of elegant biochemical and structural studies of the maltose transporter MalK, its extension to exporters¹⁰ is problematic in light of the crystal structures of these proteins.

The lipid flippase MsbA from *E. coli*¹² has emerged as the model system for defining the structural dynamics of ABC exporters^{10,13,14}. Crystal structures in detergent micelles along with extensive spin labeling EPR analysis in liposomes allow complementary views of the protein motion that couples energy expenditure to substrate translocation. Collectively, the two methods suggest ATP binding reorients MsbA from an inward- to outward-facing conformation. Apo MsbA packs in an inverted V configuration featuring a large chamber open both to the cytoplasm and inner leaflet of the bilayer¹⁰. In contrast, this chamber is closed to the cytoplasm and open to the extracellular side in AMPPNP-bound MsbA. The implied motion reverses the water accessibility of the transmembrane chamber¹⁵ which has recently been implicated in substrate binding¹⁶.

The crystal structures of MsbA define a testable model of ATP-induced conformational changes. However, the nature and amplitude of the rearrangements implied by the three crystallographic structures have raised doubts regarding their mechanistic significance. Until recently⁴ MsbA was the only ABC exporter crystallized in a nucleotide-free (Apo) open conformation. An apo intermediate is postulated in the transport cycle to allow substrate binding from the cytoplasm or the inner leaflet of the membrane. Thus, an open chamber accessible to both milieus is an expected feature of the apo structure. However, the disengagement of the two NBDs and the rather large separation between the two wings of the transporter in the crystal structure may also be a consequence of detergent environment and/or shaped by contacts in the crystal lattice. While the observation that MsbA human homolog, p-glycoprotein (p-gp) has an open conformation in the apo state⁴ alleviates these concerns, validation of this model in lipid bilayers is required to establish the mechanistic relevance of these structures.

Another central issue in the interpretation of the crystal structures is the extensive repacking of the TMD helices implied by the transition from the open to the closed conformation¹⁰. In

both structures, the TMD consists of helices contributed by both monomers, yet the outward and inward openings are mediated by two different sets of helices. The formation of the NBD dimer in the closed structure rearranges the packing of transmembrane helices thereby switching chamber accessibility.

These issues are addressed here on a comprehensive scale. Long range distance measurements between spin labels are used to define the nature and amplitude of conformational motion upon transition from the apo state to the highest energy intermediate in the ATPase cycle. The pattern of distance changes is then compared to that predicted from the open/closed crystallographic transformation with the goal of experimentally verifying its main features and placing the crystal structures in a well-defined mechanistic context. This work builds on extensive accessibility analysis^{14,15} along five transmembrane helices and segments of the intracellular helices and the extracellular loops that is qualitatively consistent with the transition from the inward- to the outward-facing crystallographic structures.

The results presented in this paper also represent one of the most extensive Double Electron Electron spectroscopy^{17,18} (DEER) analyses of distance and distance changes in a membrane protein to date. The database of distances provides an opportunity to evaluate the limitations of this approach and the pitfalls that could hamper structural interpretation and evaluation of mechanistic models based on crystal structures. One of the exciting spectroscopic conclusions is the sensitivity of distance distributions to changes in backbone order. As expected, distance changes are more readily deduced at sites where movements do not repack the spin label side chain. Changes in spin label orientation relative to the backbone can lead to significant deviations from predicted distance changes.

Results

General Methodology

The transition between the apo and the nucleotide-bound structures of MsbA has been described as alternating access with a twist¹⁰ (Figure 1). Nucleotide-free MsbA has an open structure where the two NBDs are separated by 50 Å. The transmembrane (TM) helices are arranged in two wings that form a V-shaped chamber open to the cytoplasm and the inner leaflet of the bilayer. In the AMPPNP structure, hereafter referred to as the closed structure, the two NBDs form the canonical ATP dimer while the two TM wings of the transporter pack in the cytoplasm and split in an outward-facing conformation at the extracellular side. To create this opening, a twisting motion repacks the TM helices changing the identity of the swapped helices between the two monomers. Whereas TM1-TM2 share intersubunit contacts with TM3-TM6 in the closed structure, TM4-TM5 join the other subunit in the open conformation. As a result the inward and outward openings are mediated by different sets of helices. Nucleotide-free MsbA also crystallizes in an alternative conformation (closed-apo) that has the two NBDs closer than the open-apo and the chamber partially occluded. However, it has the same helix packing in the TMD as the open-apo structure.

Spin labeling strategies to test models of conformational changes rely on the measurement of site-specific accessibilities and mobilities of spin labels and the determination of distances between pairs of spin labels^{19,20}. The crystallographic open-apo/AMPPNP-closed transformation predicts a unique pattern of distance changes across the transporter wherein symmetry-related residues in the NBD, IH and cytoplasmic end of TM helices move closer. The movement on the extracellular side of helices and loops has an opposite sign and is expected to be of smaller amplitude. TM-helix swapping changes the pattern of proximity within a single subunit. Therefore, to test the crystallographic transformation in solution, spin labels were introduced along helices 1, 3 and 4, IH1 and the NBD to measure the distance between the two MsbA monomers in apo-MsbA and the transition state of ATP hydrolysis

trapped by Vanadate (Vi) following ATP hydrolysis²¹. Helix repacking was monitored by distance measurement between pairs of spin labels introduced at neighboring helices within each monomer.

Dynamics of MsbA alternating access

a) Detergent micelles—Distances between symmetry-related pairs of spin labels (Figure 2A) were measured in the solid state using DEER spectroscopy. Raw signals (Figure 2B) for representative residues, were baseline-corrected and analyzed to obtain distance distributions (Figure 2C). At most sites, the quality of the data allowed model-free analysis using Tikhonov regularization^{22,23}. This approach may yield asymmetric distance distributions that reflect the relative orientation of the spin label as well as any static disorder of the protein backbone. For cytoplasmic helical sites, distinct dipolar oscillations in the DEER signals are often observed upon transition to the post-hydrolysis intermediate. In contrast, on the extracellular side these oscillations tend to appear in the apo intermediate. In general, dipolar oscillations are expected for pairs of spin labels related by a well-defined distance. Therefore, spin label mobility as well as the dynamics and order of the backbone are reflected in a static distribution of distances and are the primary determinants of the DEER signal shape. At site 561 in the NBD, the EPR lineshape is relatively unchanged between the two intermediates and is indicative of large amplitude, fast motion. However, trapping MsbA in the ADP-Vi intermediate results in a narrow distribution suggesting a well defined distance between the two spin labels. On the extracellular side, dipolar oscillations are observed in the apo echo amplitude at site 162. Located on the exposed surface of helix 3 in the open and closed structures, the spin label mobility at site 162 is insensitive to the transition suggesting that the change in the DEER signal shape reflects loss of backbone order in the ADP-Vi intermediate. Collectively, changes in the shape of the DEER signal likely reflect opposite alteration in the dynamics of the backbone on MsbA cytoplasmic and extracellular sides.

Each distance distribution yields an average distance, R_{av} , allowing the determination of the distance change induced by the transition between the two intermediates. Table 1, summarizing results from mutants in the NBD, helices 1,3,4, IH1 and EL (Supplementary Figures 1 and 2), provides a global perspective on the conformational changes in detergent micelles. Starting from the NBD (sites 561 and 394) and terminating in EL2, the pattern distance change unequivocally confirms opposite movements at the two sides of the transporter as a result of the transition to the high-energy post-hydrolysis intermediate. The amplitude of the movement, now reported from all of six transmembrane helices of MsbA¹³ and multiple sites in the NBD, is consistent with expectations based on the two crystal structures suggesting the AMPPNP-bound structure captures the main structural features of the ADP-Vi intermediate. This is gleaned from analysis of exposed surface sites in the NBD (e.g. 561), IH1 (e.g. 116) and the TMD (e.g. 28, 43, 144) where conformational changes do not lead to repacking of the spin label side chain. At site 121, the distance change moves the labels from 50Å to less than 15Å apart as evidenced by dipolar splitting in the CW-EPR spectrum¹⁵. At sites where the spin label is involved in tertiary contacts and thus its location relative to the α -carbon is not well-defined, the magnitudes of distance changes tend to vary.

The amplitude of the distance change is expected to be smaller at the extracellular side. Thus, the contribution of the spin label reorientation leads to variation from site to site. Nevertheless, at all sites examined the distance changes display the correct sign. The most reliable estimate of distance change, 7 Å, is reported at site 162 where the spin label has no tertiary contacts in either structure. Further, a change in its orientation relative to the backbone is not expected given the lack of change in the CW-EPR lineshape (Figure 2). Whether the discrepancy with the crystallographic distance change is indicative of differences between the structure in

solution and in the crystal lattice or are a consequence of the limited resolution of the open structure cannot be unequivocally determined.

At a subset of sites, the cysteine substitution and the subsequent labeling reduce the catalytic rates of ATP turnover and/or increase the K_m of ATP binding¹⁵. These residues are in the ABC signature motif, IH2 and in a sterically restricted environment in the closed structure. The perturbation at signature motif residues (482) is rationalized by their direct involvement in ATP binding. Distances in the apo state appear consistent with the prediction from the open crystal structure but are unchanged by trapping in the ADP-Vi intermediate. For IH2 residues, spin labels likely disrupt packing with the NBD and therefore may destabilize the dimer. At sites of tertiary contacts, the destabilization reflects the free energy cost of spin label repacking. While many of these sites have robust turnover rates, we consistently observed two populations in the DEER distance distribution (indicated by † in table 1). One component appears to represent a population of spin labeled MsbA that does undergo distance change. Neither increasing ATP concentrations nor incubation times affected the ratios of the two populations.

b) MsbA reconstituted in liposomes—The survey in table 1 confirming the outline of the crystallographic transformation suggests a lack of significant distortion from the crystal lattice. However, protein motion can be damped or enhanced in the more complex environment of a lipid bilayer. Therefore, we selected representative residues on either side of the TMD and determined distance changes in polar asolectin liposomes. The spin labeling selection criteria included location of residues on the exposed surface of the transporter, their specific activities following reconstitution and most importantly the quality of the DEER data obtained in detergent micelles. In a lipid environment, the range of the distance measurements via dipolar coupling is reduced to approximately 50Å. This is primarily the consequence of a more effective T_2 relaxation as well as the increased effective spin concentration in the pseudo 2D geometry of liposomes. The latter makes it difficult to determine the baseline, particularly for featureless DEER signals.

The samples for DEER measurements were reconstituted at a protein/lipid molar ratio of 1:2000. After ultracentrifugation to pellet the liposomes, they were resuspended in a D_2O -based buffer which lengthens T_2 at surface exposed sites. Figure 3 shows representative time domain DEER signals, the resulting distance distributions along with the positions of the spin labels in the crystallographic structures. At every site distinct oscillations were obtained in at least one of the intermediates allowing a model-free determination of distance distributions (Supplementary Figure 3). For the featureless decay-type DEER signals at sites 115 and 158, the unprocessed data clearly establishes the position of the baseline allowing a reasonable estimation of the average distance although the width of the distribution may be overestimated for distances close to the limit which, for our samples, is in the 50–55 Å range. The data at sites 103 and 561 are outside the measureable distance range.

There is an overall agreement between distances and distributions measured in liposomes and detergent micelles (Table 2). For many sites, the distances in the apo intermediate are outside the measurable range but decrease in the ADP-Vi intermediate to values similar to those obtained in detergent micelles. Although we cannot calculate the magnitude of distance change at these sites, the data is not consistent with a significant population of a closed-apo structure in liposomes (Figure 1). Furthermore, similar distance distributions reflect similar backbone order in micelles and liposomes suggesting that this particular lipid mixture does not affect the amplitude of the distance change and thus the degree of opening of the apo intermediate. Overall, the data implies that the captured crystallographic snapshots of MsbA intermediates are relevant to the functional cycle in the membrane.

Compatibility of the EPR distances with the crystal structure

Direct comparison of the EPR distances with the crystallographic data requires a model for the spin label linking arm. At solvent exposed sites, the spin label can be explicitly included to perform molecular dynamic analysis^{24,25} or conformational searches¹⁷. Alternatively, phenomenological models that use a projection along the C_{α} - C_{β} vector have lead to reasonable agreement between EPR distances and crystal structures as long as the spin labels are introduced at exposed sites devoid of tertiary contacts²⁶. In this case, the spin label is modeled as a cone projected approximately 5.5Å from the β -carbon. If the two cones point towards each other or in opposite direction, it is expected that the spin label distance differs from the α -carbon distance by ($\pm$)10–15Å. Parallel projection of the spin label can yield deviations in the 0–5Å range. At sites where the spin labels is buried, modeling is more challenging as local side chain repacking will dictate the final relative orientation and levels of static disorder which determines the average distance and the distribution.

The most complex situation involves changes in the steric environment of the label between the two intermediates. In these cases, the R_{av} deviations from the C_{α} - C_{α} distances are intermediate-specific and the ΔR_{av} can substantially differ from the crystallographic prediction. Site 146 in helix 3 is an instructive example of this class. The EPR lineshape¹⁵ indicates that the spin label is sterically unrestricted in the apo state but is packed in a buried environment in the ADP-Vi intermediate. This leads to a change in the sign of the deviation between R_{av} and $R_{C_{\alpha}-C_{\alpha}}$ with a net effect of underestimating the distance change.

Although detailed analysis of the EPR distances in the context of the crystal structures will be presented elsewhere (Alexander, N., Mchaourab, H.S., Meiler, J., unpublished results), we note the following preliminary analysis. At exposed sites such as 24, 28, 143, 144, 183, 187 and 561 (Table 1), cone projection reconciles the EPR distances with the crystal structures. For buried or tertiary contact sites, 104, 106, 110, 115, 116, 139, 142, 158, and 394, we calculated the upper and lower bound of the distance range between the two labels predicted by the cone model. The magnitude of the distance changes at these sites reported in Table 1 are all outside the calculated range. Thus these changes, induced by transition to the ADP-Vi intermediate, reflect backbone movement and are not the result of spin label re-orientation relative to the backbone.

Repacking of helices in the TMD

The transformation between the crystallographic open-apo and the AMPPNP-closed intermediates repacks helices between the two TMD wings (Figure 1) such that TM3-TM6 are pulled away from TM1-TM2 of the same subunit. To verify these rearrangements in solution, we determined distances between pairs of spin labels with each member introduced in a helix predicted to undergo relative motion. This strategy introduces four spin labels per MsbA dimer with a predicted distance distribution shaped by the contribution of six sets of pairwise dipolar couplings. The two-fold symmetry of MsbA reduces the problem to four unique distances. Two distances relate labels at distinct sites: one short range arising from pairs in the same transmembrane wing while the other is across the dimer interface and therefore is considerably longer.

Thus, in addition to considerations of ATPase activity (Supplementary table 1) and surface localization, the sites were selected to maximize the distance differences between spin labels reporting on helix proximity within a TM wing versus those expected between symmetry-related spin labels in the dimer. These selection criteria simplify the interpretation of the distance distribution and allow the identification of the distance component contributed by pairs of labels in the same TMD wing.

Figures 4 shows the location of spin labeled pairs introduced to monitor the interface between helices 1 and 3. Helix 3 is packed near helix 1 in the open structure but moves away towards helix 4 in the closed structure, which should increase the distances between spin labels in the 43/158 pair. The same motion is expected to lead to the opposite pattern of distance changes in the 146/183, 146/187 and 142/187 pairs which report on the relative proximity of helices 3 and 4 (Figure 5). For some pairs, significant broadening of the CW-EPR lineshape is observed in one of the intermediates indicating proximity in the 5–15 Å range. Non-linear least squares analysis^{26,27} provides an estimate of the distance between the labels (Supplementary Figures 4 and 5). The loss of broadening in the ADP-Vi is a qualitative indicator of a change in the distance in the monomer. However the amplitude of the distance change cannot be determined given the limited distance range of lineshape analysis.

Figures 4 and 5 show the distance distributions for the double mutants in the apo and ADP-Vi along with those of the corresponding single mutants which serve as a reference. As expected, the distance distributions of the double mutants are complex. In addition, the shapes are significantly different between the apo and ADP-Vi intermediates reflecting distances changes between symmetry related residues in the dimer and between residues on neighboring helices. For many of the double mutants, a short range component arising from interaction of spin labels in the same monomer is evident in the time domain DEER signal (Supplementary Figure 6). It can be identified in the distance distribution by careful comparison with the distance distribution from each single mutant. Qualitative analysis of double mutants in the two intermediates clearly reveals the pattern of distance changes. For the pair 43/158, monitoring helix 1/helix 3 interface, the distance increases in the ADP-Vi intermediate. In contrast, a decrease in distances is reported for pairs monitoring the interface between helices 3 and 4. The distances between sites 28 and 162 and between 24/28 and 143 are not significantly changed as expected from the crystal structures. Comparison of the average distance suggests a movement of 7–10 Å similar to the scale of the crystal structures.

Discussion

Our results here and in previous publications^{13–15} weave a global perspective of the concerted conformational rearrangements during MsbA ATPase cycle. Large amplitude motion, required to form the canonical NBD interface, propagates towards the cytoplasmic side of a chamber formed at the interface of the TMDs. Transmitted through extensive repacking of TM helices, this motion opens the extracellular side thereby switching chamber access from inward- to outward-facing. The EPR data is consistent with the crystallographic transformation between the open-apo and the AMPPNP-bound closed structures¹⁰ although a detailed quantitative comparison is hindered by the low resolution of the open conformation.

Transition to the ADP-Vi intermediate leads to profound changes in transporter dynamics. NBD dimer formation is accompanied by a reduction in the conformational entropy at the cytoplasmic side. In contrast, the concomitant opening at the extracellular side is associated with increased dynamics manifested by changes in distance distribution at surface exposed sites. A dynamic backbone may be required to accommodate the structurally and chemically dissimilar substrates that partition either from the membrane or water phases and are transported by this complex movement.

The pattern of distances, along with complementary extensive accessibility analysis, is consistent with the V-shape of the apo intermediate observed in the crystal structure. Distances between symmetry-related spin labels show the trend expected from such geometry, becoming progressively smaller towards the membrane/water interface. This conclusion is particularly important given the limited resolution of the open-apo crystal structure and, until recently⁴, the lack of a corroborating structure from another ABC exporter.

The distance between the two NBDs now sampled in different regions is inconsistent with the constant engagement model deduced from analysis of ABC importers³ and espoused by the initial interpretation of the Sav1866 closed structure⁵. Furthermore, a closed-apo conformation similar to that observed for MsbA should be manifested by a second component in the distance distribution at sites such as 103 and 561 where residues in the open conformation are outside the range of measurable distances. However, the broad distance distributions hint at a potential flexibility that may allow the sampling of more closed conformations. As a unique conformer, the apo-closed appears to be a minor population in the spectroscopic ensemble. The observation of dipolar coupling in CW-EPR spectra at several sites in the APD-Vi but not in the apo intermediate further supports this conclusion.

Our results settle one of the unanswered questions in the ABC exporter field; namely, whether the AMPPNP-bound structures of both MsbA and Sav1866 in the crystal capture the structural features of the high-energy post hydrolysis intermediate. This is the highest stable point along the energy diagram and is often referred to as the transition state of ATP hydrolysis²¹. Our results suggest that the structures of MsbA bound to AMPPNP or trapped by ADP-Vi are similar; any differences are within the resolution limit of the spin labeling EPR approach. This conclusion is consistent with cryoEM²⁸ and functional data suggesting that ATP binding is the power stroke for transport.

By providing a dynamic perspective, spin labeling and EPR spectroscopy offer a complementary tool to refute or confirm high resolution structures of membrane proteins, assess their mechanistic significance, identify regions of distortion due to crystal contacts and/or detergent solubilization and determine or test models of their conformational dynamics. The addition of DEER to the EPR tool kit significantly enhances the value of EPR restraints and facilitates their interpretations.

Materials and Methods

Reagents

Deuterium oxide (D₂O) and deuterium glycerol were obtained from Sigma-Aldrich Company. MTSSL (1-oxyl-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-methanethiosulfonate spin label was from Toronto Research Chemicals.

MsbA purification and labeling

Site-directed mutagenesis of MsbA²⁹ was carried out using QuikChange mutagenesis kit (Stratagene). The cloning, expression, purification and spin labeling^{30–32} of the MsbA mutants was described in the accompanying report¹⁵.

MsbA protein reconstitution

To prepare liposome samples for DEER measurement, purified MsbA protein was reconstituted with liposomes at protein/lipid molar ratio of 1:2000 at 4 °C for 2 hours. Thereafter detergent was removed by addition three times of Bio-beads at 80mg per ml of mixture solutions and proteoliposome pellets were resuspended in 50mM Hepes, 50mM NaCl, 2mM MgCl₂, pH 7.5 buffer. To reconstitute MsbA trapped in the ADP-Vi intermediate, mutants were incubated at 37 °C for 20 minutes in the presence of 10mM ATP, 10mM MgCl₂ and 5mM Vi prior to reconstitution. All samples were in D₂O based buffer to prolong the phase memory time.

Distance measurement by pulse EPR experiments

The detergent samples for DEER measurement were concentrated to 150–200μM with 30% glycerol (w/v). Dipolar time evolution data were obtained on a Bruker 580 pulsed EPR

spectrometer at X-band frequency (9.36GHz). All experiments were carried out at 50 or 83 K using a standard DEER four-pulse protocol³³. DEER signals were analyzed by the Tikhonov regularization of the software DeerAnalysis 2008 to determine average distances and distributions in distance $P(r)$. For MsbA samples in liposomes, the dimension of the background correction was set to 2. The error in the distance was estimated by taking half of the $P(r)$ width at 0.7 of the height.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health Grant R01-GM077659 to Hassane S. Mchaourab. We thank Dr. Hanane Koteiche and Derek Claxton for critical reading of the manuscript; Jared Godar for critical reading of the manuscript and help with figures. We also thank Dr. Geoffrey Chang (Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA) for providing the model of open apo MsbA with side chains.

Abbreviations

ABC	ATP-binding cassette
DEER	double electron-electron resonance
EPR	electron paramagnetic resonance
CW-EPR	continuous wave EPR
MTSSL	1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-methanethiosulfonate spin label
AMPPNP	5'-adenylyl imidodiphosphate
α-ddm	<i>n</i> -dodecyl- α -D-maltoside
NBD	nucleotide-binding domain
TMD	transmembrane domain
TM	transmembrane
IH	intracellular helix
EL	extracellular loop

References

- Davidson AL, Chen J. ATP-binding cassette transporters in bacteria. *Annual Review of Biochemistry* 2004;73:241–68.
- Higgins CF. ABC transporters: physiology, structure and mechanism--an overview. *Research in Microbiology* 2001;152:205–10. [PubMed: 11421269]
- Oldham ML, Davidson AL, Chen J. Structural insights into ABC transporter mechanism. *Current Opinion in Structural Biology* 2008;18:726–33. [PubMed: 18948194]
- Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* 2009;323:1718–22. [PubMed: 19325113][see comment]
- Dawson RJP, Locher KP. Structure of a bacterial multidrug ABC transporter. *Nature* 2006;443:180–5. [PubMed: 16943773][see comment]
- Khare D, Oldham ML, Orelle C, Davidson AL, Chen J. Alternating access in maltose transporter mediated by rigid-body rotations. *Molecular Cell* 2009;33:528–36. [PubMed: 19250913]
- Locher KP, Lee AT, Rees DC. The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science* 2002;296:1091–8. [PubMed: 12004122][see comment]
- Oldham ML, Khare D, Quioco FA, Davidson AL, Chen J. Crystal structure of a catalytic intermediate of the maltose transporter. *Nature* 2007;450:515–21. [PubMed: 18033289]
- Pinkett HW, Lee AT, Lum P, Locher KP, Rees DC. An inward-facing conformation of a putative metal-chelate-type ABC transporter. *Science* 2007;315:373–7. [PubMed: 17158291]
- Ward A, Reyes CL, Yu J, Roth CB, Chang G. Flexibility in the ABC transporter MsbA: Alternating access with a twist. *Proceedings of the National Academy of Sciences of the United States of America* 2007;104:19005–10. [PubMed: 18024585]
- Jardetzky O. Simple allosteric model for membrane pumps. *Nature* 1966;211:969–70. [PubMed: 5968307]
- Doerfler WT, Raetz CR. ATPase activity of the MsbA lipid flippase of Escherichia coli. *Journal of Biological Chemistry* 2002;277:36697–705. [PubMed: 12119303][erratum appears in J Biol Chem 2002 Nov 15;277(46):44588]
- Borbat PP, Surendhran K, Bortolus M, Zou P, Freed JH, Mchaourab HS. Conformational motion of the ABC transporter MsbA induced by ATP hydrolysis. *Plos Biology* 2007;5:e271. [PubMed: 17927448]
- Dong J, Yang G, Mchaourab HS. Structural basis of energy transduction in the transport cycle of MsbA. *Science* 2005;308:1023–8. [PubMed: 15890883][see comment]
- Zou P, Mchaourab HS. Alternating Access of the Putative Substrate-Binding Chamber in the ABC Transporter MsbA. *Journal of Molecular Biology*. 2009In press
- Smriti, Zou P, Mchaourab HS. Mapping Daunorubicin-binding Sites in the ATP-binding Cassette Transporter MsbA Using Site-specific Quenching by Spin Labels. *Journal of Biological Chemistry* 2009;284:13904–13. [PubMed: 19278995]
- Borbat PP, Mchaourab HS, Freed JH. Protein structure determination using long-distance constraints from double-quantum coherence ESR: study of T4 lysozyme. *Journal of the American Chemical Society* 2002;124:5304–14. [PubMed: 11996571]
- Jeschke G. Distance measurements in the nanometer range by pulse EPR. *Chemphyschem* 2002;3:927–32. [PubMed: 12503132]
- Hubbell WL, Cafiso DS, Altenbach C. Identifying conformational changes with site-directed spin labeling. *Nature Structural Biology* 2000;7:735–9.
- Hubbell WL, Mchaourab HS, Altenbach C, Lietzow MA. Watching proteins move using site-directed spin labeling. *Structure* 1996;4:779–83. [PubMed: 8805569]
- Sharma S, Davidson AL. Vanadate-induced trapping of nucleotides by purified maltose transport complex requires ATP hydrolysis. *Journal of Bacteriology* 2000;182:6570–6. [PubMed: 11073897]
- Chiang YW, Borbat PP, Freed JH. The determination of pair distance distributions by pulsed ESR using Tikhonov regularization. *J Magn Reson* 2005;172:279–95. [PubMed: 15649755]

23. Jeschke G, Koch A, Jonas U, Godt A. Direct conversion of EPR dipolar time evolution data to distance distributions. *J Magn Reson* 2002;155:72–82. [PubMed: 11945035]
24. Fajer MI, Li H, Yang W, Fajer PG. Mapping electron paramagnetic resonance spin label conformations by the simulated scaling method. *J Am Chem Soc* 2007;129:13840–6. [PubMed: 17948993]
25. Sale K, Song L, Liu YS, Perozo E, Fajer P. Explicit treatment of spin labels in modeling of distance constraints from dipolar EPR and DEER. *J Am Chem Soc* 2005;127:9334–5. [PubMed: 15984837]
26. Alexander N, Bortolus M, Al-Mestarihi A, Mchaourab H. De novo high-resolution protein structure determination from sparse spin-labeling EPR data. *Structure* 2008;16:181–95. [PubMed: 18275810]
27. Rabenstein MD, Shin YK. Determination of the distance between two spin labels attached to a macromolecule. *Proceedings of the National Academy of Sciences of the United States of America* 1995;92:8239–43. [PubMed: 7667275]
28. Ward A, Mulligan S, Carragher B, Chang G, Milligan RA. Nucleotide dependent packing differences in helical crystals of the ABC transporter MsbA. *Journal of structural biology* 2009;165:169–75. [PubMed: 19114108]
29. Koteiche HA, Reeves MD, Mchaourab HS. Structure of the substrate binding pocket of the multidrug transporter EmrE: site-directed spin labeling of transmembrane segment 1. *Biochemistry* 2003;42:6099–105. [PubMed: 12755611]
30. Mchaourab HS, Lietzow MA, Hideg K, Hubbell WL. Motion of spin-labeled side chains in T4 lysozyme. Correlation with protein structure and dynamics. *Biochemistry* 1996;35:7692–704. [PubMed: 8672470]
31. Koteiche HA, Mchaourab HS. The determinants of the oligomeric structure in Hsp16.5 are encoded in the alpha-crystallin domain. *FEBS Letters* 2002;519:16–22. [PubMed: 12023011]
32. Sathish HA, Stein RA, Yang G, Mchaourab HS. Mechanism of chaperone function in small heat-shock proteins. Fluorescence studies of the conformations of T4 lysozyme bound to alphaB-crystallin. *J Biol Chem* 2003;278:44214–44221. [PubMed: 12928430]
33. Pannier M, Veit S, Godt A, Jeschke G, Spiess HW. Dead-time free measurement of dipole-dipole interactions between electron spins. *Journal of Magnetic Resonance* 2000;142:331–40. [PubMed: 10648151]

Figure 1.

The three crystallographic conformations of MsbA A) open-apo, B) closed-apo and C) AMPPNP-bound. One monomer in each dimer is colored while the other is gray. The coordinates of the closed apo and AMPPNP-bound structures were obtained from the protein data bank, PDB ID 3B5X and 3B60 respectively. The coordinate of the open apo structure were provided to us by Dr. Geoffrey Chang, Scripps Research Institute.

Figure 2.

A) Ribbon representation of the open and closed structures highlighting representative sites selected for distance measurements in detergent micelles. B) Baseline-corrected, time-domain DEER signals demonstrating the change in distance between symmetry-related spin labels upon transition from the apo (black trace) to ADP-Vi intermediate (red trace). C) In this transition, a decrease in the average distance and narrowing of the distance distributions are observed on the cytoplasmic side. In contrast, an increase in average distance is detected on the extracellular side. The CW-EPR spectra at sites 561 and 162 demonstrate the lack of change in spin label dynamics at these sites.

Figure 3.

A) Ribbon representation of the open and closed structures highlighting representative sites selected for distance measurements in liposomes. B) & C) Baseline-corrected DEER data (apo intermediate: black trace; ADP-Vi intermediate: red trace) along with their corresponding distributions establishing the outline of conformational changes in liposomes: formation of the NBD dimer (561), closing of the cytoplasmic side (103, 115) and opening of the extracellular side (158). The DEER signal at site 561 was analyzed using a form factor only. Thus, the distribution, centered well above 50 $\text{\AA}$ does not reflect separation between the two spin labels in the dimer.

Figure 4.

A) TM helix repacking in the crystallographic open/closed transformation. For clarity, residues of TM1, TM3 and TM4 of one monomer selected for distance measurements are highlighted inside a surface rendering of the dimer. B) Distance distributions of double mutants along with their corresponding single mutants demonstrating the relative movement of TM1/TM3 upon transition to the ADP-Vi intermediate. Time domain DEER signals are shown in supplementary Figure 6. For a number of double mutants, dipolar coupling leads to spectral broadening. Details of these spectra and their analysis are in supplementary Figures 4 and 5.

Figure 5.

A) TM helix repacking in the crystallographic open/closed transformation. For clarity, residues of TM1, TM3 and TM4 of one monomer selected for distance measurements are highlighted inside a surface rendering of the dimer. B) Distance distributions of double mutants along with their corresponding single mutants demonstrating the relative movement of TM3/TM4 upon transition to the ADP-Vi intermediate. For all three double mutants, a short range distance component reflects proximity within each monomer. Time domain DEER signals are shown in the supplementary material.

Table 1
Amplitude of ATP-induced distance changes in α -ddm micelles.

Mutant	Location	Experimental Distance R_{av} ($\text{\AA}$), detergent			Calculated Distance R_{Ca-Ca} ($\text{\AA}$), MsbA _o		
		Apo	ADP·Vi	ΔR_{av}	Open Apo	AMPPNP	Closed Apo
43	TM1	35±2.5	38±3	-3	33	31	27
42		36±10	36±12	0	31	28	32
28		>70	53±4.25	>17	64	43	52
24		>70	55±1.55	>15	71	44	52
104 [†]	TM2	55±4.5	28±2.75	27	67	29	36
106 [†]		>70	47±2	>23	76	33	42
110		>70	50±1.75	>20	77	37	44
115		51±3.5	32±1	19	62	31	28
116	IH1	57±6.5	26±6.7	31	59	24	26
118		45±6	41±3	4	51	29	17
120		47±10.5	31±2	16	47	18	8
121		51±10			51	13	12
139	TM3	62±17.5	30±1.25	32	51	26	30
141 [†]		33±5.5	20±1.5	13	40	18	24
142 [*]		54±7	30±7.5	24	45	21	30
143 [*]		54±5.6	26±1.5	28	47	28	33
144 [†]		35±2.5	20±2.25	15	41	25	27
146		42±3.75	37±3.5	5	48	27	37
158		36±1.5	51±7.5	-15	41	46	43
162		44±3.25	51±6.5	-7	39	53	47
166	EL2	51±6.5	>65	>-14	35	54	49
167	TM4	37±6.5	51±11	-14	34	48	47
183		43±13.25	53±4	-10	38	44	42
187		46±6.25	51±6	-5	41	41	39
394 [*]		>70	55±4	>15	82	48	31
482	NBD		60±0.5		73	22	52
561		>70	42±1.5	>28	86	36	37

* analyzed by Gaussian fit, otherwise by Tikhonov fit.
† bimodal distance distributions
cytoplasm extracellular

Table 2
Amplitude of ATP-induced distance changes in liposomes.

Mutant	Location	Experimental Distance R_{av} (Å), liposomes			Calculated Distance R_{Ca-Ca} (Å),		
		Apo	ADP·Vi	ΔR_{av}	Open Apo	AMPNP	Closed Apo
166	EL2	47±3.75			35	54	49
158		36±2.75	47±5.5	-11	41	46	43
144		34±1.25	20±1	14	41	25	27
141		31±3	20±1.75	11	40	18	24
115	IH1	53±3	31±1	22	62	31	28
103	TM2	>55	47±2.5	>8	73	34	43
561	NBD	>55	42± 2.25	>13	86	36	37

cytoplasm

extracellular