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Characterization of the E506Q and H537A Dysfunctional Mutants in the *E. coli* ABC Transporter MsbA

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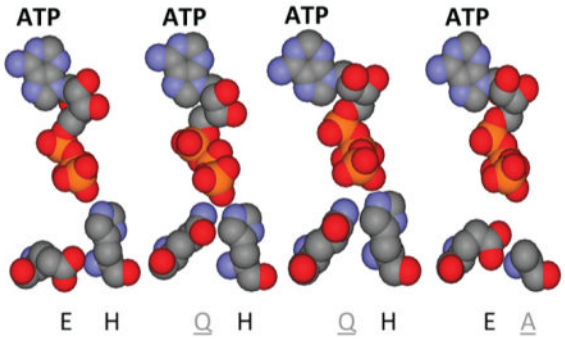
Abstract



MsbA is a member of the ABC transporter superfamily that is specifically found in Gram-negative bacteria and is homologous to proteins involved in both bacterial and human drug resistance. The E506Q and H537A mutations have been introduced and used for crystallization of other members of the ABC transporter protein family, including BmrA and the ATPase domains MalK, HlyB-NBD, and MJ0796, but have not been previously studied in detail or investigated in the MsbA lipid A exporter. We utilized an array of biochemical and EPR spectroscopy approaches to characterize the local and global effects of these nucleotide binding domain mutations on the *E. coli* MsbA homodimer. The lack of cell viability in an *in vivo* growth assay confirms that the presence of the E506Q or H537A mutations within MsbA creates a dysfunctional protein. To further investigate the mode of dysfunction, a fluorescent ATP binding assay was used and showed that both mutant proteins maintain their ability to bind ATP, but ATPase assays indicate hydrolysis is severely inhibited by each mutation. EPR spectroscopy data using previously identified and characterized reporter sites within the nucleotide binding domain along with ATP detection assays show that hydrolysis does occur over time in both mutants, though more readily in the H537A protein. DEER spectroscopy demonstrates that both proteins studied are purified in a closed dimer conformation, indicating that events within the cell can induce a stable, closed conformation of the MsbA homodimer that does not reopen even in the absence of nucleotide.

ATP-binding cassette (ABC) transporters are a large and important superfamily of proteins responsible for the membrane translocation of a diverse group of substrates including peptides, lipids, and drugs. ABC transporters play a role in a variety of human pathologies including cystic fibrosis,[1] Stargardt's macular dystrophy,[2,3] and drug resistance.[4]

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MsbA is a 65 kDa protein found in the inner membrane of Gram-negative bacteria.[5] MsbA is thought to be responsible for the transport of lipid A across the inner membrane and is homologous to bacterial and human proteins involved in antibiotic and chemotherapeutic resistance, respectively. The absence of a functional MsbA protein in the cell results in a toxic accumulation of lipid A within the membrane.[6–8] Though ABC transporters are often important in cell survival, MsbA is the only essential ABC transporter in *E. coli*.[5]

The MsbA homodimer is comprised of two monomers, each with a cytosolic nucleotide binding domain (NBD) and a transmembrane domain (TMD). The crystal structures of the apo, MgADP-V_i, and MgAMP-PNP conformations of MsbA have been determined and show that the TMD is formed by a six helical bundle that intertwines with the opposite monomer at the periplasmic tip of the protein, producing stable contact points for the two monomers.[9] The helical bundles are linked to the NBDs, which are responsible for binding and hydrolyzing ATP as the energy to transport lipid A across the inner membrane. Within the NBD there are several motifs that are conserved throughout the ABC transporter superfamily (highlighted in Figure 1). The C-loop, also known as its amino acid sequence LSGGQ, comes together with the Walker A motif from the opposite monomer to form a nucleotide sandwich when binding and/or hydrolyzing ATP.[10] Other conserved motifs within this protein family include the Walker B, H-motif, and the Q-loop.[11]

The dysfunctional NBD mutants studied here are E506Qand H537A, which have also been introduced into other members of the ABC transporter family.[10,12–14] The E506Q point mutation is located just downstream of the Walker B motif,[15] and its effect on the *E. coli* MsbA transporter will be studied in detail here. In the ATPase domains of transporter complexes such as MalK, HlyB-NBD, PDR5, and MJ0796 and in the transporter BmrA, the E to Q substituted proteins have been suggested to be able to bind ATP but lack the ability to hydrolyze to ADP and P_i.[12,14–16] This was concluded based on solved crystal structures with closed ATPase domains and ATPase activities of 0 ± 5 nmol/(mg min) for BmrA,[15] 10% of WT for HlyB-NBD,[12] 15% of WT for PDR5,[17] and not measurable (but nonzero) for MJ0796.[16] In contrast, studies on the NBDs of the intracellular peptide transporter Mdl1p containing E to Q substitutions showed that the ATP-induced dimer was able to hydrolyze ATP at both sites.[18]

The H537A point mutation is a modification of the conserved H-motif, which is suggested to interact with the γ-phosphate of ATP as well as with E506.[11,19] The H537 and E506 interaction has been proposed in a linchpin model in which H537 stabilizes ATP binding,[19] and their comparable positions with respect to each other and ATP in HlyB-NBD and MalK are shown in Figure 2. Also shown is the only known structure of a protein containing the H to A mutation, HlyB-NBD, which has no ATPase activity.[19] However, WT-like activity was reported for a similar mutation during the study of PDR5.[17] The actual mode of dysfunction is unknown and therefore was studied more thoroughly in this work using *E. coli* MsbA.

A large number of residues in or near each of the conserved ATP-binding motifs described above have been previously studied using cysteine substitution and biophysical and biochemical techniques.[20–22] Similar to studies on two D-loop mutants,[23] nine of these sites have been selected for this study to report on local changes within each motif caused by the introduction of the E506Qor H537A dysfunctional mutations.

Site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy is an advantageous technique to study the effects of dysfunctional mutations in MsbA because it does not have protein size limitations and can accommodate lipids without the issue of light scattering. Continuous wave (CW) EPR allows for the observation of changes in spin-label mobility and environment at very localized sites within the large MsbA

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homodimer.[24–30] Double electron electron resonance (DEER) spectroscopy is a pulsed EPR technique used to measure interspin distances[31–33] between MsbA monomers and is an important diagnostic tool in determining if the protein is in the open or closed conformation. In addition, *in vivo* growth assays have been utilized to assay the ability of the mutant proteins to maintain cell growth and *in vitro* ATP binding, detection, and ATPase assays have been carried out to assess the ATP binding and hydrolysis capabilities of MsbA containing E506Qand H537A.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis and Protein Preparation

Site-directed cysteine mutants were introduced into the c-less (C88S/C315S) MsbA gene in a pET28 (Novagen) plasmid using the Quik Change mutagenesis kit (Stratagene) and verified by sequencing at the Medical College of Wisconsin Protein and Nucleic Acid Facility, as described previously.[34] MsbA was overexpressed in *E. coli* NovaBlue cells (Novagen) and purified by cobalt affinity chromatography using Talon resin (BD Biosciences Clontech) as described previously[34] with the following modification: purified MsbA cysteine mutants were labeled with a 10-fold molar excess of the sulfhydryl-specific MTSL (2,2,5,5-tetramethylpyrroline-3-yl-methanethiosulfonate spin-label; Toronto Research Chemicals) or 100-fold excess of MAL-6 (4-maleimido-TEMPO; Aldrich) reagent on the column overnight at 4 °C prior to washing extensively with 50 mM $NaPO_4$, pH 7, 0.01% DM, 10% glycerol buffer. Protein concentrations were determined by the detergent-compatible BCA protein assay (Pierce) after concentration in Amicon Ultra centrifugal filters. Spin-labeled MsbA was reconstituted into inner membrane liposomes [65:25:10 phosphatidylethanolamine (PE):phosphatidylglycerol (PG):cardiolipin (CL) (Avanti Polar Lipids)] at a 250:1 lipid:protein molar ratio, as previously described.[21]

In Vivo Growth Assay

Plasmid containing WT or mutant MsbA was electroporated into electrocompetent WD2 cells [a kind gift from C. Raetz, Duke University (Durham, NC), and W. Doerrler, Louisiana State University (Baton Rouge, LA)] carrying the temperature-sensitive A270T mutation in the chromosomal copy of MsbA[8] and assayed in a Thermo Varioskan Flash microplate reader as described previously.[22] WT and c-less MsbA, and empty pET28 plasmid, were run as positive and negative controls, respectively. Each mutant was tested in triplicate per run with a minimum of two separate runs with fresh WD2 cells. Cells containing plasmid-encoded MsbA with normal function typically grew to an OD_{600} of greater than ~0.5, while cells containing no MsbA or completely inactive MsbA grew to an OD_{600} of ~0.3 or less; pET28 plasmid without the MsbA gene grows to an OD_{600} of 0.25. An $0.30 < OD_{600} < 0.50$ indicates substandard cell viability in which the cell can survive but does not flourish.

ATP Binding Assay

As described previously, MsbA (0.5 and 2.0 μM) in 50 mM $NaPO_4$, pH 7, 0.01% DM, 10% glycerol was mixed with increasing concentrations of TNP-ATP (Invitrogen) between 0.02 and 1.0 μM to determine F_{obs}.[23] The experiments were conducted on a Photon Technology International spectrofluorometer with an excitation wavelength of 407 nm and an emission wavelength recorded at 532 nm. Controls with and without 3 μM WT were used to determine F_{max} and F_0, respectively. $(F_{obs} - F_{max})/(F_{max} - F_0)$ values were plotted against TNP-ATP concentration[35] in SigmaPlot (Systat Software) to determine K_D. Experiments were repeated 2–5 times.

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ATPase Assays

ATP hydrolysis was assessed in triplicate by quantitating the release of γ-$^{32}P_i$ from ATP in the presence of MsbA, PE:PG:CL lipids containing lipid A, and MgATP containing [γ-^{32}P] ATP (Perkin-Elmer) at 37 °C using Cherenkov counting in a Tri-Carb liquid scintillation counter (Perkin-Elmer) as described previously.[22] Initial rates of hydrolysis were determined by plotting the nanomoles of P_i released per milligram of protein versus time (0.1–2.0 min). Standard errors were generated using SigmaPlot.

V_{max} and K_m values for MsbA were determined using the EnzCheck Phosphate Assay kit (Molecular Probes) following the manufacturer's instructions for measuring the continuous release of P_i as described previously.[23] For each experiment, 1 μM MsbA and seven different concentrations (0–1 mM) of ATP were analyzed in triplicate in a 200 μL reaction in a 96-well microplate at room temperature. Data were recorded over 30 min in a ThermoScientific Varioskan Flash microplate reader and quantitated using a standard P_i curve. SigmaPlot was used to analyze the resulting data.

The rates presented here for purified WT MsbA from *E. coli* in DM, and DM in the presence of lipids, are between rates previously reported for this protein.[9,36]

Nucleotide Detection Assays

The Kinase-Glo, Kinase-Glo Plus, and ADP-Glo assays (Promega) take advantage of luciferin luminescence for the detection of ATP and ADP. For each experiment, 5 μM MsbA and 10 μM ATP with excess $MgCl_2$ were used along with standards containing 1 – 10 μM ATP or ADP (Sigma-Aldrich). After incubation at RT, the protein samples were boiled for 5 min to denature the protein and release any bound nucleotide for detection in the assay, as described.[23] The manufacturer's protocol was followed for each assay, and the plate was read in a Thermo Scientific Varioskan Flash with LumiSens for luminometric spectral scanning immediately after the assay was completed.

Site-Directed Spin Labeling EPR Spectroscopy

CW EPR spectroscopy was carried out at room temperature on a Bruker ELEXSYS 500 X-band spectrometer equipped with a super high Q(SHQ) cavity (Bruker Biospin). Spectra were recorded over a 100 G scan width under nonsaturating conditions and with a 100 kHz field modulation of 1 G. Samples were typically 10–20 μL in volume and were contained in a glass capillary. The concentration of the protein reconstituted into liposomes was ~200 μM in 50 mM $NaPO_4$ (pH 7) containing 20 mM ATP or AMP-PNP, 2 mM EDTA, 20 mM $MgCl_2$, and 2 mM sodium orthovanadate, as appropriate. Some spectra, especially I385C-E506Q, showed a small population of denatured protein and/or free spin-label (sharp peaks) caused by the freeze–thaw cycles used to ensure incorporation of ligand inside the lipid vesicles.

Four-pulse DEER spectroscopy experiments were carried out on a Bruker ELEXSYS 580 X-band spectrometer equipped with a 3 mm split-ring resonator (Bruker Biospin) at a temperature of 80 K. Samples were 30 μL in 50 mM $NaPO_4$, pH 7, 0.01% DM buffer, and were contained in a sealed quartz capillary. Before insertion into the resonator, 20% deuterated glycerol was added to the sample as a cryoprotectant, and the samples were flash frozen using a dry ice–acetone slurry. The four-pulse DEER pulse sequence $(\Pi/2)_{n1}-t_1-(\Pi)_{n1}-t-(\Pi)_{n2}-t_1+t_2-t-(\Pi)_{n1}-t_2-$echo was used.[32] The ELDOR pulse (n1) was positioned at the centerfield maximum of the echo detected nitroxide spectrum, whereas the observe pulses (n2) were positioned at the low field line of the spectrum. Two-step phase cycling was applied to the first Π/2 observe pulse to remove baseline offsets. Echo decay

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data were analyzed, and the distance distribution was calculated using Deer Analysis 2008 software created by Dr. Gunnar Jeschke (ETH).[37]

RESULTS

In Vivo Growth Assay Indicates That Both E506Q and H537A Prevent Cell Growth

Upon analysis of either the E506Qor H537A mutant in the *in vivo* growth assay, the cell viability and growth falls to levels near or below the inactive mark, confirming that each of these mutations produces a protein unable to maintain cell viability. Of the nine NBD reporters, all but three (S482C, Q485C, and L504C) grow to near or above an OD_{600} = 0.50, which indicates normal cell viability (ref 22, reprinted in Figure 3). When the E506Q mutation is added to each of the reporters, the OD_{600} values fall to 0.19–0.29, while E506Q alone has an OD_{600} = 0.26. When H537A is added to the reporters, the OD_{600} values fall to 0.19–0.32, while H537A alone has an OD_{600} = 0.25. Clearly, the addition of the E506Q and H537A mutations to MsbA results in a loss of protein function within the cell. Loss of function could be attributed to deficiencies in ATP binding, ATP hydrolysis, release of hydrolysis products, or lipid flipping.

E506Q and H537A Exhibit ATP Binding Capability in Fluorescent ATP Binding Assay

In order to assess if the loss of function verified in the growth assay is due to the protein's inability to bind ATP, a fluorescent ATP analogue, TNP-ATP, was used in a fluorescence assay to determine K_d values of ATP binding. WT MsbA was previously determined to have a K_d of 0.32 μM for TNP-ATP.[23] The E506Q and H537A proteins demonstrated ATP dissociation constants similar to WT with K_d values of 0.51 and 0.44 μM, respectively (Table 1). These data clearly indicate that ATP binding is intact and is not the source of dysfunction in these mutations.

In Vitro ATPase Assay Demonstrates That E506Q and H537A Significantly Reduce the Rate of ATP Hydrolysis

To assess the ability of MsbA to hydrolyze and release the products of ATP hydrolysis, an *in vitro* ATPase assay was carried out that measured the amount of free P_i released into solution upon addition of ATP. The results shown in Figure 4 clearly indicate that the addition of the E506Q mutation to each of the reporter residues produces a protein that has a severely diminished ability to hydrolyze ATP in the presence of a high excess of ATP over 2 min at 37 °C. The majority of the reporter residues in the c-less background are near or above the ATPase activity of WT, with the exception of S482C and L504C. Upon the addition of the E506Q mutation, each of the reporter mutant pairs functions at 10% or below that of WT, as shown in Figure 4. Since the reporter–E506Q mutant pairs have such similar ATPase activity to E506Q alone (5% of WT), H537A alone was measured with the assumption that the addition of the reporter would not change the ATPase activity significantly. The H537A mutant MsbA functions at a level of 8% of WT ATPase activity. This study indicates that the E506Q and H537A mutations considerably affect the ATPase function of the MsbA protein within the short time scale of these experiments.

To follow up, K_m and V_{max} values for MgATP were determined for WT, c-less, E506Q, and H537A (Table 2). WT and c-less MsbA had hydrolysis rates of 67.6 and 47.6 nmol/(mg min), respectively, and both had K_m values close to 100 μM ATP. As expected based on the results above, E506Q and H537A again showed slowed rates of hydrolysis (5% WT). In addition, S423C and its corresponding double mutations were analyzed for comparison; the S423C MsbA protein showed a V_{max} similar to WT (68.5 nmol/(mg min)) and the S423C–E506Q and S423C–H537A proteins again showed significantly diminished rates of hydrolysis (1% and 4% WT, respectively; Table 2). Though the turnover rate for S423C is

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very similar to WT, the K_m value is somewhat increased (161 μM), indicating a potential decrease in affinity for ATP. Results of these experiments align well with those of the radioactive assay, and both clearly indicate that E506Q and H537A both have nonzero rates of ATP hydrolysis.

ATP Detection Assays Indicate Slow Hydrolysis Occurs

To determine if the E506Q and H537A mutants are capable of converting ATP to ADP, an ATP-detecting luminescent assay was carried out in the presence of added ATP using the E506Q and H537A purified proteins along with WT MsbA and BSA as positive and negative controls, respectively. These experiments were performed over 30 min using excess ATP (10 μM) and limiting protein (5 μM), and the results show that both controls functioned as expected; BSA does not convert ATP to another product, and WT MsbA converts all of the ATP to ADP (Figure 5A). Not surprisingly, H537A and E506Q showed a reduced ability to hydrolyze ATP over time. However, H537A hydrolyzed all of the available ATP within 2 h, while E506Q required 6 h to hydrolyze 10 μM ATP (Figure 5B). These results indicate that both H537A and E506Q are able to complete multiple hydrolysis cycles if given sufficient time and confirm that the rates of ATP hydrolysis reported above are indeed nonzero.

CW EPR Shows That the Introduction of the Dysfunctional Mutations Changes the Resting State and Alters Effects of ATP and MgATP Binding

CW EPR spectroscopy was chosen to assess local changes within critical nucleotide binding motifs of the MsbA NBD during the hydrolysis cycle in the presence of E506Q and H537A. Nine reporter sites within five conserved ATPase motifs of MsbA previously shown to report on local mobility changes upon addition of ATP, MgATP, or MgATP/V_i were each paired with E506Q and with H537A.

E506Q

All of the reporter–E506Q pairs studied by EPR showed mobility changes in the apo state when compared to the reporter in the c-less background (i.e., without E506Q) (top overlays, Figure 6), indicating that these sites within the NBD are experiencing an altered conformation solely due to the presence of the E506Q mutation within a 582-amino acid protein.

The Walker A motif is found at residues 378–384 in MsbA, and it has been suggested that it is involved in ATP binding by coming together with the opposite monomer's C-loop to form a "nucleotide sandwich".[10] S380C and I385C were chosen as the reporters for the Walker A motif for their changes upon the addition of ATP/EDTA (representing the ATP-bound state).[21] With the addition of the E506Q mutation to the S380C and I385C reporters, there are no longer observable motional changes upon the addition of ATP. As in the c-less background, I385C does not show further changes upon addition of Mg or V_i; however, the S380C–E506Q protein shows a significant new change in mobility upon the addition of MgATP/V_i or MgATP. This indicates that Mg affects the local environment within this motif or it induces additional ATP to bind to the protein or that hydrolysis is able to proceed.

The Q-loop (Q424) is thought to be involved in ATP binding and hydrolysis by hydrogen bonding to the Mg^{2+} and binding to the attacking water during hydrolysis or possibly in communicating with the transmembrane helices.[38] The reporters chosen to represent the Q-loop are S423C and V426C, located on each side of the motif. Each of these reporters was chosen for the changes observed in the c-less background upon the addition of MgATP/V_i, which mimics the immediate posthydrolysis state of MgADP/P_i. There is a slight change in mobility upon the addition of ATP/EDTA to both S423C-E506Q and V426C-E506Q (Figure

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6). While S423C-E506Q shows no further changes upon addition of Mg or V_i, as were seen in the c-less background, V426C-E506Q shows a significant change in mobility upon the addition of Mg. As is the case for S380C-E506Q described above, the MgATP an MgATP/V_i states are nearly identical in motion.

The C-loop (or LSGGQ motif, residues 481–485) is the signature sequence of an ABC transporter and is involved in ATP binding along with the Walker A motif.[10] Both S482C and Q485C were chosen as reporters for their changes in the MgATP/V_i state. Both mutants in the E506Q background show motional changes upon ATP binding and further changes upon the addition of Mg. Again, the MgATP and MgATP/V_i states show the same mobility. It is interesting to note that though the S482C–E506Q spectrum shows mobility changes upon the addition of Mg, the mobility increases rather than decreases upon ATP binding and then reverts to that observed in the apo state of the same protein. Again, no motional differences are observed between the MgATP and MgATP/V_i states in the C-loop.

The E506Q mutant is located just following the Walker B motif (501–505) in MsbA. The L504C reporter was chosen as the Walker B representative because it was the only site in this motif that showed observable motional changes by EPR, which were seen upon the addition of MgATP/V_i.[22] When coupled with the nearby E506Q mutation, the spin-label appears to maintain the same local environment throughout the hydrolysis cycle, indicating that this residue is no longer affected in the same way as in the c-less protein.

The final motif studied is the H-motif (H537), which is suggested to be involved in ATP binding by hydrogen bonding to its γ-phosphate. The reporters chosen for this motif are V534C and T541C, on either side of the motif, and each demonstrates small changes upon the addition of MgATP/V_i in the c-less background.[20,22] V534C–E506Q and T541C–E506Q both show no change upon addition of ATP and small changes upon the addition of MgATP or MgATP/V_i.

Briefly, the presence of the E506Q mutation results in conformational changes in the apo state of the protein at each of the NBD sites studied by CW EPR spectroscopy when compared to the c-less backgrounds previously investigated. And, new mobility changes are observed upon the addition of ATP in the Q- and C-loops, and significant changes occur in most of the motifs upon addition of Mg yet no differences are observed between the MgATP and MgATP/V_i spectra. These results could suggest that Mg affects the conformation of the protein, aids ATP in binding more tightly to the protein, or allows hydrolysis to occur without efficiently releasing the products or causing local conformational rearrangements.

H537A

As stated earlier and shown in two crystal structures, the H-motif is predicted to be involved in hydrogen bonding to the γ-phosphate of ATP, possibly in conjunction with E506.[10,13,19,39,40] Similar to the E506Q studies described above, each of the reporters was also paired with H537A and analyzed for changes in motion upon the addition of ligands. Two mutational pairs in this set, S380C–H537A and T541C–H537A, were so detrimental in combination to the protein fold or cellular function that they were unable to be expressed and purified.

With the exception of S482C, each site coupled with H537A studied by EPR showed a decrease in mobility in the apo state when compared to the reporter in the c-less background (top overlays, Figure 6). Changes in the apo states for Q485C and V534C with H537A were more drastic than seen with the E506Q, but in general the nucleotide-induced changes were more subtle than those observed in the presence of E506Q.

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V426C–H537A becomes slightly more immobile upon addition of ATP, I385C–H537A shows a very slight change in spectral breadth upon the addition of ATP, and the remaining sites are unaffected by the presence of ATP.

The Q-loop sites (S423C and V426C) are both affected by the addition of Mg, which is the step in which they were affected in the c-less background,[22] Q485C–H537A shows a subtle change in mobility upon the addition of Mg, and the remaining motifs are unaffected. However, unlike the other sites studied, V426C–H537A shows a mobility difference between the MgATP and MgATP/V_i states. This combination appears to be the only one attempting to revert to a more apo- or ATP-like conformation after the addition of MgATP, indicating that this site is reporting on changes occurring due to successful hydrolysis.

The lack of significant changes upon the addition of ATP could indicate that H537A does not bind ATP or that it requires Mg to bind ATP; however, H537A was shown to bind ATP as well as WT in the TNP-ATP assay in the absence of Mg. And, the motional changes observed in the Q- and C-loops upon addition of MgATP/V_i indicate that Mg and V_i affect this motif differently than Mg alone. Thus, it is more likely that the protein is already in an ATP-bound-like state in the presence of H537A

AMP-PNP Analysis

In order to identify whether the effects observed after addition of Mg were the result of increased ATP binding or the result of hydrolysis in E506Q and H537A, the effects of the addition of ATP and AMP-PNP, a nonhydrolyzable ATP analogue, were compared using the Q485C–E506Q and V426C–H537A pairs.

Binding of MgATP or MgAMP-PNP to MsbA induced significant changes in local mobility compared to the apo state, as observed in their EPR spectra. The mobility changes in the EPR spectra of Q485C–E506Q upon the addition of ATP/EDTA (Figure 6) or AMP-PNP/EDTA (spectrum not shown) were comparable to each other, though ATP had a very slightly greater effect than AMP-PNP. Upon addition of Mg, slight changes were observed in both spectra. However, the fact that the MgATP and MgAMP-PNP spectra are nearly identical for this pair suggests that either hydrolysis is not occurring or hydrolysis of ATP does not induce further local conformational changes at this site in the E506Q protein (Figure 8A). In contrast, significant changes were observed upon comparison of the MgATP and MgAMP-PNP spectra for V426C–H537A (Figure 8B), indicating that hydrolysis is occurring in the H537A protein and causing local conformational rearrangements. These data strongly corroborate the biochemical data presented above for time points less than 1 h (Figure 5) and indicate that H537A is able to hydrolyze more ATP than E506Q on the time scale of the EPR experiments.

DEER Spectroscopy Indicates That Some Population of the E506Q and H537A Mutants Are Purified in the Closed Conformation

The DEER technique allows for interspin distance measurements between ~20 and 55 Å. Given the predicted C_α–C_α distances of 18–37 Å between residues in this study as determined by the crystallized MsbA homodimer in the closed conformations,[9] this is an appropriate distance window in which to measure the closed homodimer conformation. Using spin-labels at S423C as reporters for the conformational status of MsbA containing the E506Q or H537A mutation, the DEER data in Figure 9 demonstrate that at least some population of both proteins is found in the closed conformation (spin-labels are 28 Å apart in all three samples) even in the apo state. This supports the altered protein conformations observed in the EPR spectra in the apo states of many of the reporter pairs and suggests that the E506Q and H537C proteins are both purified in the closed dimer conformation. As can

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be seen in Figure 9, based on the echo amplitudes of the dipolar evolutions, a very small population of dimers are in the closed conformation for the S423C mutation in the c-less background, and E506Q promotes a greater population of closed dimers than H537A.

Dipolar evolution and distance distributions did not change upon addition of nucleotide, Mg, or V_i (data not shown), indicating that the conformational changes observed through CW EPR motional analysis are local changes due to ligand binding and the apo state spectrum likely already represents a closed dimer protein conformation.

Nucleotide Detection Assays Report That No Nucleotide Is Present in the Purified Proteins

Upon discovering that at least some, if not most, of the purified E506Q and H537A proteins are in the closed dimer conformation and given the hypothesis that a nucleotide is likely required to form this conformation, we attempted to identify the nucleotide trapped within the purified proteins using the ATP and ADP luminescence detection kits. It was discovered that there is no nucleotide present within the WT (negative control), E506Q, or H537A purified proteins, suggesting that either nucleotide is not required to induce a closed conformation of E506Q or H537A MsbA or the ATP or hydrolysis products bound *in vivo* are lost during protein purification (data not shown). Yet, the protein remains in a closed conformation even without nucleotide. These results are also consistent with the ability of these proteins to bind TNP-ATP in the binding assay and the observable local conformational changes induced by further addition of ligands in the CW EPR spectroscopy experiments.

DISCUSSION

The studies completed here investigate the mechanism of dysfunction of the E506Q and H537A point mutations in the MsbA ABC transporter.

The inability of the MsbA proteins containing the E506Q or H537A point mutations to sustain cell growth is likely due to the protein binding and/or hydrolyzing ATP and closing *in vivo* and then unable to reopen and thus unable to provide the necessary transport of lipid A for cell survival. Even though hydrolysis is able to slowly occur, as could be concluded from the *in vitro* ATPase and ATP detection assay results, if the protein does not reopen, it is no longer useful as a transporter. Our data show that the proteins are still able to bind ATP and slowly hydrolyze ATP to ADP and P_i yet become locked into a closed dimer conformation even when the hydrolysis products are no longer present. If ATP can rebind the closed dimer, then it is reasonable that there is space for the nucleotide to dissociate and create an empty, closed homodimer structure. This closed conformation could also explain the low K_m values for E506Q and H537A, where ATP is not as free to dissociate as it would be in the open conformation.

The DEER data support the speculation that the protein gets trapped in the ATP-bound or posthydrolysis state with the hydrolysis products trapped in the binding pocket. This is similar to the occluded state model proposed for Pgp E552A/E1197A, which suggests that loose binding of MgATP at both NBDs (in combination with ligand binding) results in dimer closure with only one tightly bound MgATP.[41] Thus, in an effort to determine which nucleotide the purified E506Q and H537A mutants contain natively, a luminescent assay that detects the presence of ATP or ADP was carried out on purified E506Q and H537A MsbA proteins and surprisingly showed that there is no nucleotide in the protein upon purification. This indicates that the nucleotide that is trapped *in vivo* is lost sometime during the purification process and that the protein continues to be trapped closed even in the absence of the nucleotide that causes the initial trapping. Alternatively, the intrinsic

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sampling of conformational states *in vivo* even in the absence of ligand or hydrolysis maybe sufficient to generate the interactions required to maintain the closed dimer conformation.

Fluorescence data indicate that both E506Q and H537A retain their ability to bind ATP. These data also support the above conclusion that the proteins must not have nucleotide bound prior to the assay or they would not have WT-like affinity for the TNP-ATP. Compared to published binding values for other ATPase dimers (K_d examples: MalK, 150 μM;[42] MJ0796, 14 μM;[10] HlyB-NBD, 6.5–7.23 μM;[19] OpuA, 0.9 uM[43]), the entire WT MsbA transporter has a K_d for TNP-ATP of 0.32 μM. Using a different method to detect binding of TNP-ATP to MsbA, another group reported K_d values with significantly lower affinity (50 μM).[44]

ATPase data show significantly reduced hydrolysis rates for the E506Q and H537A proteins. The values, which range from 1 to 10% of WT for the reporter pairs and 5 and 8% for E506Q and H537A, respectively, are slightly above background levels. However, all but three of the double mutations, as well as E506Q and H537A, have nonzero hydrolysis rates as indicated by the standard errors in the radioactive ATPase assay as well as corroborating nonzero V_{max} values from an independent assay for E506Q and H537A. This, plus the ATP detection assay and CW EPR data, further suggests that hydrolysis does occur over time.

Apo states of each of the reporter mutant pairs studied by CW EPR motional analysis tend to reflect a conformation more in line with the MgATP/V_i state for the reporters observed in the c-less background, and of the five conserved NBD motifs studied, all were motionally affected on some level. In the case of the H537A mutation, not only are each of the five conserved motifs affected by the addition of this point mutation, but the mutation is so detrimental to protein when combined with reporter residues that are otherwise active that the S380C–H537A from the Walker A and T541C–H537A reporting for the H-motif mutants could not be purified. Because the Walker A is thought to bind ATP initially, and only upon dimer closure bring the ATP into contact with the C-loop, Walker B, and H-motifs, the fact that the mobility of the spin-labels at all nine reporters is altered in the apo state upon inclusion of E506Q or H537A indicates that either these point mutations are able to alter the conformational state of the entire NBD or the protein is in a closed state upon purification. This latter possibility is backed up by the fact that the DEER data show a distance of 28 Å between the two S423C sites within the E506Q and H537A homodimers even in the apo state.

The identical MgATP and MgATP/V_i spectra for all but one pair studied also suggests that the proteins are already locked closed and not in need of V_i to lock in the hydrolysis products in a closed dimer conformation as it is in the c-less background experiments. The data show that many of the spectra report motional differences between ATP and MgATP states, which could be due to Mg aiding ATP in binding more tightly to the protein, to changes induced by the binding of Mg itself, or to hydrolysis-induced changes in a small amount of the proteins. AMP-PNP experiments also indicated that H537A shows local conformational changes due to slow hydrolysis of ATP.

The results reported here that E506Q can hydrolyze ATP very slowly over time are consistent with the nonzero (i.e., 10%–15%) activity data reported for HlyB-NBD E631Q[12] and PDR5 E1306Q,[17] but not similar to the statements reported for E to Q mutants in BmrA, HlyB-NBD, MalK, and MJ0796 that hydrolysis is defective in these proteins.[14–16,19] In addition, the rate of hydrolysis obtained for MsbA E506Q is similar to the 4.4 nmol/(mg min) rate observed for the equivalent mutations (E552Q/E1197Q) in Pgp, which is also similar to that observed for Pgp E552A/E1197A (5.0 nmol/(mg min)).[45] And, the results reported here that H537A can also fully hydrolyze ATP, but not on the short time scale of

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the ATPase assay, are only somewhat consistent with the WT-like activity reported for PDR5 H1068A.[17] The comprehensive approach applied to the study of MsbA E506Q and H537A has provided data for the first time that unequivocally state that these point mutations are able to hydrolyze multiple ATPs in the *E. coli* MsbA transporter.

The similarities in the impaired function of the E506Q and H537A mutations that create altered resting state conformations and reduce the number of changes throughout the hydrolysis cycle suggest that, since the native E506 and H537 residues may interact, a point mutation at either residue results in a similar dysfunctional state caused by the absent interaction. It is remarkable that single point mutations can generate new inter-molecular interactions within a large protein structure that are unable to be broken even in the absence of nucleotide.

CONCLUSIONS

With these studies, we have confirmed that the NBD MsbA mutants E506Q and H537A lack cell viability and do not support growth. We report for the first time that they are able to bind ATP with similar affinity as WT and report dissociation constants. However, these proteins can conduct ATP hydrolysis if given sufficient time. The lack of cell growth and viability demonstrates that lipid A transport is not occurring, resulting in cell death. ATP binding experiments indicate that the dysfunction is not in nucleotide binding but in lack of hydrolysis or release of nucleotide. The ATPase activities show a very limited, but nonzero, ability to hydrolyze ATP, whereas if given sufficient time, the ATP detection assay reports that both E506Q and H537A are capable of hydrolyzing multiple ATP molecules to ADP. The CW EPR mobility data support these biochemical conclusions, and the DEER data provide new evidence that the protein becomes locked closed *in vivo* and does not reopen during purification despite the lack of bound nucleotide. The new local interactions between the NBD monomers in these mutant proteins that inhibit its ability to reopen suggest that active conformational changes normally occur to reopen the closed ATPase dimer structure.

Acknowledgments

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ABBREVIATIONS

ABC	ATP-binding cassette
AMP-PNP	5′-adenylyl-β,γ-imidodiphosphate
CL	cardiolipin
CW	continuous wave
DM	dodecylmaltopyranoside
DTT	dithiothreitol
EPR	electron paramagnetic resonance
MAL-6	4-maleimido-TEMPO
MTSL	2,2,5,5-tetramethylpyrroline-3-yl-methanethiosulfonate spin-label

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NBD	nucleotide binding domain
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
SDSL	site-directed spin-labeling
TNP-ATP	2′-(or 3′)-*O*-(trinitrophenyl)adenosine 5′-triphosphate, trisodium salt
WT	wild-type

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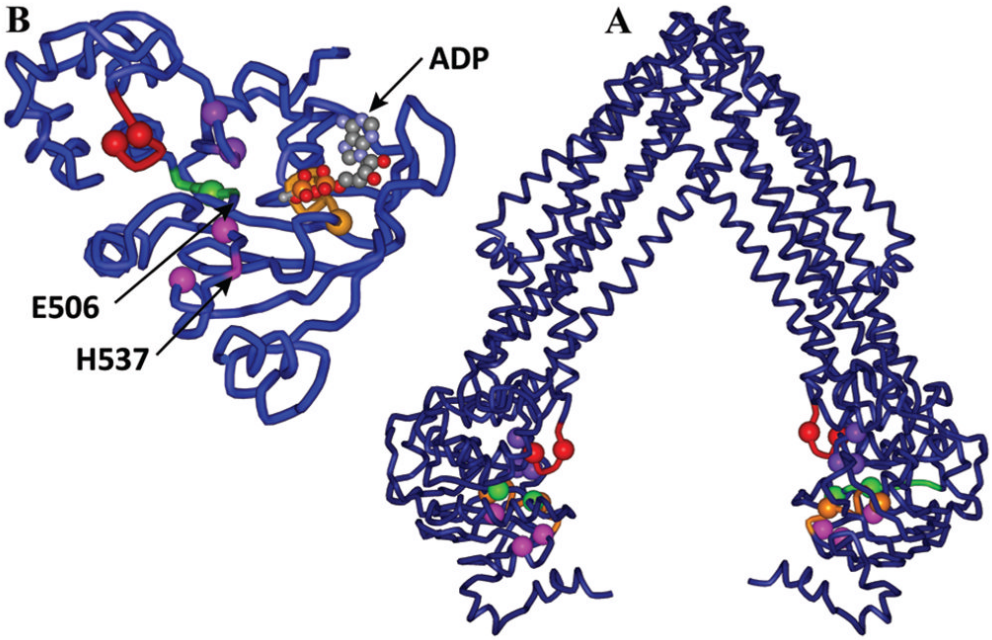

Figure 1.

Structure of MsbA. (A) Apo MsbA (PDB: 3B5W) and (B) MsbA NBD with ADP/V_i (labeled arrow and colored by element) (3B5Z).[2] Domains studied here are colored: LSGGQ (red), Walker A (orange), Walker B (green), H-motif (H537) (magenta), and Q-motif (purple). The nine reporter sites studied within and surrounding these motifs are indicated by colored spheres, and arrows point to the locations of E506 (immediately follows the Walker B) and H537 (H-motif).

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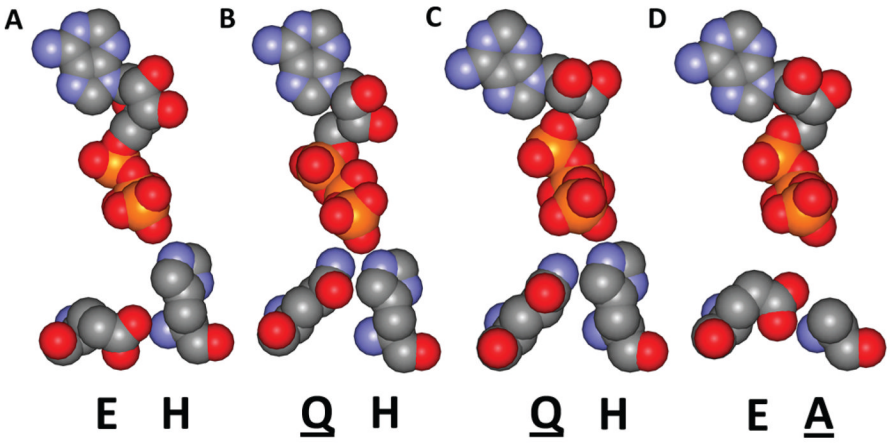

Figure 2.

ATP, E506, E506Q, H537, and H537A arrangement in the MalK and HlyB-NBD crystal structures. (A) WT MalK (1Q12), (B) E159Q MalK (2R6G), (C) E631Q HlyB-NBD (2FGK), and (D) H662A HlyB-NBD (2FGJ). When glutamate is changed to glutamine in both structures, the side chain changes orientation to point in the direction of the ATP with potential new contacts with the ATP molecule. When histidine is changed to an alanine, the interaction between histidine and ATP is lost.

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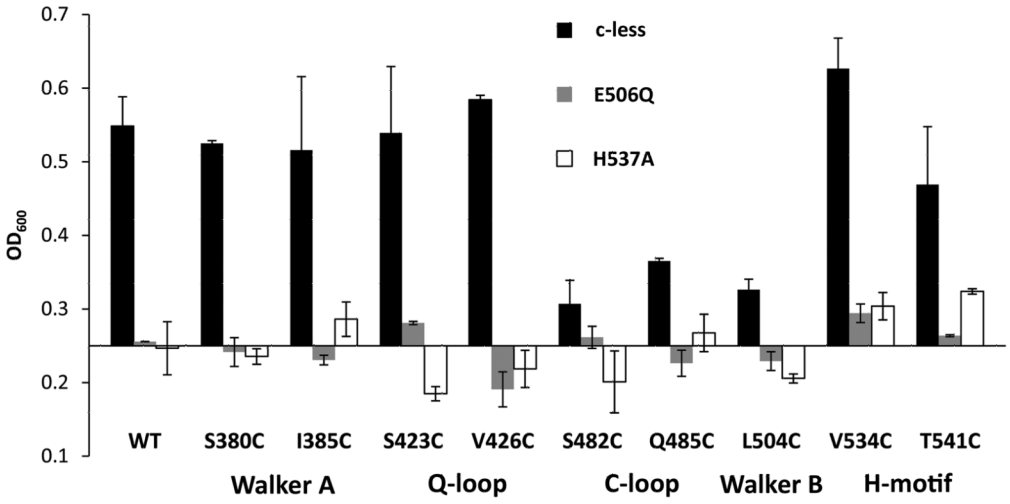

Figure 3.

In vivo growth assay results for NBD reporters alone (black, ref 22) and with E506Q (gray) and H537A (white). The *in vivo* growth assay is used to determine the ability of each plasmid-encoded mutant to maintain cell viability at 44 °C, after which the temperature sensitive chromosomal copy of MsbA is inactivated. Error bars represent the standard deviation for experiments run twice in triplicate.

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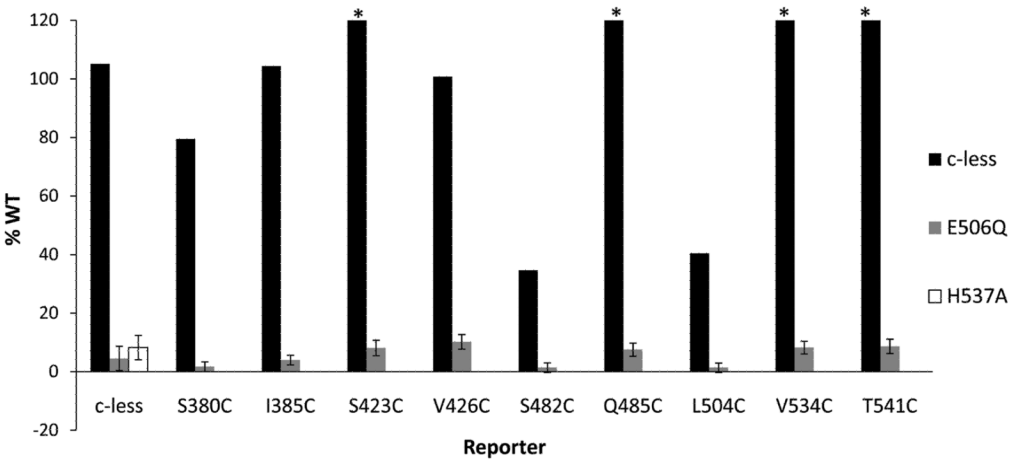

Figure 4.

In vitro ATPase activity results. Rates of hydrolysis over 2 min at 37 °C as compared to WT are reported for the NBD reporters,[22] E506Q, H537A, and the NBD reporters with E506Q. Standard deviations for the E506Q and H537A data represent results carried out in triplicate. *Values greater than 120% of WT: S423C (244%), Q485C (146%), V534C (153%), T541C (140%).

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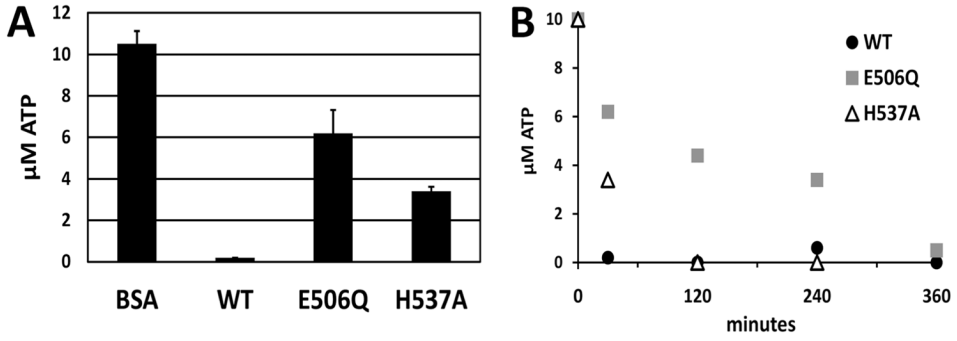

Figure 5.

Nucleotide detection assay results. (A) BSA (negative control), WT MsbA (positive control), E506Q MsbA, and H537A MsbA (5 μM) were analyzed using a luminescence assay for remaining ATP concentration after incubation with 10 μM ATP and excess Mg for 30 min at room temperature. (B) Additional time points show that the H537A and E506Q proteins hydrolyzed all available ATP after 2 and 6 h incubations, respectively.

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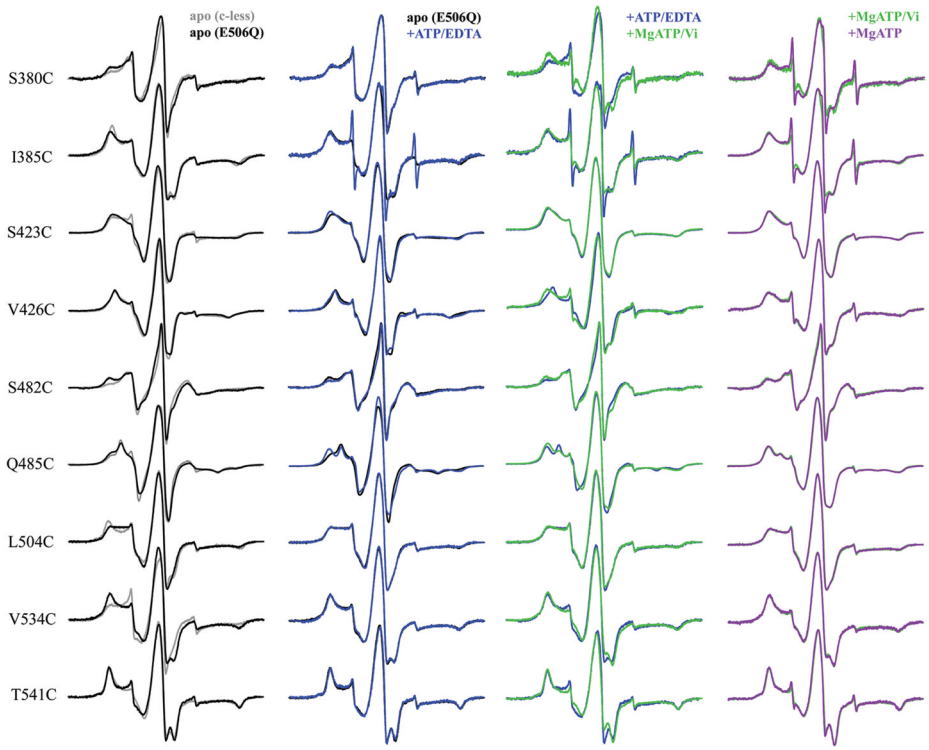

Figure 6.

X-band CW EPR spectra of MsbA reconstituted into inner membrane lipids at various stages of the hydrolysis cycle. The figure indicates the color coding of each reporter residue alone[20–22] as well as with the E506Q mutation in the apo state, in the presence of ATP, MgATP/V_i, and MgATP.

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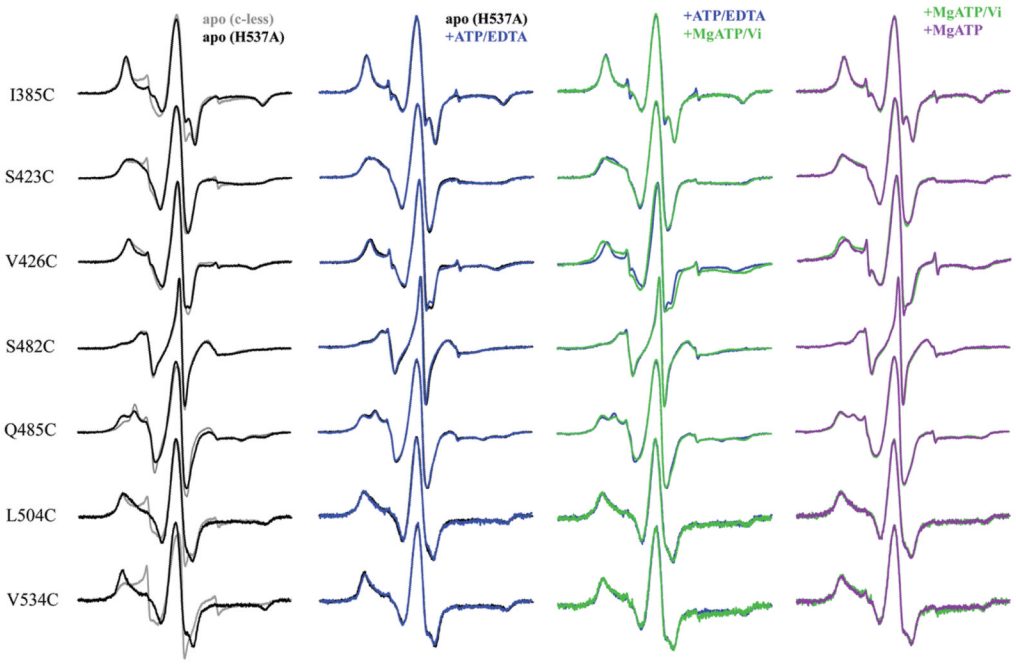

Figure 7.

X-band CW EPR spectra of MsbA reconstituted into inner membrane lipids at various stages of the hydrolysis cycle. The figure indicates the color coding of each reporter residue alone[20–22] as well as with the H537A mutation in the apo state, in the presence of ATP, MgATP/V_i, and MgATP. The S380C–H537A and T541C–H537A proteins were not able to be purified.

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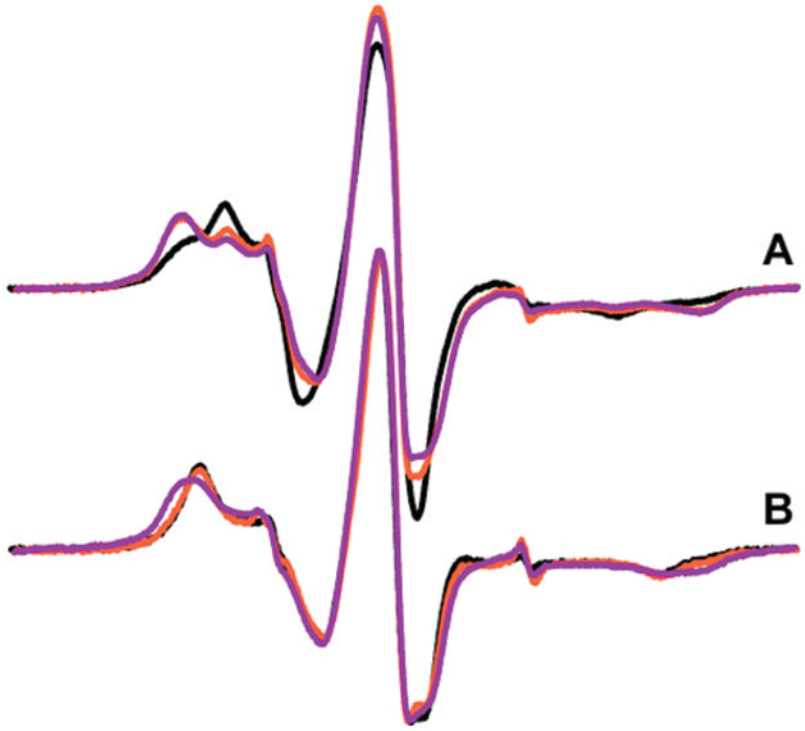

Figure 8.
X-band CW EPR spectral comparison of the MgAMP-PNP-and MgATP-bound (A) Q485C–E506Q and (B) V426C–H537A proteins. Spectra are colored as apo (black), MgATP (purple), and MgAMP-PNP (orange).

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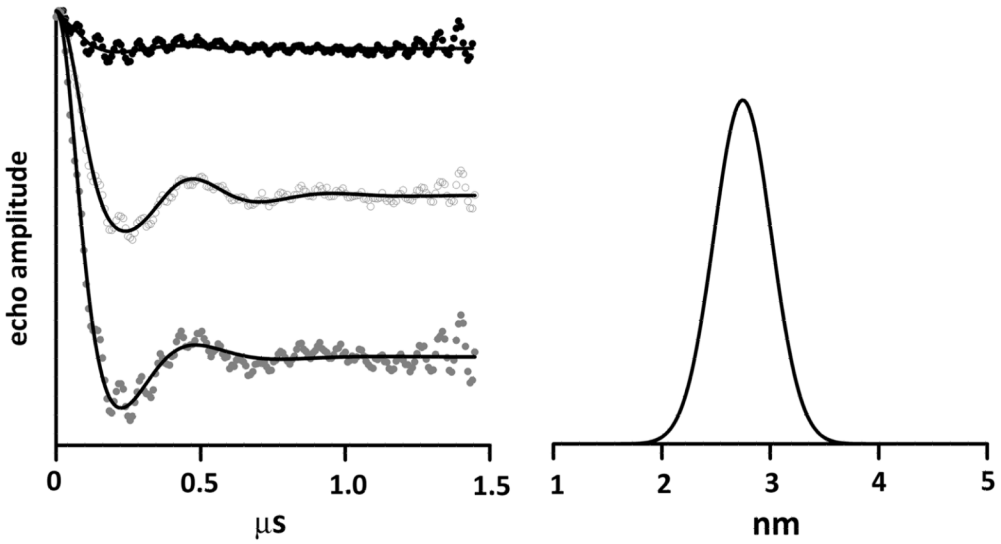

Figure 9.

X-band background-corrected DEER evolutions (left) for S423C (black), S423C–E506Q (gray), and S423C–H537A (white) and the corresponding distance distribution (right) following protein purification in the absence of added ligand. The same concentration of protein was used in each of the samples; thus, the spectral overlays represent a direct comparison of spin populations in the closed dimer conformation. All three samples gave a distance distribution centered on 28 Å.

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Table 1

Dissociation Constants for TNP-ATP Binding to MsbA

mutant	K_d (μM)	std error
WT	0.32	0.05
E506Q	0.51	0.01
H537A	0.44	0.11

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Table 2

ATP Hydrolysis Values for MsbA

mutant	V_{max} (nmol/(mg min))	K_m (μM ATP)
WT	68	117
c-less	48	91
E506Q	3.4	12
H537A	3.5	2
S423C	69	161
S423C–E506Q	0.7	<5
S423C–H537A	3.0	5