



ATP binding to two sites is necessary for dimerization of nucleotide-binding domains of ABC proteins



Maria E. Zoghbi, Guillermo A. Altenberg*

Department of Cell Physiology and Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, TX 79430-6551, USA
Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, TX 79430-6551, USA

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ABSTRACT

ATP binding cassette (ABC) transporters have a functional unit formed by two transmembrane domains and two nucleotide binding domains (NBDs). ATP-bound NBDs dimerize in a head-to-tail arrangement, with two nucleotides sandwiched at the dimer interface. Both NBDs contribute residues to each of the two nucleotide-binding sites (NBSs) in the dimer. In previous studies, we showed that the prototypical NBD MJ0796 from *Methanocaldococcus jannaschii* forms ATP-bound dimers that dissociate completely following hydrolysis of one of the two bound ATP molecules. Since hydrolysis of ATP at one NBS is sufficient to drive dimer dissociation, it is unclear why all ABC proteins contain two NBSs. Here, we used luminescence resonance energy transfer (LRET) to study ATP-induced formation of NBD homodimers containing two NBSs competent for ATP binding, and NBD heterodimers with one active NBS and one binding-defective NBS. The results showed that binding of two ATP molecules is necessary for NBD dimerization. We conclude that ATP hydrolysis at one nucleotide-binding site drives NBD dissociation, but two binding sites are required to form the ATP-sandwich NBD dimer necessary for hydrolysis.

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1. Introduction

ATP-binding cassette (ABC) proteins comprise one of the largest protein superfamilies, extending from bacteria to man, with most members mediating transmembrane transport [1,2]. Their core functional unit consists of two transmembrane domains and two nucleotide-binding domains (NBDs) [1,2]. ABC proteins include importers and exporters. Exporters have varied functions, such as multidrug-resistance proteins that pump anticancer agents out of cells, the Cl[−] channel CFTR, and channel regulators such as the sulfonylurea receptor [1–3]. NBDs are the engines of ABC proteins, responsible for nucleotide binding and hydrolysis, and their struc-

ture is well conserved despite the dissimilar functions of specific proteins [1,2].

Crystal structures have shown that NBDs form dimers in a head-to-tail arrangement, where two ATP molecules are sandwiched at the dimer interface (Fig. 1A) [1,4,5]. Each of the two nucleotide-binding sites (NBSs) in the dimer is formed by the Walker A motif, Walker B motif, A loop, H loop and Q loop of one NBD, and the D loop and signature motif of the other NBD (Fig. 1A) [1,4,5]. The Walker A motif is involved in binding of nucleotide phosphates and the Walker B, together with the Q loop, is involved in Mg²⁺ and water coordination at the catalytic site. The A loop contains a conserved aromatic residue that interacts with the adenine ring of ATP, whereas the signature motif and D and H loops are involved in the coordination of the γ phosphate of ATP.

The isolated NBD MJ0796 from the thermophile *Methanocaldococcus jannaschii* is an excellent experimental model because of the abundant structural and functional information available [5–11]. MJ0796 forms ATP-bound dimers [5,6,8–10] that dissociate following ATP hydrolysis at one of the two catalytically-active NBSs [10]. The finding that dimer dissociation is driven by a single ATP hydrolysis event brings back a long-standing question: Why do all ABC proteins have two NBSs? Although formation of a stable NBD dimer with two bound ATPs has been clearly demonstrated in 3D crystals using ATP-deficient mutants and non-hydrolyzable ATP analogs, the possibility of stable or transient dimers with only

Abbreviations: 8-azido-ATP-biotin, 8-N₃ATP-2',3'-biotin-long chain-hydrazide; ABC, ATP-binding cassette; LRET, luminescence (or lanthanide-based) resonance energy transfer; MJ, single-Cys mutant G14C based on MJ-CL; MJ-CL, Cys-less single-Trp mutant MJ0796-C53G-C128I-G174W; MJ-K44A, mutant based on MJ in which Lys44 was replaced with Ala; MJ-K44E, mutant based on MJ in which Lys44 was replaced with Glu; MJ-S42F, mutant based on MJ in which Ser42 was replaced with Phe; MJ-Y11A, mutant based on MJ in which Tyr11 was replaced with Ala; MJ1, single-Cys mutant E171Q based on MJ; NBD, nucleotide-binding domain; NBS, nucleotide-binding site.

* Corresponding author at: Department of Cell Physiology and Molecular Biophysics, Texas Tech Health Sciences Center, Lubbock, TX 79430-6551, USA.

E-mail addresses: maria.zoghbi@ttuhsc.edu (M.E. Zoghbi), g.altenberg@ttuhsc.edu (G.A. Altenberg).

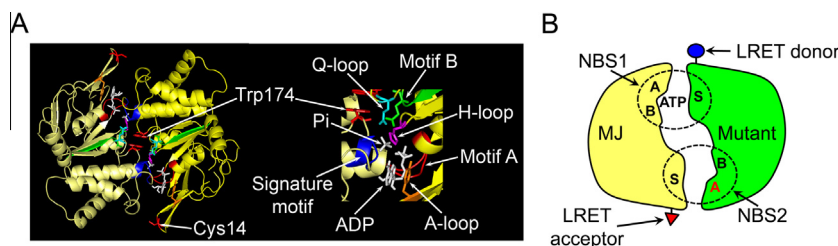


Fig. 1. Structure of the NBD dimer. (A) Ribbon representation of the two monomers (in different tones of yellow). A zoomed view of one nucleotide-binding site (NBS) is shown on the right. ADP and Pi (white, sticks); Walker A motif (red, motif A); Walker B motif (green, motif B; Gln171 in sticks); signature motif (blue); Tyr11 (orange, sticks, A-loop); His204 (magenta, sticks, H-loop); Gln90 (cyan, sticks, Q-loop); Cys14 (Gly14 to Cys mutation, red, sticks, left panel); and Trp174 (red, sticks, center of dimer interface). The figure corresponds to the nucleotide-bound MJ (based on PDB 3TIF), where the catalytic Glu171 is replaced with Gln. (B) Schematic representation of an NBD heterodimer formed by MJ and an ATP-binding-deficient mutant. The heterodimer has one intact NBS (NBS1) and an ATP-binding-deficient NBS (NBS2), where Walker A residues of one NBD monomer were mutated (red A). Letters A, B and S denote the Walker A, Walker B and signature motifs, respectively.

one ATP bound has not been explored. We speculate that binding of two ATP molecules is necessary to form a stable ATP-bound dimer, without which ATP hydrolysis at one NBS cannot occur. To address this question, we used luminescence resonance energy transfer (LRET) [12] to measure ATP-induced formation of NBD dimers with one or two binding-competent NBSs (Fig. 1B). LRET is a spectroscopic technique that allows for measurements of distance changes between a donor and an acceptor attached to a protein, with Angstrom resolution and in real time. We have proven its usefulness to study the association/dissociation process of isolated NBDs [9,10] and NBDs in a full length ABC transporter [13]. Our general strategy was to determine the association of dimers formed by an NBD with a normal NBS, and a mutant NBD with a defective NBS. NBDs were labeled with either LRET donor or acceptor probes, and mixed to produce normal/normal, mutant/mutant, and normal/mutant dimers. Under these conditions, donor/acceptor pairs are present only in the heterodimers, allowing for selective measurements from those dimers in the complex population.

2. Material and methods

2.1. Protein expression and purification

Mutants of *M. jannaschii* MJ0796 were expressed in *Escherichia coli* and purified by anion-exchange and gel-filtration chromatography as described [8,9]. The properties of the Cys-less MJ0796-G174W (MJ-CL, Cys53 and Cys128 replaced with Gly and Ile, respectively) and the two single-Cys mutants MJ0796-G174W-G14C (MJ) and MJ0796-G174W-E171Q-G14C (MJl) have also been published [8,9]. MJ is active, whereas MJl is hydrolysis deficient due to the replacement of the catalytic carboxylate Glu171 with Gln, but binds ATP with high affinity [8–10]. New mutants were generated on the MJ background and were: MJ-K44A (replacement of the conserved Lys44 of the Walker A motif with Ala), MJ-K44E (replacement of the conserved Lys44 of the Walker A motif with Glu), MJ-S42F (replacement of the Walker A motif Ser42 with Phe) and MJ-Y11A (replacement of the A-loop conserved aromatic residue Tyr11 with Ala). All the proteins have a Trp at position 174 (Gly 174 replaced with Trp). Trp174 is a good probe to assess dimerization by Trp quenching [8,9]. In size-exclusion chromatography in the absence of ATP, all mutants run as a single peak at the position of monomeric MJ.

2.2. ATPase activity, tryptophan fluorescence and LRET

Details on these methods have been published [8–10,13,14], and more details are presented under [Supplementary Material](#).

3. Results and discussion

3.1. Characterization of ATP-binding defective mutants

Dissociation of ATP-bound dimers follows a single ATP hydrolysis event [10]. However, there is still the question of the universal presence of two NBSs in ABC proteins. A possible explanation is that formation of a stable dimer, where at least one of the NBSs can hydrolyze ATP, requires two ATP molecules bound. This is the central hypothesis of this study. To test it, we generated mutants defective in ATP binding by targeting residues that have been shown to interact with ATP in NBD crystal structures [5,8]. The conserved Lys in the Walker A motif (K44 in MJ) and the non-conserved Walker A residue Ser42 interact with ATP phosphates, while Tyr11 is a conserved aromatic residue involved in π - π interactions with the ATP adenine ring, forming the A-loop upstream of the Walker A motif (Fig. 1A) [5,8,15,16]. As expected, the mutants MJ-K44A, MJ-K44E, MJ-S42F, and Y11A displayed decreased binding of 8-azido-ATP under conditions where the photolabeling of MJ was maximum (Fig. 2A). Similarly to MJl, the mutants MJ-K44A, MJ-K44E and MJ-S42F were catalytically deficient, and had activities <3% of the activity of MJ (0.31 ± 0.02 ATP/s, $n = 8$), whereas MJ-Y11A retained ~75% of the normal activity (0.24 ± 0.05 ATP/s, $n = 4$). Similar effects of equivalent mutations on ATP binding and/or ATP hydrolysis have been reported for other ABC transporters [17–22].

The ability of the mutants to dimerize in response to ATP was evaluated by the fluorescence quenching of a single Trp introduced at the center of the dimer interface (Trp174, Fig. 1A). The crystal structure of the ATP-bound dimer shows the two Trp174 residues (one from each NBD) forming a parallel π -stacking interaction that results in quenching of Trp174 fluorescence and a blue-shift of the emission peak [8]. We have shown that Trp174 is a good reporter of MJ association/dissociation [8,9], with the high fluorescence observed in absence of ATP (monomeric NBDs) decreasing in a [ATP] dependent fashion as the NBDs dimerize. From Trp174 quenching data in MJ, we determined K_d values for NaATP-induced and MgATP-induced dimerization (K_{dNaATP} and K_{dMgATP} , respectively) of ~50 and 5 μ M, respectively [8,9]. For the MJ-K44A mutant (Fig. 2B) the fluorescence decreased slightly in 5 mM NaATP (blue) compared to that in the absence of ATP (black), whereas quenching was almost complete in 1 mM MgATP (red). The absence of complete quenching by MgATP agrees with prior results for MJ, where we have observed a maximal fluorescence quenching of ~80%, under conditions that promote complete NBD dimerization, with the remaining fluorescence interpreted as the sum of emission from quenched Trp in dimers and Tyr emission [8]. The calculated K_{dMgATP} of MJ-K44A was 70 ± 13 μ M ($n = 3$), ~15-fold higher than

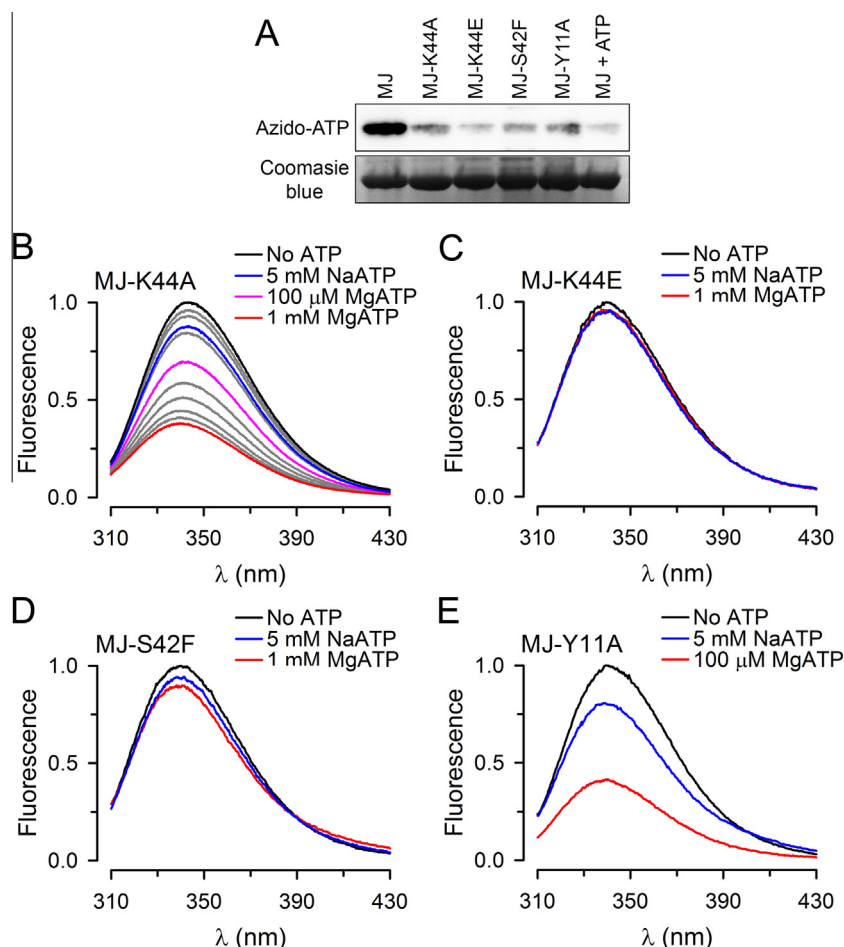


Fig. 2. Effect of mutations on ATP binding. (A) Binding of azido-ATP. Proteins (10 ng) were incubated with the 0.2 mM of the ATP analog 8- N_3 ATP-2',3'-biotin-long chain-hydrazide (8-azido-ATP-biotin, Affinity Photoprobes, USA). After 5-min incubation at room temperature the samples were irradiated at 254 nm, and crosslinking to the photo-sensitive probe products was analyzed in Western blots using a horse radish peroxidase anti-biotin antibody with chemiluminescence detection. Top: Western blot with anti-biotin antibody showing decreased binding for all mutants compared to MJ. Bottom: Coomassie blue-stained gel. Pre-incubation of MJ with 0.5 mM ATP dramatically decreased 8-azido-ATP crosslinking (MJ + ATP). (B) Effect of ATP on MJ-K44A Trp emission. For the MgATP experiments, $MgCl_2$ was kept at 10 mM and ATP was increased progressively from zero (black) to 10, 20, 50 μ M (all gray), 100 μ M (magenta), 200, 300, 400 and 500 μ M (all gray), and 1 mM (red). The response to 5 mM ATP in the absence of Mg (5 mM NaATP, blue) was obtained from an independent experiment. (C) Effect of ATP on MJ-K44E Trp emission. The traces in 5 mM NaATP (blue) and 1 mM MgATP (red) are from separate experiments. (D) Effect of ATP on MJ-S42F Trp emission. The traces in 5 mM NaATP (blue) and 1 mM MgATP (red) are from separate experiments. (E) Effect of ATP on MJ-Y11A Trp emission. Effects of 5 mM NaATP (blue) and 100 μ M MgATP (red) from separate experiments. All traces were normalized to the peak fluorescence in absence of ATP (black) and are representative of at least 3 independent experiments. The ATP-free solution was divalent cation-free and contained 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM Tris/HCl, pH 7.6.

that of MJ ($\sim 5 \mu$ M) [8]. The K_{dNaATP} was not quantified due to the small response to high [NaATP]s. These results show that the increased affinity for MgATP vs. NaATP observed for MJ [5,8] is also displayed by the MJ-K44A mutant. The mutants MJ-K44E (Fig. 2C) and MJ-S42F (Fig. 2D) showed very limited dimerization, even at high concentrations of NaATP and MgATP, indicating a severe reduction in affinity for ATP. MJ-Y11A (Fig. 3E) also showed reduced affinity for ATP compared to MJ. However, the decrease in affinity was not as pronounced as that of the other mutants (see the large response to 100 μ M MgATP, red trace), consistent with the fairly conserved ATPase. In summary, the results show that three of the tested mutants (MJ-K44A, MJ-K44E and MJ-S42F) display a major reduction in affinity for ATP. Of these mutants, MJ-K44A seemed particularly useful for our studies because it had reduced affinity for NaATP and MgATP, but still displayed significant dimerization at saturating MgATP concentrations (Fig. 2B); MJ-K44A is expected to behave as non ATP binder in the presence of NaATP or at low micromolar MgATP concentrations, but should bind MgATP at higher concentrations.

3.2. Formation of NBD heterodimers with one normal and one ATP-defective binding site

In MJ homodimers the two identical NBSs can bind two ATPs with high affinity, but heterodimers of MJ with MJ-K44A, MJ-K44E or MJ-S42F have a "normal" binding site (NBS1) and a defective binding site (NBS2) because of the Walker A motif mutations (Fig. 1B). Here, we used LRET on single-Cys NBDs labeled with either LRET donor (Tb^{3+}) or acceptor (fluorescein) probes to determine whether two ATP-binding-competent NBSs are needed to form a stable NBD dimer. Mixing of MJ and mutant NBDs produces MJ homodimers, mutant homodimers, and MJ/mutant heterodimers, but donor/acceptor pairs are present only in the heterodimers. Fig. 3A shows the typical increase in fluorescein emission after the addition of a saturating [NaATP] of 0.5 mM to a mix of Tb^{3+} -labeled MJ and fluorescein-labeled MJ. We have demonstrated that the slow increase in fluorescence that occurs during the course of minutes is the result of ATP-induced dimerization [9,10]. In the absence of ATP the intensity is low because the mono-

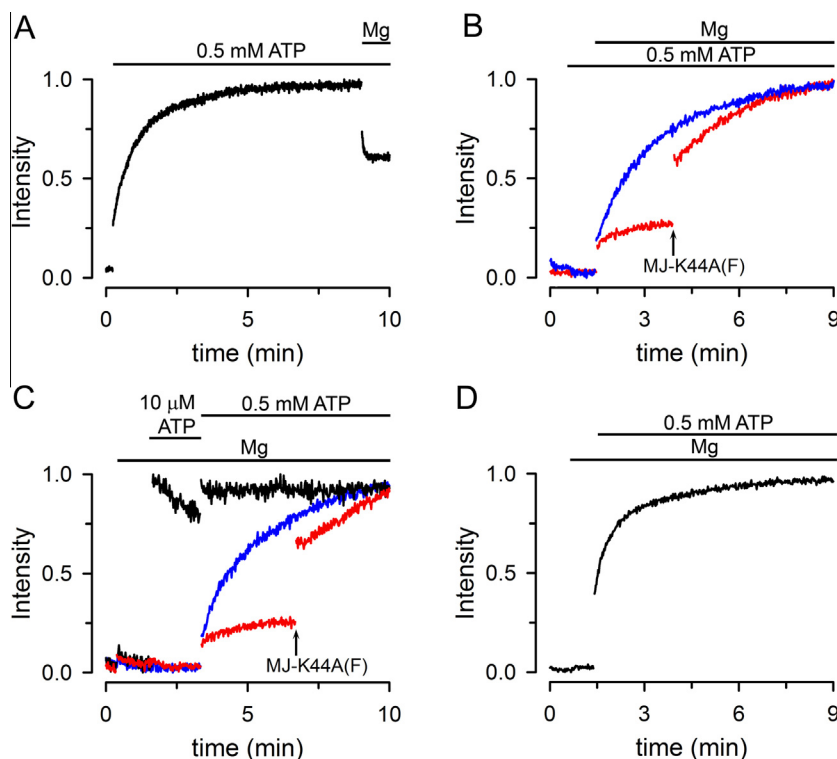


Fig. 3. Association of homodimers and heterodimers. (A) MJ association/dissociation followed by LRET. The experiment corresponds to an equimolar mix of Tb^{3+} -labeled and fluorescein-labeled MJ. (B) Formation of MJ-K44A homodimers and MJ/MJ-K44A heterodimers. MJ-K44A homodimers: blue trace, Tb^{3+} -labeled and fluorescein-labeled MJ-K44A; MJ/MJ-K44A heterodimers: red trace, Tb^{3+} -labeled MJ-K44A and fluorescein-labeled MJ. At the arrow, fluorescein-labeled MJ-K44A (MJ-K44A(F)) was added (0.5 μM). (C) Dimerization of MJ-K44A in response to MgATP. MJ homodimers: black record; MJ-K44A homodimers: blue record; MJ/MJ-K44A heterodimers: red record. MJ-K44A(F) was added at the arrow (0.5 μM). (D) Formation of MJ/MJ-K44A heterodimers. Equimolar mix of Tb^{3+} -labeled MJ-K44A and fluorescein-labeled MJ. For all panels, the starting concentration of each labeled protein was 0.5 μM . The traces were normalized to the maximum intensity recorded at the end of each experiment, but the absolute intensity values were very similar. In all panels, 0.5 mM ATP refers to 0.5 mM NaATP, and Mg to 10 mM MgCl_2 . All records are representative of at least 4 independent experiments. Measurements started in the divalent cation- and ATP-free solution (see Fig. 2).

mers are too distant from each other for energy transfer, but addition of NaATP promotes MJ dimerization, bringing the donor and acceptor closer [9,10]. In the dimer, the donor–acceptor distance of ~ 50 Å that results from labeling Cys14 is near ideal to allow energy transfer from Tb^{3+} to fluorescein, which is observed as increased sensitized fluorescence emission. Addition of Mg to the ATP-bound dimers produced a rapid decrease in intensity due to the dimer dissociation that follows ATP hydrolysis (Fig. 3A) [9,10]. The intensity of the LRET signal in 0.5 mM MgATP falls to a steady-state value intermediate between those of the nucleotide-free and ATP-bound states (Fig. 3A). This intermediate value represents a dynamic monomer/dimer equilibrium during continuous ATP hydrolysis in the presence of saturating [MgATP] [9,10]. Under these conditions, neither association nor dissociation are favored, and $\sim 50\%$ of the NBDs are monomers and $\sim 50\%$ form dimers.

Similar LRET experiments, but mixing MJ and ATP-binding mutants, allowed the study of heterodimers with a normal NBS1 and a defective NBS2. Based on the affinities of the mutants and the [NaATP] or [MgATP] (see previous section and Fig. 2), we established conditions where NBS1 is nucleotide-bound, whereas NBS2 is either nucleotide-free or nucleotide-bound, to determine whether heterodimers can be formed with only one NBS occupied by ATP or whether both NBSs need to have ATP bound. In the heterodimer mixtures of MJ labeled with donor and an ATP-binding-defective mutant labeled with acceptor, only dimers containing donor/acceptor pairs are visible by LRET, whereas homodimers formed by MJ or binding-deficient mutants are “invisible” because they do not produce sensitized fluorescence. Therefore, we

selectively measured the behavior of normal/mutant dimers within the complex population. Fig. 3B shows the response to NaATP and MgATP of an equimolar mix of Tb^{3+} -labeled and fluorescein-labeled MJ-K44A (blue; MJ-K44A homodimers) or fluorescein-labeled MJ and Tb^{3+} -labeled-MJ-K44A (red; heterodimers). Addition of 0.5 mM NaATP was insufficient to induce formation of MJ-K44A homodimers or MJ/MJ-K44A heterodimers (Fig. 3B), whereas it caused complete dimerization of MJ (Fig. 3A), confirming the lower affinity of MJ-K44A for NaATP. Subsequent addition of Mg in the continuous presence of 0.5 mM ATP promoted formation of MJ-K44A homodimers and MJ/MJ-K44A heterodimers. This [MgATP] is ~ 7 -fold the 70 μM Kd_{MgATP} estimated from Trp-fluorescence quenching experiments (Fig. 2B). Since MJ Kd_{NaATP} and Kd_{MgATP} are 50 and 5 μM , respectively [8], and MJ-K44A Kd_{NaATP} and Kd_{MgATP} are >1 mM and 70 μM , respectively, only NBS1 is expected to have full occupancy by ATP in 0.5 mM NaATP, whereas both NBS1 and NBS2 are expected to be ATP-bound in 0.5 mM MgATP. From Fig. 3B, it is also clear that the intensity of the signal corresponding to heterodimers was only a fraction ($26 \pm 2\%$; $n = 6$) of the maximal intensity observed for MJ-K44A homodimers. Although dissociation of heterodimers following ATP hydrolysis may contribute to the small proportion of MJ/MJ-K44A heterodimers, differences in the association rates between MJ and MJ-K44A are a major factor. The MJ association rate in response to saturating [MgATP] is $\sim 0.25 \text{ s}^{-1}$ (~ 30 -fold faster than the association rate in response to NaATP) [8,9], whereas the rate of association of MJ-K44A is slow (completed in minutes, Fig. 3B). This difference in dimerization rates effectively reduces the availability of MJ for heterodimerization, as MJ preferentially forms homodimers

invisible to LRET (containing only acceptor) and MJ-K44A also forms invisible homodimers (containing only Tb³⁺). In agreement with this interpretation, addition of fluorescein-labeled-MJ-K44A to the mix increased the fluorescence intensity due to the formation of MJ-K44A homodimers visible by LRET (Fig. 3B). Moreover, MJ-K44A is fully capable of forming heterodimers with the slowly dimerizing hydrolysis-deficient MJ1 (catalytic glutamate mutant E171Q). MJ1/MJ-K44A heterodimer formation in response to 0.5 mM MgATP was slow, but near-maximal (normalized to the maxima intensity for MJ-K44A dimers, Fig. 3D). MJ1 binds ATP with high affinity and dimerizes completely at low micromolar concentrations of NaATP or MgATP, but it does so slowly (time course of minutes), even in the presence of saturating [MgATP] [8,9]. In addition, since both NBSs are catalytically deficient, once formed the MJ1/MJ-K44A heterodimers do not dissociate.

Heterodimerization experiments such as those shown above, but using the ATP-binding-defective mutants MJ-K44E or MJ-S42F instead of MJ-K44A, were also performed. These mutants show a more dramatic decrease in affinity for NaATP and MgATP (Fig. 2C and D), and homodimers were not detectable by LRET in 0.5 mM NaATP or MgATP, in agreement with the Trp-fluorescence results in Fig. 2C and D. MJ/MJ-K44E and MJ/MJ-S42F heterodimers did not form in 0.5 mM NaATP, and only a very small proportion was detected in 0.5 mM MgATP (<10% of the MJ homodimers maximal signal; $n = 2$ for each mutant).

The data shown in Fig. 3B suggest that MJ-K44A can form heterodimers only when the 2 NBSs are occupied by nucleotide. To explore this in more detail we studied dimerization in 10 μ M MgATP, a concentration significantly below the K_d MgATP of MJ-K44A (70 μ M, Fig. 2B). Since the K_d MgATP of MJ is ~ 5 μ M [8], at 10 μ M MgATP NBS1 is expected to bind ATP, but no significant ATP binding is expected to NBS2. Fig. 3C shows that MJ-K44A was unable to form homodimers (blue) or heterodimers with MJ (red) in 10 μ M MgATP, whereas MJ homodimers were rapidly formed (black). When [MgATP] was increased to 0.5 mM, several-fold above the MJ-K44A K_d MgATP, MJ-K44A homodimers and MJ/MJ-K44A heterodimers were formed (Fig. 3C). Under these conditions, both NBSs in the MJ/MJ-K44A heterodimers are expected to be bound to ATP. As mentioned above, at a saturating [MgATP] of 0.5 mM MJ homodimers produce a stable LRET signal that results from monomer/dimer dynamic equilibrium during continuous ATP hydrolysis. The stable signal occurs because MJ ATPase activity is insufficient to decrease [ATP] below saturation (Fig. 3A and C, black trace under 0.5 mM ATP) [8,9]. However, at [MgATP] below the K_d the LRET signal decreases continuously after the initial peak, due to reduction of [ATP] below saturation levels that occurs as the nucleotide is consumed (Fig. 3C, black record under 10 μ M ATP) [9].

Together, the results presented here suggest that formation of a stable NBD dimer requires ATP bound to NBS1 and NBS2 because NBD dimers were not formed at nucleotide concentrations at which the low-affinity mutant NBS2 does not bind ATP. A summary of the main results is presented in Table 1.

Table 1
Relationship between NBD dimerization and ATP binding to NBS1 and NBS2.

		NBS1	NBS2	Dimerization
MJ homodimers	0.5 mM NaATP	Yes	Yes	Yes
	0.5 mM MgATP	Yes	Yes	Yes
	10 μ M MgATP	Yes	Yes	Yes
MJ-K44A homodimers	0.5 mM NaATP	No	No	No
	0.5 mM MgATP	Yes	Yes	Yes
	10 μ M MgATP	No	No	No
MJ/MJ-K44A heterodimers	0.5 mM NaATP	Yes	No	No
	0.5 mM MgATP	Yes	Yes	Yes
	10 μ M MgATP	Yes	No	No

In the prevailing model of transport by ABC exporters, the alternate accessibility of the drug-binding pocket is controlled by the association and dissociation of the NBDs [1]. In the nucleotide-free conformation (dissociated NBDs, open state), the drug-binding pocket is accessible to the NBD side of the membrane, and the accessibility of the pocket switches to the opposite side as a result of NBDs association (closed state). Our results indicate that binding of two ATPs is required for the formation of a NBD dimer, suggesting that two NBSs competent for nucleotide binding are necessary for the stabilization of the closed state.

Based on the results presented here and our recent observations [10], we propose that there is a universal need of ABC proteins for two NBSs that bind ATP because the ATP-sandwich dimer provides sufficient stability to allow for ATP hydrolysis at one of the sites, which drives the dissociation of the NBD dimer. Since the functional unit of all ABC proteins includes two NBSs capable of ATP binding, and at least one of those sites is able to hydrolyze the nucleotide, it is possible that ABC transporters with two hydrolysis-competent NBSs (like P-glycoprotein) and only one such site (like CFTR) have a similar basic molecular mechanism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.11.050>.

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