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

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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 structure 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 and H loops are involved in 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
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 (MJI) have also been published [8,9]. MJ is active, whereas MJI 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, MJ-S42F (replacement of the conserved Lys44 of the Walker A motif with Glu), MJ-S42F (replacement of the conserved Lys44 of the Walker A motif with Ala), MJ-K44A, MJ-K44E, and Y11A displayed decreased binding of 8-azido-ATP under conditions where the photolabeling of MJ was maximum (Fig. 2A). Similarly to MJI, 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 p-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 Kd values for NaATP-induced and MgATP-induced dimerization (KdNaATP and KdMgATP, respectively) of ~50 and 5 μM, 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 constant in 1 mM MgATP (red). The absence of complete quenching by MgATP agrees with prior results for MJ, were 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 KdMgATP of MJ-K44A was 70 ± 13 μM (n = 3), ~15-fold higher than

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 p–p 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).

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; Glu171 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 a NBD heterodimer formed by MJ and an ATP-binding-deficient mutant. The heterodimer consists of an 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.
that of MJ ($\sim$5 $\mu$M) [8]. The $K_{\text{d,NaATP}}$ was not quantified due to the small response to high [NaATP]. 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 $\mu$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 fluorescence 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-
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 \( \frac{50}{\text{Å}} \) that results from labeling Cys14 is near ideal to allow energy transfer from Tb\(^{3+} \) to fluorescein, which is observed as increased sensitized fluorescein 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 homodimers: black record; MJ/K44A homodimers: blue record; MJ/MJ-K44A heterodimers: red record. MJ-K44A(F) was added at the arrow (0.5 \( \mu \text{M} \)). (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 \( \mu \text{M} \). 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).

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 \( \mu \text{M} \)). (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 \( \mu \text{M} \)). (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 \( \mu \text{M} \). 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).
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 dimersizing hydrolysis-deficient MJ (catalytic glutamate mutant E171Q). MJ/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). MJ 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 MJ/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 μM MgATP, a concentration significantly below the KdMgATP of MJ-K44A (70 μM, Fig. 2B). Since the KdMgATP of MJ is ~5 μM [8], at 10 μM 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 μM MgATP, whereas MJ homodimers were rapidly formed (black). When [MgATP] was increased to 0.5 mM, several-fold above the MJ-K44A KdMgATP, 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 saturation [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 Kd 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 μM 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.

In the prevailing model of transport by ABC exporters, the alternate accessbility 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 accessbility 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 NBDS 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 NBDS 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 NBDS 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 hydrolys-sis-competent NBDS (like P-glycoprotein) and only one such site (like CFTR) have a similar basic molecular mechanism.

Acknowledgments

We thank Drs. Luis Reuss and Ina Urbatsch for comments on the paper, and Dr. Urbatsch for helping with the 8-azido-ATP binding assay. This work was supported in part by Grants from the National Institutes of Health (R01GM79629 and 3R01GM079629-03S1) and Cancer Prevention & Research Institute of Texas (RP101073). Some images and data were generated in the Imaging Analysis & Molecular Biology Core Facilities supported by TTUHSC.

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.

References


