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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.

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 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 Mg^{2+} 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-N_3ATP-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; MJI, single-Cys mutant E171Q based on MJ; NBD, nucleotide-binding domain; NBS, nucleotide-binding site.

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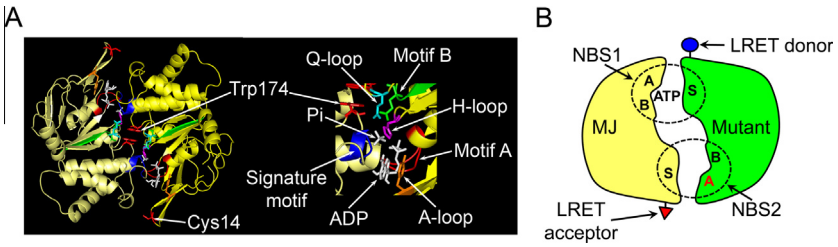

Fig. 1. Structure of the NBD dimer. (A) Ribbon representation of the two monomers (in different tones of yellow). A zoomed view of one nucleotide-binding site (NBS) is shown on the right. ADP and Pi (white, sticks); Walker A motif (red, motif A); Walker B motif (green, motif B; Gln171 in sticks); signature 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 MJI (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 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 (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 (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 reside 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 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 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 Kd values for NaATP-induced and MgATP-induced dimerization (Kd_{NaATP} and Kd_{MgATP}, 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 complete 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 Kd_{MgATP} of MJ-K44A was 70 ± 13 μM ($n = 3$), ~15-fold higher than