Kinetics of the ATP Hydrolysis Cycle of the Nucleotide-binding Domain of Mdl1 Studied by a Novel Site-specific Labeling Technique*

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We have recently proposed a “processive clamp” model for the ATP hydrolysis cycle of the nucleotide-binding domain (NBD) of the mitochondrial ABC transporter Mdl1 (Janas, E., Hofacker, M., Chen, M., Gompf, S., van der Does, C., and Tampé, R. (2003) J. Biol. Chem. 278, 26862–26869). In this model, ATP binding to two monomeric NBDs leads to formation of an NBD dimer that, after hydrolysis of both ATPs, dissociates and releases ADP. Here, we set out to follow the association and dissociation of NBDs using a novel minimally invasive site-specific labeling technique, which provides stable and stoichiometric attachment of fluorophores. The association and dissociation kinetics of the E599Q-NBD dimer upon addition and removal of ATP were determined by fluorescence self-quenching. Remarkably, the rate of ATP hydrolysis of the wild type NBD is determined by the rate of NBD dimerization. In the E599Q-NBD, however, in which the ATP hydrolysis is 250-fold reduced, the ATP hydrolysis reaction controls dimer dissociation and the overall ATPase cycle. These data explain contradicting observations on the rate-limiting step of various ABC proteins and further demonstrate that dimer formation is an important step in the ATP hydrolysis cycle.

ATP-binding cassette (ABC)4 transporters drive the translocation of substrates by the hydrolysis of ATP. They represent one of the largest transporter families and are found in all phyla of life (1, 2). ABC transporters have a conserved domain structure, which consists of two cytosolic nucleotide-binding domains (NBDs) and two membrane-spanning domains. The membrane-spanning domains build the translocation pathway, whereas the NBDs function as motor energizing the transport. The membrane-spanning domains typically contain six transmembrane helices (TMHs), but the amino acid sequences are very diverse, and transporters with more or less transmembrane helices have been found. The highly conserved NBDs contain the Walker A, Walker B, and C-loop motifs. In eukaryotes and procaryotes, the domains are most commonly fused to one protein with the membrane-spanning domain and the NBD domains in an alternating order or as two half-size transporters where each transporter contains a membrane-spanning domain and an NBD. In Archaea and eubacteria, the domains are mostly found in four distinct units, although they also have been found fused in almost any possible combination. ABC transporters function as exporters; nevertheless, in prokaryotes and Archaea, some ABC transporters include a fifth domain, the substrate-binding protein, which turns the transporter into an importer.

The structures of two distinct full-length ABC transporters and several isolated NBDs have been solved and showed that the structures of the NBDs are highly conserved (for reviews see Refs. 3 and 4). The NBD monomer forms an L-shaped molecule with two domains. The first, RecA-like domain includes the ATP-binding core with the Walker A and B motifs, whereas the characteristic ABC signature (C-loop) motif is located in the second α-helical domain. In the monomeric NBD, the ABC signature motif is far away from the nucleotide, but in the dimeric state, the two monomers are oriented in a head-to-tail configuration, with two ATPs bound at the interface of the two monomers. The ATPs are sandwiched between the Walker A and B motifs from one monomer and the C-loop of the other, the C-loop thus complementing the nucleotide-binding site. ATP occupies a significant part of the dimer interface and thus stabilizes the dimeric state. Analyses of different nucleotide-bound and free states showed that ATP binding induced a rigid body movement of the two domains of the NBD, which aligns the monomers in such an “induced-fit” manner that the dimer can be formed.

How ATP is exactly hydrolyzed is currently a matter of intense debate, and many different models have been proposed (reviewed in Ref. 5). The “alternating-site” model suggests that ATP hydrolysis occurs alternating in the two nucleotide-binding sites without dissociation of the dimer (6, 7). ATP hydrolysis in the first catalytic site is followed by opening of this site, whereas the second catalytic site remains closed. Release of ADP and re-binding of ATP then induces closure of the site and leads to ATP hydrolysis in the second catalytic site.

We have recently studied the ATP hydrolysis cycle of the NBD of Mdl1 (Multidrug resistance-like) (8), an intracellular peptide transporter of Saccharomyces cerevisiae localized in the inner mitochondrial membrane. This homodimeric half-size ABC transporter is involved in the export of peptides with a length of 6–21 amino acids from the matrix (9). These peptides are derived from the degradation of non-assembled complexes of the inner mitochondrial membrane generated by ATP-dependent m-AAA (Matrix-oriented ATPases Associated with a variety of cellular Activities) proteases (10). The NBD of Mdl1 was overexpressed and purified to homogeneity (8). The isolated NBD was active in ATP binding and hydrolysis. Three different catalytic intermediates of the NBD dimer were trapped either by incubation with orthovanadate or beryllium fluoride, or by exchange of the conserved glutamate downstream of the Walker B (E599Q). The nucleotide composition of intermediate states was determined using [α-32P]ATP and [γ-32P]ATP. The three isolated dimeric intermediate states contain two ATPs, one ATP and one ADP, or two ADPs. Based on our and other biochemical and structural data (11–14), the “processive clamp” model for the catalytic cycle was proposed, in which association and dissociation...
tion of the NBDs depend on the status of bound nucleotides (5, 8). In this model, two ATPs are bound to two monomers, which results in formation of the dimer. Both ATPs are therefore hydrolyzed, finally leading to dissociation of the dimer. Association and dissociation of the NBDs are therefore important characteristics of the processive clamp model.

We set out to study the relationship between the kinetics of ATP hydrolysis and the association and dissociation rates of the NBDs. We designed His-tag-specific fluorescent probes, which allow a site-specific, stable, stoichiometric, and minimally invasive labeling of the NBD. This strategy was successfully applied to resolve the dynamic interaction of the NBDs within the ATP hydrolysis cycle. Based on the association and dissociation kinetics, we identified the rate-limiting step in ATP hydrolysis cycle for the NBDs of Mdl1 and discussed the consequences for the current models for ATP hydrolysis in ABC proteins.

**MATERIALS AND METHODS**

**Tris-NTA(ATTO565) Synthesis**—To prevent quenching of the fluorophore by complexed nickel ions, tris-NTA was coupled on solid phase to the N terminus of the proline linker peptide, PPPPCA. TrisNTA was synthesized as described (15). After deprotection and cleavage from the resin, the cysteine residue was labeled with ATTO565-maleimide (ATTO-TEC GmbH, Siegen, Germany) via standard thiol-chemistry. Products were purified via reversed-phase C18 HPLC. The trisNTA(ATTO565) was loaded with nickel as described (15), and the concentration was determined via the absorption of the fluorophore. Details of the synthesis will be described elsewhere.5

**Expression and Purification of Mdl1-NBD in Escherichia coli**—Over-expression and purification of the WT-NBD and the E599Q-NBD using nickel-affinity and gel filtration chromatography were performed as described (8).

**Tris-NTA(ATTO565) labeling**—To determine stable and stoichiometric labeling with tris-NTA(ATTO565), 8 μM of E599Q-NBD were mixed with various ratios of tris-NTA(ATTO565) in assay buffer (100 mM NaCl, 20 mM Tris, pH 8.0) and applied to a Superdex 200 PC 3.2 (Amersham Biosciences) gel filtration column. To study the dimer formation, 8 μM E599Q-NBD labeled with equimolar tris-NTA(ATTO565) were incubated with 1 mM ATP for 5 min on ice and then applied to the gel filtration column (50-μl injection volume). The Superdex 200 column was equilibrated with assay buffer and run at 6 °C at a flow rate of 50 μl/min. Dimer formation was monitored at 280 and 565 nm.

**Kinetics of Dimer Association and Dissociation**—To analyze the rate of dimer association, 10 μM E599Q-NBD were labeled with an equimolar amount of tris-NTA(ATTO565) in assay buffer and applied to a Superdex 200 PC 3.2 gel filtration column equilibrated with assay buffer at a flow rate of 50 μl/min at 6 °C (50-μl injection volume). Fractions containing the labeled NBD were collected, and the protein concentration was determined using the micro BCA assay (Pierce). The labeled NBD was transferred to a 150-μl quartz cuvette and diluted to the appropriate concentrations with the assay buffer plus 5 mM Mg2+. After addition of nucleotides (1 mM), self-quenching of the fluorophore was followed over time in a temperature-controlled Cary Eclipse fluorometer (Varian Instruments; λex/em = 565/595 nm, slit-widths 5 nm). The association rate constant (kassoc) was determined from the slope of the plot of the reciprocal value of the monomeric NBD concentration versus time, whereas the dissociation rate constant (kdiss) was obtained from the slope of the plot of natural log of the dimeric NBD concentration versus time. Experiments were performed at 65, 125, 250, and 375 mM NBD, at 10, 15, 20, and 25 °C, and in the presence of 5, 10, and 15% (w/v) sucrose. As controls, similar experiments were performed with the WT-NBD or the E599Q-NBD in the presence of 1 mM ATP and 10 μM of unlabeled E599Q-NBD.

To analyze dimer dissociation, 10 μM E599Q-NBD were incubated with 10 μM tris-NTA(ATTO565) and 2 mM ATP. After isolation of the labeled dimer by gel filtration, the decrease in fluorescence self-quenching was determined using the micro BCA assay (Pierce). The labeled dimer was transferred to a 150-μl quartz cuvette and diluted to the appropriate concentrations with the assay buffer plus 5 mM Mg2+. After addition of nucleotides (1 mM), self-quenching of the fluorophore was followed over time in a temperature-controlled Cary Eclipse fluorometer (Varian Instruments; λex/em = 565/595 nm, slit-widths 5 nm). The dissociation rate constant (kdiss) was determined from the slope of the plot of the reciprocal value of the monomeric NBD concentration versus time, whereas the association rate constant (kassoc) was obtained from the slope of the plot of natural log of the dimeric NBD concentration versus time.

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FIGURE 2. Dimer formation and dissociation followed by self-quenching of the tris-NTA- (ATTO565)-labeled NBD. A, the E599Q-NBD (8 μM) was labeled with equimolar tris-NTA- (ATTO565). After incubation for 5 min on ice, dimer formation was monitored in the presence (solid line) and absence (dashed line) of 1 mM ATP by gel filtration at 565 nm. B, model of the dimerization of two ATP bound monomers. The ATP-bound monomer and dimer are derived from the ATP-bound dimer of MJ0796 (PDB entry: 1L2T (13)) using PYMOL. The red star depicts the position of the tris-NTA (ATTO565) bound at the N terminus. The bar indicates a 6-nm distance. C, fluorescence self-quenching of the labeled E599Q-NBD (1 μM) was followed after addition of 1 mM ATP, ADP, AMP, or 1 mM ATP in the presence of unlabeled E599Q-NBD (10 μM). All experiments were performed at 20 °C. D, after isolation of the labeled E599Q-NBD dimer, the dissociation kinetics were examined by fluorescence increase.

ing upon dimer dissociation was measured at 10, 15, 20, and 25 °C. Again, experiments were performed in the presence of 5, 10, and 15% (w/v) sucrose.

**Results**

**Determination of ATP Hydrolysis Rate—**ATPase activities were measured by the malachite green assay as described (16). The ATPase assay was performed with 5 μM NBD in assay buffer plus 5 mM Mg2+ at 10, 15, 20, and 25 °C in the presence of 0–2 mM ATP or with 0–150 μM NBD in assay buffer plus 5 mM Mg2+ in the presence of 2 mM ATP at 25 °C. The activity of the E599Q-NBD was measured in the same way but with longer incubation times. Similar experiments were performed in the assay buffer plus 5 mM Mg2+ containing 5, 10, and 15% (w/v) sucrose. To determine the ATPase activity of the NBD labeled with tris-NTA (ATTO565), a radioactive ATPase assay was used (17). Briefly, 5 μM NBD were incubated with 2 mM ATP supplemented with 13.5 μM [γ-32P]ATP in a total volume of 50 μl for 10 min at 25 °C. The reaction was stopped with 1 ml of 10 mM ammonium molybdate in 1 M HCl. Subsequently, 15 μl of 20 mM H3PO4 and 2 ml of a butanol/acyclohexane/acetone (5:5:1) mixture were added. The organic phase was extracted, and the radioactivity in this phase was quantified by liquid scintillation counting.

**RESULTS**

Self-quenching of two fluorescently labeled proteins has been extensively applied to determine the kinetics of association and dissociation of interacting proteins. For protein labeling, often thiol-specific fluorescent probes are reacted with single cysteine mutants. The NBD contains three cysteines, which could be mutated to serines without affecting the ATPase activity of the purified NBD (data not shown). However, specific labeling of single cysteine mutants with maleimide-specific probes strongly impaired the ATPase activity and dimer formation (data not shown). We therefore searched for a minimally invasive approach to label the NBD site-specifically. We developed multivalent chelator heads, which bind with nanomolar affinities to histidine tags (15).

To follow the association and dissociation of NBDs during their ATP hydrolysis cycle in real time, we coupled the tris-NTA chelator head to the ATTO565 fluorophore. In addition, a polyproline spacer was introduced between the multivalent chelator head and the fluorophore to prevent fluorescence quenching by metal binding (Fig. 1A). The His-tagged NBD was stably labeled by tris-NTA(ATTO565) with (1:1) stoichiometry as shown by gel filtration (Fig. 1B). Strikingly, in contrast to all other attempts in labeling ABC proteins without affecting their function, the labeled NBD showed the same ATPase activity as the unlabeled one (Fig. 1C).

To study the association and dissociation of the NBDs, the E599Q-NBD was used. In this mutant, the conserved glutamate downstream of the Walker B motif was mutated to a glutamine resulting in a stable ATP-dependent dimer formation (8). Indeed, after incubation of the tris-NTA (ATTO565)-labeled E599Q-NBD with ATP, the dimeric species was observed after gel filtration (Fig. 2A), demonstrating that dimer formation is not affected by the attachment of the fluorophore. Based on homology modeling on the ATP-bound dimer of MJ0796, the two fluorophores are in the Mdr1-NBD dimer at a distance of 6 nm, approximately the Förster radius of the ATTO565 probe (Fig. 2B). Indeed, incubation of the E599Q-NBD with ATP resulted in a specific self-quenching of the fluorescence (Fig. 2C). Thus, dimer formation of the tris-NTA (ATTO565)-labeled E599Q-NBD could be followed over time. For the monomeric NBD, fluorescence quenching was observed after addition of ATP, whereas, for the dimer, a recovery in fluorescence intensity to the level of the monomeric species was observed over time (Fig. 2D). This indicates a specific self-quenching of the fluorescent probes after association of the NBDs. However, no change in fluorescence was detected when ADP, AMP (Fig. 2C), or the WT-NBD was used (data not shown). Furthermore, the fluorescence quenching could be specifically competed with an excess of unlabeled E599Q-NBD (Fig. 2C), demonstrating that the self-quenching is dimer-specific and not caused by a conformational change. Worth mentioning, similar results
were obtained with tris-NTA(OregonGreen)-labeled NBDs or with different resonance energy transfer pairs. However, to avoid any asymmetric situation within a homodimeric complex, all experiments were performed with the tris-NTA(ATTO565)-labeled NBD. Taken together, these data demonstrate that these novel tris-NTA-fluorophores label the ABC protein stably and stoichiometrically and that association of the E599Q-NBD dimer results in self-quenching of the ATTO565 probe.

We next derived the rate of dimer formation from the decrease of fluorescence after addition of 1 mM ATP (Fig. 3A). At this ATP concentration, ATP binding is very fast, and does not influence the rate of the dimerization process (see “Discussion”). As expected for a dimerization with a slow backward reaction, a linear relation was observed when the reciprocal value of the monomeric NBD concentration was plotted against the reaction time (Fig. 3B). This allowed us to calculate the rates ($k_{on}$) for dimer association. The $k_{on}$ values were determined at different temperatures and are summarized in Table 1. From the temperature dependence of the association rate constant (Fig. 3E), an activation energy for dimer association of 68 kJ/mol was determined.

After isolation of the dimer by gel filtration, we followed the dissociation of the labeled NBD dimer over time (Fig. 3C). The fluorescence reached a similar final value after prolonged incubation at the different temperatures (data not shown). As expected for a dissociation process where no reassociation occurs, a linear relationship was observed when the natural log of the dimeric NBD concentration was plotted against the reaction time (Fig. 3D). This allowed us to calculate the rate con-

![Figure 3: Kinetics of dimer formation and dissociation.](https://example.com/figure3)

**TABLE 1**

| Temperature | $k_{on}$ | $k_{off}$ |
|-------------|---------|----------|
| °C          | $\text{min}^{-1} \mu\text{M}^{-1}$ | $\text{min}^{-1}$ |
| 10          | $0.18 \pm 0.035$       | $0.012 \pm 0.002$ |
| 15          | $0.32 \pm 0.050$       | $0.024 \pm 0.004$ |
| 20          | $0.50 \pm 0.048$       | $0.036 \pm 0.005$ |
| 25          | $0.80 \pm 0.210$       | $0.054 \pm 0.004$ |

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stants for dimer dissociation ($k_{\text{off}}$) at different temperatures (Table 1). From the temperature dependence of the dissociation rate constants, an activation energy of 69 kJ/mol was calculated (Fig. 3E).

We next related the rates identified for dimer association and dissociation to the rate of ATP hydrolysis. The ATP hydrolysis activity of the WT-NBD and the E599Q-NBD were determined at different temperatures as a function of the ATP concentration (Fig. 4A and C) and as a function of the NBD concentration (Fig. 4B and D). The WT-NBD showed normal ATP-dependent Michaelis-Menten kinetics with a $K_m$ of 0.6 mM (Fig. 4A), whereas the ATPase activity was non-linearly dependent on the protein concentration (Fig. 4B). Compared with the WT-NBD, the E599Q-NBD has a 250-fold reduced ATPase activity and a slightly lowered $K_m$ of 0.2 mM (Fig. 4C). The ATPase activity of the E599Q-NBD showed normal Michaelis-Menten kinetics. However, contrasting with our observation of the WT-NBD, the ATPase activity of the E599Q-NBD was independent of the NBD concentration (Fig. 4D). This shows that different steps in the ATP hydrolysis cycle are rate-limiting for the WT-NBD and the E599Q-NBD. A shift in the rate-limiting step is often associated with a change in activation energy. The WT and the E599Q-NBD however showed rather similar temperature dependences of the ATP hydrolysis rates (Fig. 4E), with activation energies of 73 and 69 kJ/mol for the WT-NBD and the E599Q-NBD, respectively. Within the margin of error, these activation energies are similar to those for association and dissociation of the E599Q-NBD dimer, and we were therefore unable to determine the rate-limiting step based on the activation energies of the different steps.

To further examine the difference in the concentration dependence of the ATPase activity of the WT-NBD and the E599Q-NBD, the rates of the different steps were determined in solutions of different viscosities. The molecular diffusion coefficient depends inversely on the viscosity of the solution, and diffusion-controlled reactions are slowed down at higher viscosities (18, 19). The rate of dimer association (Fig. 5A) and the ATPase activity of the WT-NBD (Fig. 5B) were both dependent on the viscosity, whereas the rate of dimer dissociation (Fig. 5A) and the ATPase activity of the E599Q-NBD (Fig. 5B) did not show any dependence. These results further demonstrated that ATP hydroly-

![Figure 4. ATP hydrolysis by the WT-NBD and the E599Q-NBD.](image-url)
ATP Hydrolysis Cycle of ATP-binding Cassette Proteins

In this study, the His-tagged NBD of Mdl1 was labeled with a new fluorescent probe linked to a multivalent tris-NTA group. Tethering of three NTA moieties substantially increased the binding affinity (from a $K_d$ value of 14 μM to 20 nM), which led to a stable, stoichiometric, and site-specific labeling of His-tagged proteins (15). We further improved the quantum yield of the fluorescence label by introducing a polyproline sequence between the tris-NTA group and the fluorophore, which functions as a rigid spacer to prevent quenching by nickel bound to tris-NTA. Strikingly, after site-specific labeling with tris-NTA (ATTO565), the activity of the NBD was fully retained. The labeled NBD was therefore excellently suited to study the dynamic association and dissociation of the NBDs in real time. The facts that the His-tags are currently the most widely applied affinity tags and that labeling is site-specific, stoichiometric, and takes place under mild conditions with nanomolar affinity, make this new labeling technique widely applicable for the analysis of dynamic protein interactions within macromolecular assemblies even in cell lysates or at the surfaces of living cells.

Here, this new labeling technique was used to investigate the kinetics of the ATP hydrolysis cycle of the mitochondrial ABC transporter Mdl1. It was previously shown that ATP hydrolysis by the NBD occurs via the processive clamp model (8). In this model, one catalytic cycle consists of ATP binding to two monomers, followed by formation of an NBD dimer, where, after both ATPs are hydrolyzed, the dimer dissociates. This demonstrates that either dimer dissociation or a step before is rate-limiting. Because ATP binding to the NBD occurs very fast (20, 21) (see “Discussion”), this suggests that the dimer association step is the rate-limiting step in the ATP hydrolysis cycle of the WT-NBD. Contrary to the WT-NBD, the rate of ATP hydrolysis of the E599Q-NBD was very similar to the rate of E599Q dimer dissociation. This demonstrates that either dimer dissociation or a step before is rate-limiting. In the WT-NBD, where ATP hydrolysis is much faster, dimer dissociation is not rate-limiting, indicating that dimer dissociation (at least after ATP hydrolysis) is also a very rapid step. We can therefore conclude that in the E599Q mutant, the ATP hydrolysis reaction is the rate-limiting step in the ATP hydrolysis cycle.

**DISCUSSION**

Analyses of protein networks and domain interactions within complex machineries are of emerging interest in modern biology. Fluorescence quenching and Förster resonance energy transfer are frequently applied to follow the kinetics of and structural rearrangements within such assemblies. A widely used technique is to introduce fluorescent probes to single cysteine residues. Unfortunately, single cysteine mutants often do not have the activity of the WT protein, or, likewise after labeling with thiol-specific probes, show a reduced activity. Also the labeling of each of the three single cysteine mutants of Mdl1 resulted in a drastic reduction of the ATPase activity.

In this study, the His-tagged NBD of Mdl1 was labeled with a new fluorescent probe linked to a multivalent tris-NTA group. Tethering of three NTA moieties substantially increased the binding affinity (from a $K_d$ value of 14 μM to 20 nM), which led to a stable, stoichiometric, and site-specific labeling of His-tagged proteins (15). We further improved the quantum yield of the fluorescence label by introducing a polyproline sequence between the tris-NTA group and the fluorophore, which functions as a rigid spacer to prevent quenching by nickel bound to tris-NTA. Strikingly, after site-specific labeling with tris-NTA (ATTO565), the activity of the NBD was fully retained. The labeled NBD was therefore excellently suited to study the dynamic association and dissociation of the NBDs in real time. The facts that the His-tags are currently the most widely applied affinity tags and that labeling is site-specific, stoichiometric, and takes place under mild conditions with nanomolar affinity, make this new labeling technique widely applicable for the analysis of dynamic protein interactions within macromolecular assemblies even in cell lysates or at the surfaces of living cells.

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binding of nucleotides to different NBDs (13, 24–26). Also in the NBD of Mdl1, the introduced single tryptophan directly sensed the bound nucleotide and resulted in 30% ATP and ADP-dependent quenching of the fluorescence for both the WT and the E599Q mutant (data not shown). Time-dependent experiments demonstrated that nucleotide binding occurred within the mixing time of the experiments, with apparent rate constants faster than 30 min−1 nM−1.6 These rate constants are at least two orders of magnitude faster than ATP hydrolysis by the NDB of Mdl1 or the dimer formation of the E599Q mutant, thus excluding the possibility that nucleotide binding is rate-limiting under the conditions used in our experiments.

Because ATP binding is not the rate-limiting step, we focused on the kinetics of the association and dissociation of the NBDs. The concentration of the transiently formed WT dimer is far below the detectable limit, and only the monomeric species is normally observed. Several attempts have been made to stabilize the WT ATP dimer, such as using non-hydrolyzable ATP analogues or experiments in the absence of Mg2+, but these attempts were unsuccessful. The ATP-dependent dimer was only observed after mutagenesis of the NBD or after trapping with vanadate or beryllium fluoride (8). Vanadate or beryllium fluoride trapping occurs, however, after ATP hydrolysis and possibly after several rounds dimer association and dissociation and therefore cannot be used to study the association process. We therefore determined the rate of dimer association for the E599Q-NBD. Remarkably, the rates of dimer association of the E599Q-NBD and the rate of ATP hydrolysis in the WT-NBD were comparable and showed a similar dependence on the diffusion rate and the NBD concentration, whereas the rate of association of the E599Q-NBD was equal to the rate of ATP hydrolysis of the WT protein. Under the assumption that the rates of dimer association in the WT and the E599Q mutant are similar, the rate of dimer association is the rate-limiting step in the ATP hydrolysis cycle of the WT-NBD of Mdl1. ADP release is often discussed as the rate-limiting step, but our experiments demonstrate that this is not the case, because ADP release would be a concentration-independent process. Furthermore, competition assays with non-labeled and fluorescent nucleotides showed that under the conditions used in our experiments, the ADP release rate was at least 1 s−1.6 Thus, steps like the ATP hydrolysis reaction, dimer dissociation, and ADP release occur faster than the rate-limiting step of dimer association. Similar dependences of the ATPase activities on the NBD concentration (with Hill coefficients of ~1.7) have previously been reported for isolated NBDs like HisP (27), NBD-Mdl1 (8), and NBD-HlyB (25). These data can be interpreted by a monomer-dimer equilibrium. We however propose that the concentration dependence can also be explained by a shift in the rate-limiting step when at higher protein concentrations with an increasing rate of dimerization, a step that is not concentration-dependent like the ATP hydrolysis reaction, dissociation of the dimer or the release of ADP becomes rate-limiting. This has indeed been observed for HisP, the NBD of the histidine permease (28) and full-length Mdl1.7 Noticeably, in the E599Q-NBD, ATP hydrolysis is independent on the viscosity and the NBD concentration, suggesting that here another step is slower than the dimerization of the NBDs. Because the steady-state nucleotide binding affinities of the WT-NBD and the E599Q-NBD are similar, it is very likely that the rate of ADP release is also similar in both proteins, signifying that ADP release from the monomeric E599Q-NBD is not the rate-limiting step. Indeed, in the E599Q-NBD, ATP hydrolysis is strictly coupled to the rate of E599Q-NBD dissociation. Because we previously proven that in the isolated E599Q-NBD dimers dissociation can occur only after hydrolysis of the ATPs, it is very likely that the ATP hydrolysis in the NBD is the rate-limiting step.

Several models have previously been proposed for the ATP hydrolysis cycle of ABC proteins. Importantly, the concentration dependence of the ATPase activity of the Mdl1-NBD fits well with the expected kinetics for the processive clamp model with association and dissociation steps for the NBD in every turn-over but cannot be explained with an "alternating site" model, because this model assumes that the NBDs remain in contact during consecutive ATP hydrolysis cycles. The processive clamp model is further supported by the crystal structures of isolated NBDs, which always showed the symmetry of the two monomers with equal occupation of both nucleotide-binding sites (11, 13, 25, 29), and our data on the isolated Mdl1-NBD, which contained two nucleotides in the beryllium fluoride-trapped state (8). Notably, a negative cooperativity as expected for an alternating site model was never observed for any soluble NBD, suggesting that the ATP hydrolysis cycle of soluble NBDs occurs via the processive clamp model.

Data obtained for full-length transporters, like P-gp and MalFGK2, which showed that 8-azido-ATP photolabeling after beryllium fluoride or orthovanadate trapping resulted in ~1(a-32P)ADP bound per dimer (7, 30–32) conflicts, however, with the processive clamp model. Furthermore, the ATPase activity of many full-length ABC transporters is stimulated by their substrate, demonstrating that in the full-length transporter, the rate-limiting step can be altered when substrates are present. Apparently, the ATP hydrolysis cycle of full-length transporters is more complicated than observed in isolated NBDs. Our data suggest that at higher (local) concentrations of the NBD, which are found, e.g., in full-length transporters, another step, e.g. ATP hydrolysis, becomes rate-limiting and possibly can be stimulated by substrates. How far the ATP hydrolysis cycle of full-length transporters differs from the cycle in soluble NBDs still has to be determined. Therefore, determination of the rate-limiting step(s) in the ATP hydrolysis cycle of a full-length protein and the effects of substrates on the steps in the ATP hydrolysis cycle will also be essential in our understanding of how ATP hydrolysis is coupled to the transport of substrates. In this respect, the new site-specific labeling technique can be very useful to resolve the dynamics of the NBDs within these translocation machineries.

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