The Allosteric Regulatory Mechanism of the *Escherichia coli* MetNI Methionine ATP Binding Cassette (ABC) Transporter

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**Background:** The MetNI transporter drives methionine import against its concentration gradient and regulates intracellular methionine levels.

**Results:** Methionine is a noncompetitive inhibitor of MetNI ATPase activity, binding the transporter at two allosteric sites.

**Conclusion:** MetNI regulates intracellular methionine concentrations via allosteric regulation.

**Significance:** Regulation of methionine import at the protein level may minimize wasteful consumption of ATP when adequate intracellular supplies are available.

The MetNI methionine importer of *Escherichia coli*, an ATP binding cassette (ABC) transporter, uses the energy of ATP binding and hydrolysis to catalyze the high affinity uptake of D- and L-methionine. Early *in vivo* studies showed that the uptake of external methionine is repressed by the level of the internal methionine pool, a phenomenon termed transinhibition. Our understanding of the MetNI mechanism has thus far been limited to a series of crystal structures in an inward-facing conformation. To understand the molecular mechanism of transinhibition, we studied the kinetics of ATP hydrolysis using detergent-solubilized MetNI. We find that transinhibition is due to noncompetitive inhibition by L-methionine, much like a negative feedback loop. Thermodynamic analyses revealed two allosteric methionine binding sites per transporter. This quantitative analysis of transinhibition, the first to our knowledge for a structurally defined transporter, builds upon the previously proposed structurally based model for regulation. This mechanism of regulation at the transporter activity level could be applicable to not only ABC transporters but other types of membrane transporters as well.

Early *in vivo* studies by Kadner and colleagues (1, 2) established that *Escherichia coli* utilizes a high affinity transport system that mediates the energy-dependent uptake of D- and L-methionine into cells. These elegant studies further demonstrated that uptake of external methionine is repressed by the level of the internal methionine pool, a phenomenon called transinhibition (3). As a result, the cell is able to regulate uptake of extracellular methionine to maintain an appropriate intracellular concentration, thereby minimizing the wasteful consumption of energy.

The high affinity import system of *E. coli* was subsequently identified as MetNI, a member of the ABC binding cassette (ABC) family of transporters (4–6). The MetNI importer, as with all ABC transporters, consists of two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) (7). ATP binding and hydrolysis occur at the interface between the highly conserved NBDs, where ATP is sandwiched between the Walker A motif of one monomer and the ABC motif (LSGGQ) on the other (8–10). Based on the TMD fold observed in the crystal structure (see Fig. 1A), MetNI was categorized as a Type I importer, with other structurally characterized members including the ModBC molybdate transporter and the MalFGK2, maltose transporter (11–14). For this class of importers, the alternating access transport mechanism proposes that ATP binding at the NBD interface induces the closure, or “dimerization” of the two domains, thereby promoting an outward-facing conformation of the TMDs (15). The internal cavity of the transporter is thus open to receive substrate from the cognate periplasmic binding protein. Subsequent ATP hydrolysis releases NBD dimerization and converts the TMDs to an inward-facing conformation where substrate can be released to the cytoplasm (see Fig. 1B).

In addition to this common domain architecture, the MetNI NBDs are fused to two carboxyl-terminal domains, termed C2, which are believed to be crucial for transinhibition (11, 12). The MetNI C2 domains are distinct from those found on the ModBC and MalFGK2 transporters, although all are implicated in regulatory roles of transport (13, 16). The high-resolution crystal structure of MetNI shows the NBDs widely separated due to dimerization of their C2 domains (see Fig. 1A). Sel-enomethionine has been shown to bind the C2 domains and inhibit ATPase activity. Based on these findings, it has been proposed that ligand-dependent association of the C2 domains prevents dimerization of the NBDs. Because the two ATP bind-

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**References:**

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2. This article contains supplemental Figs. S1–S3, Table S1, and Modeling of MetNI Inhibition.

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*The abbreviations used are: ABC, ATP binding cassette; NBD, nucleotide binding domain; TMD, transmembrane domain; C2, carboxy-terminal domains; DDM, D-dodecyl-β-D-maltopyranoside; TAPS, 3-[(2-hydroxy-1,1-bis(hydroxyethyl)ethyl]amino]-1-propanesulfonic acid; ATP-γ-S, adenosine 5′-O-(thiotriphosphate); AMPPNP, 5′-adenyl-β,γ-imidodiphosphate; AMP-PCP, adenosine 5′-(β,γ-methylene)triphosphate; Elia, glucose-specific Elia.*
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MetNI provides a rare opportunity to develop detailed kinetic and thermodynamic analyses to study the mechanism of a structurally characterized ABC transporter. As a starting point, we examine the kinetics of ATP hydrolysis to elucidate the mechanism of MetNI transinhibition. To focus on the inhibitory effects of methionine, we employed a basic experimental system consisting of detergent-solubilized MetNI in the absence of MetQ, the substrate-containing cognate periplasmic binding protein. Although MetQ will clearly influence the structure and activity of MetNI (see Ref. 17 for an informative discussion of the maltose transport system), it will also complicate an analysis of the role of methionine because the stimulating effect of MetQ on the ATPase activity is opposite of the allosteric effect of methionine. As a consequence, we have not included MetQ in these studies. Additionally, the basal ATPase activity of detergent-solubilized ABC transporters typically increases and the coupling efficiency decreases, relative to the behavior in a phospholipid bilayer, as again exemplified by the maltose transporter (see Ref. 18). Although ATPase activity uncoupled to transport is not the physiological reaction of ABC transporters, comparisons of ABC subunits in isolation and in transporters are suggestive of a common mechanism for ATP hydrolysis (19). Consequently, meaningful kinetic interpretations can be derived from the effects of methionine on the relative ATPase activity of MetNI in our simplified system. We find that transinhibition is due to noncompetitive inhibition by L-methionine and that there are two allosteric methionine binding sites per transporter. These findings are consistent with the structurally based model for transinhibition and could be extended to understanding the regulation of the broader ABC family.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The wild-type Histagged MetNI expression plasmid (11) was transformed into BL21-Gold (DE3) cells (Novagen). Cell growth was carried out at 37 °C in Terrific Broth containing 1% glucose and 100 μg/ml ampicillin. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside to 1 mM for 1 h. Cells were harvested by centrifugation and stored at −80 °C.

Frozen cell pellets were homogenized in purification buffer (50 mM TAPS, pH 8.5, 250 mM NaCl, 0.05% n-dodecyl-β-D-maltopyranoside (DDM, Anatrace)) supplemented with DNase, lysozyme, and protease inhibitor tablets (Roche Diagnostics GmbH). To extract MetNI protein, DDM was added to 1.0% final concentration. Cells were stirred at 4 °C for 20 min and then passed through a Microfluidizer three times. The cell lysate was stirred for 20 min and then spun for 20 min at 16,000 rpm in a JLA 17 rotor. The supernatant was recovered, and imidazole was added to 25 mM final concentration. The supernatant was then flowed over three tandem 5-ml HiTrap FF columns (GE Healthcare) and washed with 2 column volumes of purification buffer containing 25 mM imidazole and 5 column volumes of purification buffer containing 75 mM imidazole. MetNI protein was eluted with purification buffer containing 350 mM imidazole. Eluate was then buffer-exchanged into purification buffer without imidazole using a HiPrep 26/10 desalting column (GE Healthcare). The protein was then sized over a Superdex 200 column (GE Healthcare) and concentrated to ~60 mM in an Amicon Ultra 100-kDa molecular mass cutoff centrifugal filter (Millipore) prewashed in purification buffer. Purified protein was flash-frozen in LN2 and stored at −80 °C.

ATPase Assays—The amount of inorganic phosphate generated in solution was measured in real time using the Enzchek phosphate assay kit (Molecular Probes). Each 100-μl reaction contained 60 mM Tris, pH 7.5, 5 mM TAPS, pH 8.5, 0.055 M DDM, 55 mM NaCl, 200 μM 2-amino-6-mercaptop-7-methylpyrimidine riboside, 0.1 units of purine nucleoside phosphorylase, equimolar amounts of MgCl2 and ATP and/or ADP, and 1-methionine as indicated. Data were collected on an Infinite M200 microplate reader (Tecan Group) at 33 °C. Reactions were incubated for 2 min and then initiated by automatic injection of MetNI (supplemental Fig. 51). For measurement of $K_{\text{R,ADP}}$, 3 μM MetNI final concentration was injected into reactions containing 100 μM MgATP. For all other measurements, reactions contained 410 nM MetNI. Initial rates were obtained by calculating the linear portion of the change in absorbance at 360 nm as a function of time using Magellan software. Error bars represent S.E. from at least three independent experiments.

All data were fit using KaleidaGraph (Synergy Software). For determination of $K_i$ values, the initial rate was plotted against inhibitor concentration and fit to the following equation

$$v = \frac{V_{\text{app}}}{1 + \left(\frac{[I]}{K_i}\right)^n}$$

(Eq. 1)

where $[I]$ is the inhibitor concentration, $K_i$ is the inhibition constant, and $n$ is the Hill coefficient for the inhibitor.

For determination of $K_{\text{m,ATP}}$, $V_{\text{max}}$, and the Hill coefficient $n_{\text{ATP}}$, the initial rate was plotted against ATP concentration and fit to the following equation

$$v = \frac{V_{\text{max}} 	imes [\text{ATP}]_{\text{app}}}{K_{\text{m,ATP}} + [\text{ATP}]_{\text{app}}}$$

(Eq. 2)

Isothermal Titration Calorimetry—MetNI was further concentrated to 350 – 450 μM in an Amicon Ultra 100-kDa molecular mass cutoff centrifugal filter (Millipore) prewashed in purification buffer. Samples were then dialyzed overnight in purification buffer in 100-kDa molecular mass cutoff Slide-a-Lyzer MINI dialysis devices (Thermo Scientific). The next day, samples were recovered and cleaned at 95,000 rpm for 10 min in a TLA100 rotor. 1-Methionine (BioUltra, Sigma) was reconstituted in the purification buffer used for dialysis. Binding data were collected on a MicroCal iTC-200 calorimeter at 25 °C. 300 – 450 μM MetNI was titrated with 8 – 10 mM L-methionine.

For each experiment, an initial injection of 0.4 μl was followed by 19 injections of 2 μl each. The sample cell was allowed to equilibrate for 180 s between titrations. Data were processed using Origin v7.0 (OriginLab). Error bars represent S.E. from at least three independent experiments.
RESULTS

To establish the kinetic mechanism of transinhibition, we measured the ATPase activity of detergent-solubilized, wild-type MetNI using a continuous spectrophotometric coupled enzymatic assay (see “Experimental Procedures”). To define the basic parameters of the assay, MetNI was automatically injected into reactions containing varying concentrations of MgATP. The initial rates were plotted and fit to a sigmoidal curve with $K_m = 330 \pm 30 \mu M$ and a Hill coefficient of $1.7 \pm 0.1$, indicating positive cooperativity between the two ATP binding pockets (Fig. 1C and supplemental Fig. S1). Cooperative binding of ATP is a common feature of several ABC transporters, including the vitamin B12, maltose, and histidine transporters (20–23).

We then verified the competitive nature of nucleotide inhibition of MetNI ATPase activity. Because canonical nucleotide analogs for ATP, including AMPPNP, AMPPCP, and ATPγS, did not completely inhibit ATPase activity in the concentration range tested (data not shown), we measured ATPase inhibition using ADP. Under subsaturating ATP concentrations, ADP inhibited MetNI ATPase activity with a $K_i(ADP)$ of $41 \pm 2 \mu M$ and a Hill coefficient of $1.3 \pm 0.1$, indicating low cooperativity for the binding of ADP (Fig. 2A and Table 1). Next, the ATP concentration was varied under constant ADP concentrations both above and below the measured $K_i(ADP)$. In the presence of ADP, the apparent $V_{\text{max}}$ remained unchanged, whereas the apparent $K_m$ increased with increasing inhibitor concentration (Fig. 2B and Table 2). As expected, these experiments confirmed that ADP is indeed a competitive inhibitor.

We next tested l-methionine inhibition of MetNI ATPase activity. Inhibition experiments under saturating ATP concentrations were fit to a $K_i(l\text{-Met})$ of $41 \pm 1 \mu M$ and a Hill coefficient of $1.4 \pm 0.1$, indicating that there is more than one methionine binding site per MetNI transporter and low cooperativity between the sites (Fig. 3A and Table 1). Furthermore, with increasing l-methionine concentrations, the apparent $V_{\text{max}}$ significantly decreased, whereas the apparent $K_m$ remained constant (Fig. 3B and Table 2). These data suggest that l-methionine is a noncompetitive inhibitor of MetNI ATPase activity. To further support this model, we conducted isothermal titration calorimetry experiments using l-methionine as the ligand (Fig. 3C). l-Methionine bound to MetNI at $2.2 \pm 0.1$ sites, with a $K_i(l\text{-Met})$ of $33 \pm 4 \mu M$. Although the error estimates provided by the software analysis are artificially low (see reduced $x$-squared values for fixed values of stoichiometry, Fig. 3C, inset), the measured value for the $K_i(l\text{-Met})$ is in the same order of magnitude of the $K_i(l\text{-Met})$ for the ATPase reaction ($41 \pm 1 \mu M$).

DISCUSSION

We have demonstrated that l-methionine can bind to MetNI at allosteric sites, resulting in the noncompetitive inhibition of
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ATP hydrolysis. This direct modulation of MetNI activity is consistent with the model based on the crystallographically observed ATPase-inactive state of MetNI. Our data suggest that the transinhibition phenomenon observed by Kadner (3) can be attributed to the regulation of methionine import at the protein activity level. The de novo biosynthesis of methionine in E. coli is costly, requiring 7 ATPs and 8 NADPHs per molecule of methionine (24). If extracellular methionine is available, uptake of the exogenous methionine at an estimated cost of ~2 ATPs per methionine molecule would be energetically preferable to de novo biosynthesis. The homostatic intracellular concentration of methionine is estimated to be 150 \( \mu \text{M} \) (25), maintained by the balance between the rate of appearance of methionine via the MetNI import system and/or the methionine biosynthesis pathway and the rate of disappearance via protein synthesis and other processes. Given the \( K_f \) for methionine inhibition measured in this study (~40 \( \mu \text{M} \)), uptake should occur under conditions when the intracellular levels of methionine are well below 150 \( \mu \text{M} \), with the rate of transport decreasing as the interior concentration increases.

Several aspects of our study are worth detailing. We have kinetically analyzed the influence of methionine on the ATPase activity of detergent-solubilized MetNI in the absence of the MetQ binding protein and in the absence of phospholipid. For the kinetic analysis, we have used the simplest models for non-competitive and competitive inhibition to treat the effects of methionine and ADP, respectively, on ATPase activity. These models do not include binding interactions between ligands. We have explored more complex kinetic models (see the supplemental text), which suggest that little interaction occurs in the binding of these ligands. Given the correlation of parameters intrinsic to these binding models (26), it is not possible to confidently assess the magnitudes of these effects. The MetQ binding protein was not utilized in these studies to avoid the opposing effects of methionine on ATPase activity due to transport (requiring methionine-bound MetQ) and to transinhibition (requiring methionine acting as an allosteric ligand). We have focused on detergent-solubilized MetNI to directly characterize the influence of methionine on the ATPase activity of the form of MetNI that has been crystallographically characterized. Although the physiological function of ABC transporters requires membranes, detergent-solubilized transporters can capture important functional properties. The most extensive mechanistic studies of ABC importers have been conducted for the MalFGK2 transporter; although the detailed properties differ, the cooperativity of ATP hydrolysis can be observed for both detergent-solubilized and proteoliposome-reconstituted ABC transporters (22). Furthermore, complexes between the maltose transporter and binding protein can be trapped using ATP + vanadate with either membrane-bound or detergent-solubilized transporter (27). Unquestionably, the structural studies on detergent-solubilized maltose transporter from the Chen group (14, 17) and on detergent-solubilized vitamin B12 transporter from the Locher group (20, 28) have provided an invaluable framework for the interpretation of mechanistic studies of transport in proteoliposomes.

ABC transporters have adopted a wide range of mechanisms to regulate their activity. The molybdate/tungstate transporter ModBC shares a similar architecture with MetNI and also demonstrates transinhibitory behavior at higher concentrations of substrate (13). The maltose transporter, in contrast, is regulated by an additional protein that responds to nutrient availability. Unphosphorylated EIIAGlc binds MalFGK2 when preferred sources of carbon, such as glucose, are available (29). Two EIIAGlc molecules bind the NBDs and promote the inward-facing conformation reminiscent of the MetNI inhibited

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**TABLE 1**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( K_f ) (( \mu \text{M} ))</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>41 ± 2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>t-Met</td>
<td>41 ± 1</td>
<td>1.4 ± 0.1</td>
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</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( V_{\text{max}} ) (min(^{-1}))</th>
<th>( K_{\text{app}} ) (( \mu \text{M} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>17 ± 1</td>
<td>330 ± 20</td>
</tr>
<tr>
<td>30 ( \mu \text{M} ) ADP</td>
<td>19 ± 1</td>
<td>660 ± 40</td>
</tr>
<tr>
<td>75 ( \mu \text{M} ) ADP</td>
<td>21 ± 1</td>
<td>1170 ± 80</td>
</tr>
<tr>
<td>120 ( \mu \text{M} ) ADP</td>
<td>20 ± 1</td>
<td>1490 ± 140</td>
</tr>
<tr>
<td>25 ( \mu \text{M} ) t-Met</td>
<td>13 ± 1</td>
<td>309 ± 19</td>
</tr>
<tr>
<td>50 ( \mu \text{M} ) t-Met</td>
<td>7 ± 1</td>
<td>325 ± 15</td>
</tr>
</tbody>
</table>

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**FIGURE 2.** ADP is a competitive inhibitor of MetNI ATPase activity. A, inhibition of MetNI ATPase activity as a function of ADP concentration. Inset, observed rate constant at low concentrations of MgATP. B, ATPase activity as a function of ATP concentration in the presence of different concentrations of ADP. Reaction conditions were identical with those in Fig. 1. Error bars represent S.E. from at least three independent experiments.
Interestingly, it has been demonstrated that the binding of EIIAGlc does not affect the binding of ATP but rather inhibits ATP hydrolysis by MalK (30). Quantitative modeling of our MetNI data suggests that methionine may similarly inhibit ATP hydrolysis but not ATP binding (see the supplemental text).

Although the above examples highlight inhibition as the result of a stabilized inward-facing conformation, ABC transporters are also regulated at the level of transcription, glycosylation, and direct phosphorylation. For example, the osmoregulatory transporter OpuA is regulated by the transcription factor OpuR in response to osmotic stress (31). CFTR (cystic fibrosis transmembrane conductance regulator), an ABC-type chloride channel whose malfunction is linked to cystic fibrosis, is gated by PKA-mediated phosphorylation of its cytoplasmic regulatory domain (32–35). Lastly, it is hypothesized that glycosylation may help stabilize the rod photoreceptor ABC transporter (ABCR) against degradation while in the endoplasmic reticulum (36).

The regulation of ABC transporters, whether through accessory proteins, regulatory domains, or posttranslational modifications, is critical to balance the transport of metabolites for optimal growth. The MetNI transporter, through transinhibition, is effectively able to shut off import when adequate intracellular methionine is available for crucial cellular processes. A possible mechanism for this phenomenon envisioned nearly 40 years ago by Kadner (3) on the basis of transport studies in E. coli cells ("... internal methionine binds to the carrier on the internal face of the membrane. This methionine-carrier complex might be unable, or at least less able, to undergo the energy-dependent change necessary for active transport...") was prescient, as the present studies have established using purified components. Significantly, the underlying mechanism (ligand binding to regulatory domains inhibits NBD dimerization, thereby preventing ATP hydrolysis and effectively "turning off the engine") exemplifies a broadly relevant mechanism for regulating the activity of ABC transporters at the protein level.

FIGURE 3. L-Methionine is a noncompetitive inhibitor. A, inhibition of MetNI ATPase activity as a function of L-Met concentration. Inset, observed rate constant at low concentrations of MgATP. B, ATPase activity as a function of ATP concentration in the presence of different concentrations of L-Met. Reaction conditions for ATPase assays were identical with those in Fig. 1. Error bars represent S.E. from at least three independent experiments. C, top, representative ITC thermogram profile for association of L-Met ligand with MetNI reactant. Bottom, binding curve from plot of heats from each injection as a function of the ratio of L-Met to MetNI, c = 14. Buffer conditions for ITC experiments consisted of 50 mM TAPS, pH 8.5, 250 mM NaCl, 0.05% n-dodecyl-β-D-maltopyranoside at 25 °C. Binding of L-methionine was exothermic with \( \Delta H = -2140 \pm 40 \text{cal mol}^{-1} \). Inset: reduced \( \chi \)-squared value for ITC data fit as function of stoichiometry, \( N \).
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REFERENCES

Supplemental Material

The allosteric regulatory mechanism of the E. coli MetNI methionine ABC transporter

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Figures S1-S3
Table S1
Modeling of MetNI inhibition
**Figure S1. Representative raw data for ATPase assays.** The amount of inorganic phosphate generated in solution was measured in real-time using the EnzChek Phosphate Assay Kit (Molecular Probes). Each 100 µl reaction contained 60 mM Tris, pH 7.5, 5 mM TAPS, pH 8.5, 0.055% DDM, 55 mM NaCl, 200 µM 2-amino-6-mercapto-7-methylpurine riboside, 0.1 units of purine nucleoside phosphorylase, equimolar amounts of MgCl₂ and ATP. Data were collected on an Infinite M200 microplate reader (Tecan Group) at 33°C. Reactions were incubated for 2 minutes and then initiated by automatic injection of MetNI to a final concentration of 410 nM. Initial rates were obtained by calculating the linear portion of the change (200–400 sec) in absorbance at 360 nm as a function of time.
Figure S2. Graphical representations of positive cooperativity for MgATP. Data from Figure 1C replotted as (A) Eadie-Hofstee plot and (B) Lineweaver-Burk plot.
Figure S3. Theoretical models for competitive and noncompetitive inhibition.
All values represent intrinsic dissociation constants. A, Thermodynamic scheme for competitive inhibition by ADP. MetNI is represented by “E”; ATP by “T”, and ADP by “D”. B, Fit of ADP data using model depicted in (A). Blue, no ADP; red, 30 μM ADP; green, 75 μM ADP; yellow, 120 μM ADP. C, Thermodynamic scheme for noncompetitive inhibition by L-methionine. MetNI is represented by “E”; ATP by “T”, and L-met by “M”. D, Fit of L-met data using model depicted in (C). Blue, no L-met; red, 25 μM L-met; green, 50 μM L-met.
Modeling of MetNI inhibition

Using the kinetic data, we generated several models to quantitatively test different thermodynamic schemes for inhibition. Nonlinear regression analysis was performed using Mathematica’s NonlinearModelFit function. Equations for fitting were derived as per Segel (see below) (1).

All models require the binding of two ATP molecules per transporter for hydrolysis, as supported by the cooperativity observed in Fig 1B. Additionally, crystallographic studies of ABC subunits suggest that two bound ATP molecules are needed to stabilize the interface between the TMDs (2,3). While ATP hydrolysis may occur with only 1 bound ATP, as suggested by the Hill coefficient of 1.7, we chose to not include this species in our modeling studies to minimize the number of refined parameters. For ADP inhibition, the data best fit a model where one MetNI transporter (“E”) can bind one molecule of ATP (“T”) and one molecule of ADP (“D”) simultaneously (Fig S3, A-B and Table S1). The presence of the mixed state, DET and TED, is not unexpected; it is conceivable that MetNI could hydrolyze only one ATP at a time, although this condition was not required to fit the kinetic model. The doubly bound DED state has been observed crystallographically in an inward facing conformation (4). More detailed comparisons of binding affinities were difficult to infer due to the large standard errors, a consequence of the highly correlative nature of the model parameters (5).

The model for L-methionine inhibition is more complicated, as there are two binding sites for ATP and two allosteric binding sites for L-methionine (“M”) per one transporter (Fig S3, C-D and Table S1). The data best fit a model in which only one species is able to hydrolyze ATP. The single catalytically active species contains two ATP and no L-methionine (TET) and represents ~24% of the population at intracellular concentrations of 150 µM L-met and 9.6 mM ATP (6). Conversely, when MetNI is bound to two ATP and one L-methionine, TEMT and TMET, hydrolysis cannot occur (~74% of population). Intriguingly, we found that the presence of one bound L-methionine does not affect the binding affinity of either the first or second molecule of ATP. For example, the binding affinity for $E + T \rightarrow ET$ is the same as that of $EM + T \rightarrow EMT$. Cooperative binding for ATP is maintained as well; $ET + T \rightarrow TET$ is the same as that for $EMT + T \rightarrow TEMT$.

These two observations suggest that the binding of one methionine may not induce a substantial enough conformational change at the NBD interface to affect nucleotide binding, and yet is able to disrupt catalysis.
Derivation of basic equations for global modeling

An ATPase reaction for a unireactant system can be visualized as:

\[ E + S \xrightleftharpoons[K_S]{k_{cat}} ES \rightarrow E + P \]

The Michaelis-Menten equation can be used to describe the ATPase reaction under rapid equilibrium conditions:

\[ v = k_{cat} \frac{[E][S]}{K_S + [S]} = V_{max} \frac{[S]}{K_S + [S]} \]

where \( v \) is the initial velocity, \([S]\) is a fixed ATP concentration, \([E]\)_t is the total concentration of enzyme, \( k_{cat} \) is the catalytic rate constant, and \( K_S \) is the dissociation constant for the ATP bound enzyme.

This can be rewritten in an alternate form:

\[ \frac{v}{[E]_t} = k_{obs} = k_{cat} \frac{[S]}{K_S} \frac{1}{1 + \frac{[S]}{K_S}} \]

where \( k_{obs} \) is the observed rate constant. In this form, the numerator contains only one term, indicating that there exists only one product forming species. There are two terms in the denominator, indicating that there are a total of two species, free E and ES complex.
Derivation of basic equations, continued

In the presence of an inhibitor, the Michaelis-Menten equation includes an additional term for the enzyme-inhibitor complex:

\[ v = k_{cat} \frac{[E]_t [S]}{K_S \left(1 + \frac{[I]}{K_i}\right) + [S]} \]

where \([I]\) is a fixed concentration of inhibitor, and \(K_i\) is the dissociation constant for the inhibitor bound enzyme.

This can be rewritten in an alternate form:

\[ k_{obs} = k_{cat} \frac{[S]}{K_S \left(1 + \frac{[S]}{K_S} + \frac{[I]}{K_i}\right)} \]
Modeling of ADP inhibition data:

1. To obtain values for $K_T, a, k_{cat}$ in the global model, we first fit the data ($k_{obs}$ as a function of [ATP]) in the absence of inhibitor (data points shown in Figure 1c). Based on the Hill coefficient discussed in the main text, we assume that only the doubly ATP bound species can hydrolyze ATP (shown in the numerator). Thus the model presented here differs from that from the main text, since here it explicitly depends on $T^2$, while the equations in the main text are dependent on $T^n$. There are a total of three possible species of enzyme, enzyme alone, singly bound by ATP, and doubly bound by ATP (represented in the denominator). If the identical ATP binding sites are cooperative, the binding of one molecule of ATP can alter the intrinsic dissociation constant of the vacant ATP site by the factor “$a$”. When the value of “$a$” is between 0 and 1, the binding of ATP exhibits positive cooperativity. The equation is as follows:

$$k_{obs} = k_{cat} \frac{\frac{T^2}{aK_T^2}}{1 + \frac{2T}{K_T} + \frac{T^2}{aK_T^2}}$$

where

- $T$ — [ATP]
- $K_T$ — intrinsic dissociation constant for ATP
- $a$ — interaction factor for binding of second ATP
- $k_{cat}$ — catalytic rate constant

Parameter estimates are as follows:

- $K_T = 560 \pm 160 \mu$M
- $a = 0.17 \pm 0.08$
- $k_{cat} = 17.5 \pm 0.5 \text{ min}^{-1}$
Modeling of ADP inhibition data, continued:

2. To obtain $K_D$ and $c$ for the global fit, we then fit the data observed at subsaturating, constant [ATP] and varying [ADP]. These data points are shown in Figure 2A.

$$k_{obs} = \frac{V_{max}^{app}}{1 + \frac{2D}{K_D} + \frac{D^2}{cK_D^2}}$$

where

- $V_{max}^{app}$ — apparent maximal velocity at subsaturating ATP
- $D$ — [ADP]
- $K_D$ — intrinsic dissociation constant for ADP
- $c$ — interaction factor for binding of second ADP

Parameter estimates are as follows:

- $K_D = 105 \pm 5 \, \mu$M
- $c = 0.68 \pm 0.12$
- $V_{max}^{app} = 0.85 \pm 0.01 \, \text{min}^{-1}$
Modeling of ADP inhibition data, continued:

3. From the parameter estimates obtained in Steps #1 and #2, we fixed the values for the following parameters:

\[ K_T, a, K_D, c, k_{cat} \]

and globally fit the four data sets observed at constant [ADP] and varying [ATP] (data points shown in Figure 2b) using the following equation:

\[
k_{obs} = k_{cat} \times \frac{\frac{\tau^2}{aK_T^2}}{1 + \frac{2\tau}{K_T} + \frac{\tau^2}{aK_T^2} + \frac{2TD}{bK_TK_D} + \frac{2D}{K_D} + \frac{D^2}{cK_D^2}}
\]

and fitting only one floating parameter:

\[ b \quad \text{— interaction factor for binding of ATP with one bound ADP or binding of ADP with one bound ATP (these are thermodynamically equivalent)} \]

The parameter estimate is as follows:

\[ b = 0.34 \pm 0.03 \]
Modeling of L-Methionine inhibition data

4. To fix parameters $K_i$ and $d$, we fit the data observed at saturating, constant [ATP] and varying [L-met] (data points show in Figure 3A).

\[
k_{obs} = \frac{V_{max}}{1 + \frac{2I}{K_I} + \frac{I^2}{dK_I^2}}
\]

where

- $I$ — [L-Methionine]
- $K_I$ — intrinsic dissociation constant for L-Met
- $d$ — interaction factor for binding of second L-Met
- $V_{max}$ — maximal velocity

Parameter estimates are as follows:

- $K_I = 100 \pm 4 \, \mu M$
- $d = 0.91 \pm 0.15$
- $V_{max} = 20.5 \pm 0.16 \, \text{min}^{-1}$
Modeling of L-Methionine inhibition data, continued

5. From the parameter estimates obtained in Steps #1 and #4, we fixed the values for the following parameters:

\[ K_T, a, K_I, d, k_{cat} \]

and globally fit the four data sets observed at constant [L-met] and varying [ATP] (data points shown in Figure 3B) using the following equation:

\[
k_{obs} = k_{cat} \times \frac{T^2}{aK_I^2} \left( 1 + \frac{2T}{K_T} + \frac{T^2}{aK_I^2} + \frac{2l^2}{dK_i^2} + \frac{4Tl}{eK_TK_I} + \frac{2T^2l}{fK_T^2K_I} + \frac{2Tl^2}{gK_TK_I^2} + \frac{T^2l^2}{hK_T^2K_I^2} \right)
\]

where

- \( e \) — interaction factor for binding of one ATP with one bound L-Met or binding of one L-Met with one bound ATP
- \( f \) — interaction factor for binding of second ATP with one bound L-Met and one bound ATP
- \( g \) — interaction factor for binding of second L-Met with one bound ATP and one bound L-Met
- \( h \) — interaction factor for binding of second ATP with two bound L-Met and one bound ATP, or binding of second L-Met with two bound ATP and one bound L-Met

6. We first chose to eliminate the doubly bound ATP, doubly bound L-Met species as this is the least likely to be physiologically relevant. The equation was simplified to:

\[
k_{obs} = k_{cat} \times \frac{T^2}{aK_I^2} \left( 1 + \frac{2T}{K_T} + \frac{T^2}{aK_I^2} + \frac{2l^2}{dK_i^2} + \frac{4Tl}{eK_TK_I} + \frac{2T^2l}{fK_T^2K_I} + \frac{2Tl^2}{gK_TK_I^2} \right)
\]
Modeling of L-Methionine inhibition data, continued

7. Next, as \( f \) and \( g \) depend on \( e \), we chose to focus on the \( e \) parameter. We fixed \( e \) at successive values and determined that the best fit was achieved when \( e = 1 \). The equation was further simplified to the following:

\[
k_{obs} = k_{cat} \times \frac{T^2}{aK_T^2} \frac{1}{1 + \frac{2T}{K_T} + \frac{T^2}{aK_T^2} + \frac{2I}{K_I} + \frac{I^2}{aK_I^2} + \frac{4TI}{K_TK_I} + \frac{2T^2I}{fK_T^2K_I} + \frac{2I^2}{gK_IK_T^2}}
\]

8. There were several combinations of values for \( f \) and \( g \) that resulted in same goodness-of-fit.

9. We next tried to eliminate an additional species – either the doubly bound ATP, singly bound L-met species or the doubly bound L-met, singly bound ATP species. We were able to obtain a similar goodness-of-fit as Step #8 only when the doubly bound L-met, singly bound ATP species was eliminated. Thus the final equation for the global fitting is as follows:

\[
k_{obs} = k_{cat} \times \frac{T^2}{aK_T^2} \frac{1}{1 + \frac{2T}{K_T} + \frac{T^2}{aK_T^2} + \frac{2I}{K_I} + \frac{I^2}{aK_I^2} + \frac{4TI}{K_TK_I} + \frac{2T^2I}{fK_T^2K_I}}
\]

and fitting only one floating parameter:

\( f \) — interaction factor for binding of second ATP when one ATP and one L-Met are bound

The parameter estimate is as follows:

\( f = 0.16 \pm 0.01 \)
Modeling of L-Methionine inhibition data, continued

10. The goodness-of-fit in the above steps was measured using the following equation:

\[
\% \text{RMSD} = \sqrt{\frac{\sum_{t=1}^{n}(k_{\text{obs}_t}-k_{\text{mod}_t})^2}{k_{\text{obs}_t}^2/n}}
\]

where

- \( n \) — number of average values from 3 independent experiments
- \( k_{\text{obs}} \) — observed rate constant
- \( k_{\text{mod}} \) — modeled rate constant

11. The \% RMSD for the final fit (equation shown in Step #9) was 2.3%.

References

Table S1. Intrinsic dissociation constants

<table>
<thead>
<tr>
<th>Constant</th>
<th>Description</th>
<th>Value (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_D^{apo\rightarrow ATP}$</td>
<td>apo MetNI binds one ATP</td>
<td>560 ± 170</td>
</tr>
<tr>
<td>$K_D^{ATP\rightarrow ATP\ast}$</td>
<td>ATP-MetNI binds second ATP</td>
<td>93 ± 55</td>
</tr>
<tr>
<td>$K_D^{apo\rightarrow ADP}$</td>
<td>Apo MetNI binds one ADP</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>$K_D^{ADP\rightarrow ADP}$</td>
<td>ADP-MetNI binds second ADP</td>
<td>72 ± 12</td>
</tr>
<tr>
<td>$K_D^{ATP\rightarrow ADP}$</td>
<td>ATP-MetNI binds one ADP</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>$K_D^{ADP\rightarrow ATP}$</td>
<td>ADP-MetNI binds one ATP</td>
<td>190 ± 60</td>
</tr>
<tr>
<td>$K_D^{apo\rightarrow Met}$</td>
<td>Apo MetNI binds one Met</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>$K_D^{Met\rightarrow Met}$</td>
<td>Met-MetNI binds second Met</td>
<td>92 ± 15</td>
</tr>
<tr>
<td>$K_D^{Met\rightarrow ATP}$</td>
<td>Met-MetNI binds one ATP</td>
<td>560 ± 170</td>
</tr>
<tr>
<td>$K_D^{ATP\rightarrow Met}$</td>
<td>ATP-MetNI binds one Met</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>$K_D^{ATP/Met\rightarrow ATP}$</td>
<td>ATP-Met-MetNI binds second ATP</td>
<td>92 ± 28</td>
</tr>
</tbody>
</table>

* $k_{cat} = 18 ± 1 \text{ min}^{-1}$ in the presence of two ATPs

The above intrinsic dissociation constants are defined in terms of binding to a single site (rather than a single molecule of MetNI). Numerical differences in constants between these values and those in the main text are due to differences in equations used for fitting, as described in Step #1.
Enzymology:
The Allosteric Regulatory Mechanism of the *Escherichia coli* MetNI Methionine ATP Binding Cassette (ABC) Transporter

Janet G. Yang and Douglas C. Rees
doi: 10.1074/jbc.M114.603365 originally published online February 12, 2015

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