Inward facing conformations of the MetNI methionine ABC transporter: Implications for the mechanism of transinhibition

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Abstract: Two new crystal structures of the *Escherichia coli* high affinity methionine uptake ATP Binding Cassette (ABC) transporter MetNI, purified in the detergents cyclohexyl-pentyl- β -D-maltoside (CY5) and *n*-decyl- β -D-maltopyranoside (DM), have been solved in inward facing conformations to resolutions of 2.9 and 4.0 Å, respectively. Compared to the previously reported 3.7 Å resolution structure of MetNI purified in *n*-dodecyl- β -D-maltopyranoside (DDM), the higher resolution of the CY5 data enabled significant improvements to the structural model in several regions, including corrections to the sequence registry, and identification of ADP in the nucleotide binding site. CY5 crystals soaked with selenomethionine established details of the methionine binding site in the C2 regulatory domain of the ABC subunit, including the displacement of the side chain of MetN residue methionine 301 by the exogenous ligand. When compared to the CY5 or DDM structures, the DM structure exhibits a significant repositioning of the dimeric C2 domains, including an unexpected register shift in the intermolecular β -sheet hydrogen bonding between monomers, and a narrowing of the nucleotide binding space. The immediate proximity of the exogenous methionine binding site to the conformationally variable dimeric interface provides an indication of how methionine binding to the regulatory domains might mediate the phenomenon of transinhibition.

Keywords: membrane protein structure; importers; ATP binding cassette; allosteric regulation

Introduction

The MetNI methionine importer catalyzes the high affinity uptake of D- and L-methionine by *Escherichia coli* and other bacteria.^{1,2} An ATP Binding Cassette (ABC) transporter,^{3–5} MetNI consists of two copies of the conserved ABC subunit MetN that is the hallmark of this transporter superfamily, in complex with two copies of the transmembrane domain (TMD) subunit MetI.^{6–8} Each MetN

ABC subunit can be further divided into two subdomains⁹; the nucleotide binding domain (NBD; residues 1–245) and a C-terminal domain (C2; residues 265–343) connected by a linker spanning residues 246–264. The MetI subunits contain five transmembrane (TM) helices that define a conserved core present in the Type I family of ABC importers,^{10,11} including the previously solved structures of the ModBC molybdate¹² and MalFGK₂ maltose¹³ transporters. In addition to the transporter subunits, importers require a periplasmic binding protein (MetQ for the MetNI system) to deliver the proper substrate.^{14,15}

The mechanism of ABC transporters is generally interpreted in terms of an alternating access model^{16,17} where substrate translocation is coupled

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to the ATP dependent interconversion of inward and outward facing conformations.^{11,12,18} The binding and hydrolysis of ATP requires association of the NBDs to form a closed dimer, with the nucleotide binding site positioned at the dimer interface.^{19–22} In the conformation competent for ATP hydrolysis, the nucleotides are sandwiched between the conserved P-loop and ABC signature motifs from opposing ABC subunits. The conformational changes accompanying nucleotide binding and hydrolysis are then transmitted to the membrane spanning domains through coupling helices^{23,24} present in cytoplasmic loops between transmembrane helices. The mechanistic understanding of the transporter cycle is most advanced for Type I importers, particularly the maltose MalFGK₂ importer, where a combination of biochemical, biophysical, and structural studies have provided important insights.^{25–28} These studies have revealed a range of conformational states corresponding to inward and outward facing stabilized with various nucleotides, binding proteins, and mutations.

Transporters contribute significantly to the basal energy requirement of cells,^{29–30} and so regulatory mechanisms are essential for controlling their activities. In pioneering studies, Kadner demonstrated that the *in vivo* uptake of methionine by the *E. coli* high affinity uptake system (now recognized as MetNI) was inhibited by the intracellular methionine pool.³¹ This phenomenon of transinhibition, by which an intracellular ligand inhibits uptake of that ligand from the extracellular pool, likely represents a regulatory mechanism to minimize wasteful consumption of ATP when adequate intracellular supplies are available. The structure of MetNI suggested a plausible structural mechanism for this process,⁹ whereby binding of methionine to the carboxy-terminal C2 domain of MetN stabilized an ATPase inactive conformation of the transporter by sterically preventing association of the ABC subunits essential for ATP hydrolysis. Parallel observations on the molybdate transporter, with a distinct fold for the regulatory domain,³² indicates that this is a general phenomenon.

The modest resolution (3.7 Å) of the original crystallographic analysis of MetNI solubilized in DDM precluded a detailed analysis of the structure. In this article, we describe two distinct crystal forms of MetNI solubilized in the detergents cyclohexylpentyl-β-D-maltoside (CY5) and *n*-decyl-β-D-maltopyranoside (DM), and solved to resolutions of 2.9 Å and 4.0 Å, respectively. The higher resolution of the CY5 form enabled significant improvements to be made to the original structure and a detailed model to be developed for the binding of free methionine to the allosteric site. Comparisons of the available structures reveal variants of inward facing conformations that may be relevant to the structural basis of the transinhibition mechanism.

Results

Overall molecular architecture and nomenclature

We first describe the transporter structure and introduce the nomenclature used to identify the subunits and domain organization, and then discuss the relationships between the different structures. The two copies of MetI and MetN present in one transporter are assigned chain IDs A, B and C, D, respectively. For the DDM and CY5 structures with two transporters/asymmetric unit, subunits in the second copy of the transporter are assigned chain IDs E, F (MetI) and G, H (MetN). A total of five complete MetNI transporter structures are available based on data obtained from crystals grown with transporter purified in three detergents; CY5 (2.9 Å resolution, two transporters/asymmetric unit (designated CY5-ABCD and CY5-EFGH, PDB 3TUI), DM (4.0 Å resolution, one transporter/asymmetric unit, PDB 3TUIJ), and the originally solved DDM solubilized transporter (3.7 Å resolution, two transporters/asymmetric unit,⁹ PDB 3DHW).

Although the higher resolution data from CY5 crystals largely validates the original DDM model, improvements to the fit and sequence registry were achieved in several regions; for MetI, these changes include corrections to residues 35–51 and 118–143 in the loops between TM1–TM2 and TM3–TM4, and inclusion of residues 1–5 and 209–216 at the amino- and carboxy- termini, respectively. For MetN, the most significant adjustments were made to residues 36–47 (including the P-loop) and 229–251 in the transition from the NBD to the linker region.

Comparisons of MetNI structures and quaternary structure changes

The available MetNI structures all adopt inward facing conformations with splayed TMDs opening towards the cytoplasmic side and separated NBDs [Fig. 1(A)]. The folds of the corresponding domains (TMD or NBD) in these structures are similar, as reflected in the rms deviations of ~0.8 and 0.9 Å, respectively. The rms deviations between intact transporters exhibit more substantial variation [2.3 Å (CY5-ABCD to CY5-EFGH), 4.1 Å (CY5-ABCD to DM), and 4.7 Å (CY5-EFGH to DM)], however, due to differences in the rigid body orientations of domains between structures, particularly the dimeric C2 domains. Internal rearrangements in the C2 dimer are also evident, and are discussed later. Comparison of the three independent transporter molecules from the CY5 and DM structures reveals the C2 dimers exhibit three alternate positions relative to the reference molecular twofold axis defined by the TMDs and NBDs [Fig. 1(B–D)]. For the C2 dimer of the CY5 subunits C and D, the twofold axis relating these domains is nearly parallel (within

Figure 1. Cartoon ribbon models depicting the structures of CY5 and DM forms of MetNI. A: The CY5-ABCD transporter illustrating the inward facing conformation of the TMDs and separated NBDs. B: CY5-ABCD rotated 90° about the vertical axis from panel A. C: The CY5-EFGH molecule having a significantly different relative orientation of the C2 dimer with respect to the molecular twofold symmetry axis when compared to the CY5-ABCD molecule. D: The single transporter in the DM asymmetric unit having an additional alternate orientation of the C2 dimer. Schematic at far right indicates the (sub)domain organization of MetNI. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

~4°) to the reference axis (the “symmetric” orientation). In the second, crystallographically independent CY5 transporter, a distinct orientation is exhibited by the C2 dimer of subunits G and H, which corresponds to a rotation by ~15° about an axis approximately perpendicular to the reference twofold axis [and oriented normal to the plane of the page in Fig. 1(B)]. Similar orientations of the C2 dimers are observed in the two transporters in the DDM MetNI. A third distinct orientation for the C2 dimer is observed for the DM MetNI transporter, which, relative to the symmetric CY5 orientation, is rotated by ~30° about an axis nearly parallel (within ~5°) to the reference molecular twofold axis. In comparison to the CY5 structures, a major rearrangement of the linker region connecting the NBDs and C2 domains is observed in the DM solubilized MetNI structure, with a concomitant narrowing of the separation between NBDs (see following).

Compared to the C2 domains, the differences observed in the relative positions of the NBDs between the CY5 and DM structures are more modest, with a smaller separation found in the latter. The separation between NBDs has been characterized by the distance between the P-loops and signature motifs of different ABC subunits in a transporter.³³ Using the C α positions of MetN residues Gly43 and Gly143 to define the positions of the P-loop and signature motifs, respectively, the inter-subunit distances in the CY5 transporter are found to be ~26 Å for the CD subunits (average of 25.1 Å and 26.6 Å) and ~24 Å for the GH subunits (average of 22.7 Å and 25.6 Å). The corresponding value for the DM transporter is ~23 Å (average of 21.6 Å and 23.8 Å). The range of distances reflects the asymmetry present in these structures, as noted above for

the orientation of the C2 dimer. A more pronounced difference is observed in the spacing between equivalent “D-loop” residues (Asp172) of the CY5 and DM NBDs. The D-loops of the two distinct CY5 transporters are separated by 21.1 Å and 18.7 Å. In the DM structure, this distance closes to 11.5 Å; the D-loop is in proximity to the C2 domain, and the narrowing observed in this spacing from CY5 to DM is perhaps coupled to the repositioning of the C2 dimer and linker region between these structures.

The translocation pathway of MetNI

The translocation pathway between the MetI subunits is closed at the periplasmic surface by the convergence of TM helices 2 and 4 from opposing subunits [Figs. 1(A) and 2], with very similar conformations exhibited for all the available MetNI structures. Despite low sequence conservation (Fig. S2), the MetI translocation pathway resembles that observed in the inward facing conformation of other Type I structures. Periplasmic gating regions proposed for the *A. fulgidus* ModBC transporter²⁴ are structurally conserved with MetI; a conserved proline (Pro67 in MetI) forms a kink at the periplasmic surface of TM2 in Type I structures (Figs. 2 and S2), while Met163 located in TM 4 of MetI replaces the conserved Phe200 in ModB. The structurally equivalent residues to Met163 in the maltose transporter are Val442 and Val230 of MalF and MalG, respectively.¹³ In the methionine transporter, the Met163 side chains from opposing subunits pack together in a gate like configuration [Fig. 2(B)]. The gating methionines are surrounded on both the periplasmic and cytoplasmic boundaries with amino acid residues having aromatic or hydrophobic side chains.

Figure 2. MetI architecture and proposed gating regions based on structural conservation with ModB: A: Schematic of the MetI transmembrane subunit topology. TM helices are numbered sequentially from the N-terminus. Structurally conserved gate regions 1 and 2 are colored as green and red, respectively. B: View of the MetI transmembrane domains looking down the translocation pathway from the periplasmic side. One MetI subunit is drawn as a yellow ribbon and the opposing subunit as a cyan tube. Gate regions are colored as in A, and the gating methionine and conserved proline residues are shown as stick figures. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The side chains of aromatic residues (Tyr177, Tyr181 periplasmic side; and Phe68, Tyr160 cytoplasmic side), known to form strong interactions with the side chain sulfur of methionine,³⁴ dominate these boundaries.

Interface between the MetI TMD and the MetN NBD

Communication between the nucleotide binding sites and translocation pathway involves the coupling helices (3a and 3b; also known as the EAA loop³⁵) located in the cytoplasmic loop between TM3 and TM4 of MetI and their interface with a groove formed between the RecA and helical sub-domains on the upper surface of the NBD [Figs. 2(A), 3(A), and S3]. A detailed examination of the MetNI interface, especially near the nucleotide binding site, reveals certain interacting residues as candidates for a role in coupling transport to ATPase activity. Most notable is the salt bridge interaction of the Glu121 side chain, located in the MetI 3a coupling helix, with the Arg49 side chain located proximal to the P-loop of MetN. Comparable interactions are observed in other type I importers. This interaction extends through a network of salt bridges and hydrogen bonds in MetN connecting Arg49 to Asp165 in the Walker B motif [Fig. 3(A)]. Given the proximity of Asp165 to the site of ATP hydrolysis, the catalytically critical Glu/Gln166, and the D-loop Asp172 of MetN, it seems likely this network of interactions provides a pathway for the transmission of signals related to conformational changes in the TMDs or NBDs which couple ATP hydrolysis to transport activity.

Nucleotide binding domains and bound ADP

Inspection of 2.9 Å resolution $F_o - F_c$ maps calculated for CY5 ATP co-crystals revealed large peaks (>6 sigma) of positive electron density in the vicinity of the MetN P-loop, suggesting the presence of bound nucleotide [Figs. 3(B) and S4]. To distinguish between ATP or ADP, refinements were separately conducted with the two nucleotides; inspection of the resulting density maps clearly indicated that ADP best fit the crystallographic data. Peaks of negative density were consistently observed surrounding the γ -phosphate position when ATP was included in model refinement, whereas ADP models consistently resulted in flat $F_o - F_c$ maps with clear density for the ligand appearing in the $2F_o - F_c$ map. Native crystals, grown in the absence of any added nucleotide, also revealed similar peaks of positive electron density at the MetN nucleotide binding site, indicating co-purification of ADP with the transporter. Based on the crystallographic analysis, we can not exclude, however, the presence at this site of ATP with a disordering of the γ -phosphate, as proposed in a recent structural analysis of MalFGK₂.²⁷

Changes in the C2 domains between the CY5 and DM transporter structures

Although the TMDs and NBDs of the different MetNI structures are similar, more pronounced differences are evident in the C2 domains. To shed light on the relationship between conformational changes occurring in the C2 domain and reorganization of the NBD pairs, we first compared C2 domains from the crystal structures of intact transporters and from the structure of the isolated C2

Figure 3. Transmission interface between the TMD (MetI) and NBD (MetN). A: A detailed view of interactions potentially involved in transmission of conformational changes between the TMDs and the ATPase active site. The catalytic core/RecA-like and alpha helical subdomains of MetN are indicated by light gray and blue ribbon coloring, respectively. Conserved motifs are colored as follows: P-loop/Walker A, cyan; Walker B, magenta; and D-loop, orange. ADP is shown in ball and stick form, and the MetN C2 domain has been removed for clarity. B: Detailed view of the nucleotide binding site with bound ADP, and the $2F_o - F_c$ electron density map contoured at 1.2σ . ADP and interacting residues are drawn in ball and stick representation. The catalytically relevant Asp165, Gln166, and His199 do not interact with ADP but are shown for reference. P-loop residues are drawn in a cyan cartoon representation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

domain (iso-C2, residues 247–343; PDB ID 3DHX) solved at $2.1 \text{ \AA}$ resolution.⁹ Although the individual (i.e., monomeric) C2 domains of the DM transporter and iso-C2 are similar (RMSD = $0.9 \text{ \AA}$; C α atoms 250–340), more pronounced changes are evident when these are compared to the C2 domains of CY5 derived C2 monomers (RMSDs of 2.2 and $1.9 \text{ \AA}$, respectively). Excluding residues in the NBD to C2 linker, superimposed C α atoms that deviate by more

Figure 4. Conformational states of the C2 domain dimer. A: Ribbon figure of the selenomethionine soaked CY5 C2 dimer with transparent surface representation and selenomethionine ligands shown as spheres. C α atoms separated by $<1 \text{ \AA}$ from the corresponding C α atoms of a superimposed iso-C2 dimer are colored in blue to indicate structurally similar regions. C α atoms separated by $>2 \text{ \AA}$ are shown in red and indicate areas of relatively high conformational variability. Superimposed C α atoms separated by >1.0 and $<2.0 \text{ \AA}$ are drawn in green. Lower model is rotated 90° about the vertical axis with respect to the upper model. B: Plot of C α atom RMSD ($\text{\AA}$) versus residue number for superpositioning of C2 dimer domains from different crystal structures using LSQMAN. Values represent the average RMSD from both chains in the dimer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5. Methionine binding in the MetN C2 domain. A: Detailed view illustrating the proposed mode of (seleno)methionine interaction at the C2 dimer interface. Interacting residues from the chain C and D monomers are drawn as green and cyan sticks, respectively. Peak from a phased anomalous Fourier difference map (contoured at 8σ) obtained from CY5 SeMet soak crystals indicated by red mesh. B: Ligand binding site of superimposed C2 monomers from the CY5 native (green), CY5 SeMet soak (cyan), DM (purple), and iso-C2 (orange) structures. Protein side chains are drawn as stick figures on a ribbon-style $C\alpha$ trace. Selenomethionine (CY5 SeMet soak) appears in ball and stick form with black bonds for clarity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

than 2.0 Å between DM or iso-C2 and CY5 are confined to two stretches (residues 273–280 and 303–310). Conspicuously, these conformationally variable regions are positioned precisely such that they overlap or directly flank the set of amino acid residues that define the C2 ligand binding pocket (see the following section).

The above observations regarding the individual C2 domains also apply to the corresponding dimers; the DM and iso-C2 dimers are structurally comparable but both differ significantly from the CY5 form [Fig. 4(A,B)]. Conformational differences are largely restricted to the “upper” portion of the C2 dimer (proximal to its interface with the NBD pair), while distal interfacial elements residing at the domain’s “lower” surface remain conformationally equivalent. In the upper part of the C2 dimer, a closer look at the differences between the multiple conformations reveals an approximately 7 Å relative displacement of the two monomers at the interface where they form a short anti-parallel beta sheet (residues 297–302). The alternate conformations correspond to a register shift in the intermolecular β -sheet hydrogen bonding (Fig. S5). In the CY5 structure, the local axis of twofold symmetry between C2 monomers falls between equivalent copies of Ala299, whereas the symmetry axis falls between equivalent copies of Gln300 in the DM and iso-C2 structures.

Selenomethionine binding to the C2 domains of the CY5 transporter

To identify the methionine binding site on the C2 domain, CY5 transporter crystals were soaked with selenomethionine. Following data collection at the

selenium-edge to 3.4 Å resolution, the structure was solved by molecular replacement using the CY5 model. Selenomethionine was found bound to all four crystallographically independent C2 domains, at equivalent sites positioned near the C2 dimer interface [Figs. 4(A) and S6]. The binding pocket is defined by five elements: the side chain of Phe273, and four stretches of polypeptide including residues 277–283, 300–301 and 310–312 from the same subunit, and residues 294–296 of the opposing subunit. The ligand Se atom is positioned adjacent to the phenyl ring of the Phe273 side chain, and is sandwiched between the protein methionine side chains of Met301 and Met312. At 3.4 Å resolution, a precise assignment of protein-ligand interactions is problematic, but the ligand appears to be stabilized by a network of hydrogen bonds formed with main and side chain atoms from both C2 domains. The ligand is modeled such that the selenomethionine amino group interacts with the backbone carbonyl oxygen atoms of Val278 and Ala280 of chain C and the Asn295 OD1 from the opposing chain D. The selenomethionine carboxylate group interacts with the amide NH groups of Leu282 and Leu283 from chain C and Ile296 from chain D [Fig. 5(A)].

The conformational changes which occur upon soaking of CY5 crystals with selenomethionine are subtle. Superpositioning of individual C2 monomers ($C\alpha$ atoms 270–330) from native or soaked crystals yields an average RMSD of 0.7 Å. In soaked crystals, a slight inward movement of the protein backbone occurs at residues 277–283 just surrounding the bound ligand. In the native crystals, the Met301 side chains adopt different conformations in

different subunits; the side chain ranges from being positioned such that the sulfur atom resides at a position that nearly coincides with the ligand Se atom in the soaked crystals, to a conformation where the side chain swings outward and away from the ligand binding site. In the soaked crystals, the Met301 side chains in all four crystallographically independent C2 domains are displaced by ligand and assume a conformation that corresponds to the outwardly positioned arrangement in the native form [Fig. 5(B)]. The minimal changes observed between native and SeMet soaked C2 domains, in addition to the observation of an outwardly positioned Met 301 side chain in a native C2 domain [Fig. 5(B)], both suggest the possibility of low occupancy binding of co-purified methionine ligand in the native crystals. The observation of small peaks of positive difference density in the native C2 ligand binding site is also consistent with this idea. Attempts to model ligand in the native C2 domain, however, were unsuccessful.

In contrast to the results with the selenomethionine soaked CY5 transporter crystals, no binding of selenomethionine has been crystallographically observed in either crystal soaking or cocrystallization experiments using the DM solubilized transporter or the iso-C2 domain. The positioning of the Met 301 side chain into the binding pocket in the DM and iso-C2 structures makes occupancy of these sites with co-purified substrate unlikely.

Discussion

A key mechanistic challenge with ABC importers is to establish how nucleotide, ligand, binding protein, and regulatory effectors interact with the transporter to mediate the unidirectional translocation of ligand across the membrane. The structural foundations for this analysis are most advanced for Type I importers,¹⁰ particularly for the maltose MalFGK₂ transporter,^{25–28} and provide an important framework for interpreting the mechanistic significance of the current MetNI structures. Structurally characterized type I importers include MalFGK₂ in outward facing (PDB IDs 2R6G¹³ and 3PUY²⁷), pre-translocation (PDB IDs 3PV0 and 3PUZ²⁷), and inward facing (3FH6³⁶) conformations, ModABC in the inward and inward/inhibited conformations (PDB IDs 2ONK²⁴ and 3D31,³² respectively), and the CY5 and DM forms of MetNI (PDB IDs 3TUI and 3TUJ, respectively) presented in this work. Although the TMDs of Type I importers are organized around a common core of five TM helices, a subset of three of these helices, TM2-4 in MetNI, are highly conserved and exhibit a one to one residue correspondence that can be used for quantitative comparisons of the relationships of the TMDs between different structures; a conserved core can also be identified for the NBDs (that is, of course, general to the entire ABC transporter superfamily).

Equivalent residues in the different structures are detailed in the Methods.

For identifying the relationships between structures, a principal components analysis was implemented based on the “Essential Dynamics” algorithm of Amadei *et al.*³⁷ The outward facing 3PUY maltose transporter coordinates were used as the reference structure²⁷ (this structure is similar to the originally determined 2R6G structure¹³); core residues in TMDs from the different Type I importer structures were superimposed onto the equivalent residues in subunit F of 3PUY [Fig. 6(A)]. Using this reference frame, the principal components were calculated that interrelate either the other TMDs of the different transporters to subunit MalG (TMD-EV1), or that interrelate the NBDs associated with the superimposed TMD (NBD-EV1). In these calculations, the first component dominated the analysis and accounted for ~90% of the differences between a particular structure and the reference, and the following discussion will be based on this dominant component. Figure 6(B) illustrates the variation of the dominant component inter-relating the TMDs and the NBDs of the various structures (labeled TM-EV1 and NBD-EV1, respectively), relative to the reference outward facing conformation of the maltose transporter. The transitions from outward facing through pretranslocation (pre-T) to inward facing conformation of the TMD are associated with an increasing magnitude for TM-EV1. The numerical values can be used to order the transporter structures in a sequence along a reaction coordinate for the transporter cycle, much like Dunitz developed the “structural correlation principle” to deduce reaction pathways by identifying correlated shifts in atomic positions between related structures.³⁸ As the TMDs move apart in the transition to the inward facing conformation, the NBDs rotate towards the subunit interface. The changes in the nucleotide-binding domains (NBD-EV1) generally parallel those of the TMDs, until the CY5 and 3D31 ModBC conformations. The decreased NBD-EV1 components for the CY5 and 3D31 structures indicates that their NBDs shift to a smaller extent relative to the TMDs than for the DM and ModBC 2ONK structures, corresponding to a greater distance between these NBDs as the TMDs separate.

To the extent that the principal components are dominated by rigid body motions, the rotation axes can be identified that dominate the interconversion of the TMDs (red axis) and NBDs (cyan axis) between the 3PUY and CY5 transporter structures [Fig. 6(A)]. The axis interconverting the TMDs corresponds to a hinge motion perpendicular to the two-fold axis of the transporter, oriented along the interface between the membrane spanning subunits. Although the hinge motion described by this transformation is a mathematical construct that need not

Figure 6. Principal component analysis of Type I importer structures. A: Stereoview illustrating the relationship between the TMDs and P-loops of the NBDs. The structures listed in B were superimposed on the MalF subunit (right side) of the outward facing 3PUY conformation of the maltose transporter (see Methods for details); the green chain traces detail the positions of “TM2” in MalF and MalG (TM5 and TM3, respectively). The thin trace for the TMDs on the upper right illustrates the locations of MalF TM6-7 with the coupling helices (3a and 3b in MetI). On the left hand side are the traces corresponding to TM2’s of the other subunit, showing the transition from outward facing to inward facing conformations in various structures. The thick green trace in the lower part of the stereofigure indicates the position of the P-loop of the MalK subunit associated with MalF, whereas the thin, colored traces show the relative positions of the P-loop in the different structures. The rigid body rotation axes describing the hinge motion dominating the interconversions of different conformations of the TMD and NBDs are illustrated in red (top) and cyan (bottom), respectively; these axes pass near the Pro67 kink in TM2 and the 3a/3b coupling helices, respectively. The black vertical axis depicts the molecular twofold axis of the reference MalFGK₂ structure. B: The magnitudes of the principal component interrelating different conformational states of the TMDs [blue circles (TMD-EV1)] and NBDs [red triangles (NBD-EV1)] of Type I importers. The transitions from outward facing through pretranslocation (pre-T) to inward facing conformation of the TMD are associated with an increasing magnitude for TM-EV1. [†]The related 2R6G structure has ATP; [‡]The related 3PV0 structure is nucleotide free (nf); [±]The ADP density in the DM structure is weaker than for the CY5 structure. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

represent the actual motion, it is intriguing that the rotation axis passes close to the conserved Pro67 of both subunits found at the kink on the periplasmic side of TM2; this region corresponds to the periplasmic gate identified in the MalFGK₂ and ModBC structures, and further is adjacent to the internal binding site for maltose found in the MalFGK₂ structure.¹³ The rotation axis corresponding to the rigid body change between the TMD and NBD also passes through elements conserved in Type I importers, including the coupling helices connecting the TMDs and NBDs.

Although the overall arrangements of the TMDs in the CY5 and DM solubilized forms of MetNI are similar, distinct differences between these structures are evident in the orientation and dimerization mode of the C2 domains (and the ability of these domains to bind selenomethionine), as well as differences in the position of the NBDs. Comparisons to other type I importers [Figs. 6(B) and 7] suggest that the CY5 structure likely represents an allosterically inhibited form of the transporter with reduced ATPase activity (more comparable to ModBC structure³² PDB 3D31), whereas the DM structure reflects an uninhibited, resting state similar to MalFGK₂ structure²⁷ PDB 3FH6. A compelling mechanistic understanding of the contribution of the detergent to the observed differences between the CY5 and DM MetNI structures is not apparent at present.

An informative comparison of the progression of conformational states of Type I importers may be made for MetNI and MalFGK₂ (Fig. 7), providing a structural representation of the principal component analysis depicted in Figure 6(B). As the conformational states change from outward to inward facing, the cytoplasmic regions of the two TMDs converge [especially evident in the spacing between the pairs of coupling helices, Fig. 7(A)] as the NBDs draw together to sandwich the nucleotide between the conserved sequence motifs on different domains [Fig. 7(B)]. For MetNI, twisting of the C2 domains between the CY5 and DM structures [Fig. 7(C)] is associated with a change in spacing between the NBDs. While the C-terminal domains of the MalK subunits (structurally distinct from the C2 domains of MetN) remain basically fixed with respect to the NBDs, it is possible that in the presence of suitable ligands, rearrangements of these domains can be used to regulate maltose transport.²⁶

The observation of the C2 dimer in alternate discrete states related by a β -sheet register shift is notable (Fig. S5). Similar shifts in β -sheet registration have been postulated before to be sources of either biologically advantageous^{39,40} or detrimental protein subunit associations,⁴¹ or mechanisms for conformational switching within a protein domain.⁴² Discrete states offer potential advantages for propagating conformational changes. In the case of the

Figure 7. Conformational states of type I ABC transporters. A: The structure of the MetI and MalFG translocation pathway in five conformational states. For clarity, only the coupling helices and two flanking TM helices are shown as yellow and cyan ribbons. Proposed gating residues are shown as ball and stick figures. The PDB ID of each structure is noted in parentheses. B: The NBD C α -trace is shown in red and green under a transparent surface representation. Residues located within 4 Å of the bound nucleotide or coupling helix are indicated by yellow and blue surface coloring, respectively (location of nucleotide for MetNI is inferred from CY5 structure). Dashed lines illustrate separation of C α atoms in conserved motifs as follows: P-loop Gly43, red spheres; ABC signature Gly143, blue spheres; D-loop Asp172, green spheres; and H-loop His199, orange spheres (numbering from MetN). C: Regulatory domains are shown as red and green cartoon ribbons beneath a transparent surface representation of the NBDs. The black line corresponds roughly to the regulatory domain dimer interface and illustrates its orientation relative to the NBDs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MetNI transporter, alternate states of the C2 dimer could help propagate a signal arising from binding of cytoplasmic methionine. The observation that the methionine binding site is formed in part by residues from the β -strand at the dimeric C2 interface suggests the potential for coupling between the ligand binding and conformational switching events. Further studies will be required to define the atomic details and energetic basis for a potential coupling mechanism of this type.

Finally, it is an intriguing observation that the allosteric binding site for methionine in the C2 domain involves protein methionine side chains [Met301; Fig. 5(B)], while Met163 of MetI participates in the periplasmic gate of the TMD [Fig. 2(B)] through which transported methionine needs to pass. It seems plausible that the methionine side chains of the protein residues could interact with these sites in a similar way as the exogenous methionine, so that the presence of the latter could displace the endogenous side chain, perhaps serving as a trigger for conformational rearrangements. In this regard, the positioning of the Met301 side chain in the regulatory binding pocket of the C2 domains of the DM and iso-C2 structures [Fig. 5(B)] may sterically block the binding of exogenous ligand necessary for the allosteric transition. The functional consequences associated with the removal or introduction of methionines at these positions of MetNI should be informative for addressing this hypothesis.

Materials and Methods

MetN E166Q mutagenesis

Mutagenesis reactions were performed on the metNI-expression plasmid (as described in Ref. 9) to replace the *metN* sequence encoding E166 with a sequence encoding Q166, to create the MetIN E166Q construct. The reactions were performed using the Quickchange Mutagenesis Kit (Stratagene). As a result, the final plasmid is identical to the final wild-type metNI-expression plasmid except for the changes introduced by the site directed mutagenesis described above.

Cloning and expression

The appropriate plasmid was transformed into BL21-gold (λ DE3) cells (Novagen) and protein expression was carried out at 37°C in Terrific Broth containing 0.5% glycerol and 100 μ g/mL ampicillin in 6 L flasks. Three hours of target gene expression was induced by the addition of IPTG to a final concentration of 1 mM. Cells were cooled in an ice slurry and harvested via centrifugation at 4°C before freezing at -80°C . Selenomethionine substituted protein was produced using the expression strain described above with the method of metabolic inhibition.⁴³

MetNI protein purification

Ten grams of frozen cell pellet were resuspended in 80 mL of ice cold lysis buffer (50 mM TAPS, 250 mM NaCl, pH 8.5, DNase and lysozyme each at 10 μ g/mL, and one Complete Protease Inhibitor Cocktail Tablet [Roche]). Homogenization was performed on ice for 5 min, followed by gentle stirring on ice for an additional 5 min before addition of 1 g *n*-dodecyl- β -D-maltopyranoside (DDM) and ice cold lysis buffer to reach 1% β -DDM and a total volume of 100 mL. The cell homogenates containing detergent were then stirred on ice for an additional 5 min. Cell lysis was performed by sonication on ice using a Misonix S-4000 ultrasonic processor, flat tip, power 80, 5 s on/20 s off for a total of 6 min process time with pauses for cooling. The homogenates were then allowed to stir at 4°C for 20 min. Clearing of cell lysates was performed by centrifugation at 30,000g, 20 min, 4°C. Cleared lysates containing 70 mM imidazole were then loaded to a 5 mL Ni-sepharose HP column (GE Healthcare) equilibrated with 93% buffer A (50 mM TAPS, 250 mM NaCl, 0.3% CY5, pH 8.5) and 7% buffer B (50 mM TAPS, 250 mM NaCl, 1M imidazole, 0.3% CY5, pH 8.5). Following binding of MetNI to the affinity column, 10 column volumes 7% buffer B were flowed through to wash away contaminants and allow for detergent exchange. The target protein was then eluted by stepping to 40% buffer B. As indicated by A280, the eluted protein peak was loaded directly to a HiPrep 26/10 desalt column (GE Healthcare) pre-equilibrated with 100% buffer A. Protein concentration was performed in 15 mL centrifugal concentrators (Millipore) with a 100 kDa molecular weight cutoff. Aliquots of the purified concentrated protein were then immediately flash frozen in LN2 and stored at -80°C . Purification of the *n*-decyl- β -D-maltopyranoside (DM) solubilized MetNI proceeded exactly as the CY5 material except that all chromatography buffers contained 0.3% DM.

Crystallizations

Crystallization details are provided in the Supporting Information.

Data collection and structure determination

Data processing proceeded using XDS,⁴⁴ for integration followed by scaling with SCALA.⁴⁵ Unless otherwise noted, the CCP4 suite of programs was used for crystallographic calculations.⁴⁶ To correct for the presence of strong to severe anisotropy in the data, scaled reflections were submitted to the UCLA MBI Diffraction Anisotropy Server for ellipsoidal truncation and anisotropic scaling⁴⁷ (<http://services.mbi.ucla.edu/anisotryscale>). Data were truncated at the resolution limits along the directions a^* , b^* , and c^* , respectively, for reflections at which F/σ fell below 3.0 (2.7, 3.2, and 2.8 Å, CY5 native; 3.2, 3.4,

and 3.2 Å, CY5 SeMet soak; and 4.2, 3.9, and 3.9 Å, DM). An anisotropic B factor correction was also applied to the data. Initial phases were determined using PHASER⁴⁸ to obtain a molecular replacement solution based on the DDM derived model (PDB ID 3DHW). However, phases from molecular replacement produced poor quality electron density maps with model bias and subsequent model building and structure refinement failed to produce satisfactory R factors. Selenomethionine derivative protein crystals were produced and experimental phase information was obtained through collection of SeMet peak SAD datasets. The initial molecular replacement phases and SAD data were then used to generate anomalous difference Fourier maps. Heavy atom positions were determined by automatic placement of Se atoms in anomalous peaks > 4 sigma. Experimental phasing then proceeded using only the heavy atom positions and the Se peak SAD data followed by density modification in PARROT.⁴⁹ Based on selenomethionine positions from Se SAD anomalous difference maps and relatively unbiased $F_o - F_c$ and $2F_o - F_c$ maps produced from experimental phases, model building (COOT⁵⁰) and structure refinement [REFMAC 5⁵¹ and PHENIX (phenix.refine)⁵²] then proceeded in a fairly typical fashion. Improved models were used to calculate phases for the higher resolution native dataset of the CY5 form. Iterative cycles of model building and refinement were then carried out alternatively using Se peak SAD data, and higher resolution native data. Solvent atoms were located using a combination of “find waters” in COOT and “update waters” in PHENIX. Data collection and refinement statistics appear in Tables S1 and S2. Although the use of TLS restraints in refinement gave small improvements (1–2%) in R_{work} and R_{free} , the deposited coordinates were refined without them.

At the conclusion of refinement, the strongest positive residual peaks in $F_o - F_c$ maps were associated with roughly spherical features displaced ~5 Å from the peptide backbone of several residues in the MetI transmembrane helices. These peaks were generally found to follow the non-crystallographic symmetry. Although it is plausible that they arise from disordered detergent or lipids, the shape was more like an ion or solvent species. Although this interpretation seemed “far-fetched”, the possibility was tested through soaks with NaBr, NaI, CsCl, and RbCl; inspection of anomalous Fourier maps failed to reveal any significant density at these positions. In the absence of a more suitable explanation, these sites are modeled as waters (HOH) and have 5000 added to the residue numbers in the deposited coordinates.

Principal components analysis of structures

A principal components analysis of the conformational changes relating a set of transporter struc-

tures was performed using locally written programs based on methods developed by Berendsen and co-workers.^{37,53} The algorithm involves the calculation of the covariance matrix of the fluctuations of each atomic coordinate from a reference structure, taken in these calculations as residues in the conserved core of the MalG or MalK subunit (for the TM or NBD analysis, respectively), following superposition onto the MalF core of the 3PUY structure.²⁷ The eigenvectors of this matrix represent directions with correlated changes in coordinates between all structures. The value of an eigenvalue relative to the total squared displacement of all structures from the reference indicates the proportion of the total deviation contributed by that eigenvector.

To calculate the rigid body rotation/transformation corresponding to a particular eigenvector, the displacement along that eigenvector corresponding to the structure with the largest change from the reference structure is calculated, and the rigid body transformation corresponding to this displacement is extracted with an algorithm devised by Kabsch.⁵⁴ Equivalent residues for this analysis were defined as follows:

TMD

- MetI, TM2-4 (51–75; 101–114; 135–160)
- MalF, TM5-7 (314–336; 381–394; 415–440); MalG, TM3-5 (116–140; 170–183; 204–229)
- ModB, TM3-5 (2ONK) (82–106; 140–153; 174–199)
- ModB, TM3-5 (3D31) (92–116; 152–165; 186–211)
- NBD
- MetN (3–10; 24–30; 32–50; 60–65; 83–89; 160–166; 192–198; 210–216; 220–224)
- MalK (5–12; 22–28; 30–48; 58–63; 76–82; 153–159; 185–191; 203–209; 213–217)
- ModC (2ONK) (4–11; 18–43; 53–58; 71–77; 146–152; 178–184; 196–202; 206–210)
- ModC (3D31) (3–10; 19–25; 27–45; 55–60; 73–79; 147–153; 179–185; 197–203; 207–211)
- Reference subunits for superposition: MalF (chain F); MetI (chain A or E); ModB (chain C).
- TMD subunits for comparison: MalG (chain G); MetI (chain B or F); ModB (chain D).
- NBD subunits for comparison: MalK (chain B); MetN (chain C or G); ModC (chain A).

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