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Structural basis for heavy metal detoxification by an Atm1-type ABC exporter

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Abstract

While significant progress has been achieved in the structural analysis of exporters from the superfamily of ATP Binding Cassette (ABC) transporters, much less is known about how they selectively recognize substrates and how substrate binding is coupled to ATP hydrolysis. We have addressed these questions through the crystallographic analysis at 2.4 Å resolution of the Atm1/ABCB7/HMT1/ABCB6 ortholog from *Novosphingobium aromaticivorans* DSM 12444. Consistent with a physiological role in cellular detoxification processes, functional studies demonstrate that glutathione derivatives can serve as substrates for NaAtm1 and overexpression in *E. coli* confers protection against silver and mercury toxicity. The glutathione binding site highlights the articulated design of ABC exporters, with ligands and nucleotides spanning structurally conserved elements to create adaptable interfaces accommodating conformational rearrangements during the transport cycle.

The Atm1/ABCB7/HMT1/ABCB6 family of ATP Binding Cassette (ABC) exporters has been implicated in transition metal homeostasis and detoxification processes (1-4). In eukaryotes, the Atm1/ABCB7 orthologue is present in the inner membrane of mitochondria and is required for the formation of cytosolic iron sulfur cluster containing proteins (5); mutations of this protein result in mitochondrial accumulation of iron (6) and are responsible for X-linked sideroblastic anemia (7). ABCB6 was first identified as a porphyrin transporter present in the outer membrane of mitochondria (8), but it may also serve in arsenic resistance (9), and is present in the Golgi (10), lysosomal (11), and plasma membranes (12). Other homologs have been implicated in heavy metal detoxification to vacuoles, including cadmium resistance by Heavy Metal Tolerance Factor-1 (HMT1) in *C. elegans* and *D. melanogaster* (13-15). The available evidence (16) suggests that for some of these transporters, the substrates may be related to the thiol containing glutathione (GSH), a

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Materials and Methods are detailed in the Supplementary Materials.

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tripeptide of sequence γ -Glu-Cys-Gly. GSH is an abundant cellular antioxidant that mediates resistance to electrophiles, heavy metals, and xenobiotics (17-19).

As a starting point for addressing the molecular mechanism of this family of ABC transporters and their physiological role(s) in metal homeostasis, we have solved the structure of the Atm1-family ABC exporter from *Novosphingobium aromaticivorans* (*NaAtm1*) at 2.4 Å resolution (20). *NaAtm1* shares ~45% sequence identity with *S. cerevisiae* Atm1, human ABCB7 and HMT-1 (Fig. S1), and hence should provide a useful model for these eukaryotic transporters (21). Reflecting the basic architecture of the ABC exporter family that was first elucidated for the Sav1866 transporter (22) and subsequently found in other ABC exporters (23-27), *NaAtm1* forms a homodimer with each subunit containing 6 transmembrane (TM) helices fused to the nucleotide binding domain (NBD) (Fig. 1). *NaAtm1* adopts an inward facing conformation that most closely resembles (Fig. S2) the structures of a *T. maritima* ABC transporter (24) and the human ABCB10 transporter (26).

Since functional studies for *NaAtm1* have not been reported, we implemented three approaches to identify potential substrates for transport: (i) the ability of ligands to stimulate ATPase activity (16, 28, 29); (ii) *in vitro* transport assays measuring uptake into *E. coli* membrane vesicles; and (iii) *in vivo* susceptibility assays to assess the ability of *NaAtm1* to restore tolerance to heavy metals in sensitive *E. coli* strains (30, 31). As observed for *S. cerevisiae* Atm1p (16), the ATPase activity of *NaAtm1* is stimulated by GSH and related compounds, with the highest activity (defined by k_{cat}/K_m) observed for metallated, aromatic hydrocarbon conjugated, and oxidized GSH derivatives (Figs. 2A, S3, S4). For the most active substrates, the ATPase activity was stimulated by 3 to 7-fold over the basal rate. Effective stimulation of ATPase activity by ligands appears to require both free α -carboxyl and α -amino groups, as glycine and the γ -peptides GSH, γ -Glu-Cys, and ophthalmic acid (glutathione with the thiol group replaced with methyl) exhibit stronger ATPase activities relative to Cys-Gly and mono-carboxylic acids (Fig. 2A); these properties contrast with yeast Atm1 where the GSH thiol group is critical for efficient ATPase activity (16). However, the presence of α -amino and carboxyl groups in the transported ligand, while apparently necessary, is not sufficient for ATPase activity as evidenced by the low activity of many amino acids (Fig. S5). Further demonstration of the substrate specificity of *NaAtm1* was provided by direct transport assays where we observed ATP-driven, *NaAtm1*-dependent uptake of oxidized glutathione (GSSG) into *E. coli* membrane vesicles (Fig. 2B). The ability of *NaAtm1* to transport GSSG distinguishes it from the *E. coli* CydDC ABC transporter that is selective for reduced glutathione (32).

The greatest stimulation of ATP hydrolysis by *NaAtm1* was observed for the Ag and Hg complexes of GSH (Fig. 2A). In view of the toxicity of these metals at low intracellular concentrations, we tested the *in vivo* relevance of the ATPase results using metal susceptibility assays (30, 31). Consistent with a role for *NaAtm1* in exporting Ag-GSH and/or Hg-GSH, expression of *NaAtm1* in metal sensitive *E. coli* strains conferred protection against otherwise toxic concentrations of Ag⁺ and Hg²⁺ (Figs. 2C-D). Taken together, these results suggest that *NaAtm1* mediates resistance to heavy metal toxicity, likely through export of metallated GSH complexes, although other functional properties likely remain to be identified.

As illustrated with GSSG, the major binding site is located ~5 Å into the transmembrane region, dominated by interactions from TM5 and 6 and accessible from the cytoplasmic side (Figs. 3A-B, S6). This location is ~15 Å closer to the cytoplasmic surface than the ligand binding sites in the mouse P-glycoprotein structure (23, 33), but the majority of those interactions also involve TM5-6, and the dimeric equivalent TM11-12. Key interactions are formed between the free amino and carboxyl groups of GSSG and the protein. The α -carboxyl group of the γ -Glu forms hydrogen bonds with the amide NHs of Gly 319 and Met 320 in TM6, and the side chain of Gln 272 (TM5), while the α -amino group of Glu interacts with the peptide carbonyl of Asp 316 (TM6) and the side chains of Asn 269 and Gln 272 (TM5). At the other end of the glutathione tripeptide, the α -carboxylate of the Gly hydrogen bonds to the side chain of Tyr 156 (TM3) on the opposing subunit from the one interacting with the γ -Glu residue of GSH (Fig. S7). The residues on TM3 and 6 interacting with GSSG are positioned near helical irregularities, including a stretch of α helix between residues 314-317 following a kink at the conserved Pro 314 in TM6, and a π helix between residues 158-162 in TM3; these may represent regions that undergo conformational changes during the transport cycle (34). The non-polar contacts between the protein and ligand are primarily formed by the side chains of the broadly conserved residues Leu 265 and Leu 268 packing against the Gly end of GSSG, and by the more variable side chains of Met 317 and Met 320 interacting near the disulfide of GSSG (Fig. S8).

A second, lower occupancy, binding site for GSSG is positioned ~5 Å from the primary site towards the cytoplasmic surface. The relative positions of the two GSSG sites are suggestive of a potential binding mode for a [2Fe:2S](GS-)₄ complex (35), although the actual physiological relevance of this site is not known. The binding site involves conserved arginine residues 206 and 323, along with the more variable Arg 210 (Figs. 1B and 3B). Residue Thr 324 is the counterpart of residue Glu 433 in human ABCB7 that is mutated to Lys in X-linked sideroblastic anemia (7). Two additional pairs of Arg residues are positioned in the translocation pathway towards the periplasmic side, the conserved Arg 91 and the more variable Arg 313 (Fig. S9) that could perhaps represent a transient binding site for ligands in the outward facing conformation.

The functional significance of these interactions was assessed by evaluating the consequences of site directed mutagenesis and substrate variation on the ATPase activity. Residues Asn 269, Gln 272, and Gly 319 that hydrogen bond to the α -amino and carboxylate groups of the γ -Glu are highly conserved, and substitution of these residues alters the ATPase activity (Figs. 3C-D). While mutations of these residues (Asn 269 to Ala, Gln 272 to Ala, Gly 319 to Leu) result in varying degrees of ATPase activity, they all effectively minimize GSH stimulation. Substitution of Asn 269 to Ala both enhances the basal ATPase activity and eliminates GSH sensitivity, while incorporation of a bulky residue at position 319 (Gly to Leu) or Gln 272 to Ala mutation significantly reduces both the GSH sensitivity and the basal ATPase activity. The minimal ATPase activity of the Gln 272 to Ala variant, together with the ability of the free amino acids Gly and Met to stimulate ATPase activity as effectively as GSH, suggests that the binding of ligands with α -amino and carboxyl groups to the Gln 272 site in TM5 can trigger the conformational changes associated with ATP hydrolysis (Figs. 3C, S10). As a potential coupling mechanism, Gln 272 is adjacent to the side chain of Tyr 195 in TM4 that hydrogen bonds to the backbone

carbonyl of Leu 315 in TM6, thereby linking TM4-5 to TM6 and the following NBD. Substitution of these residues yields interesting phenotypes (Fig. 3D); the Tyr 195 to Phe variant exhibits substrate insensitive ATPase hyperactivity, suggesting that the native Tyr 195 contact to TM6 may stabilize the inward facing conformation. Conversely, since the Gln 272 to Ala variant is essentially ATPase inactive, interactions of this residue with the binding of an amino acid motif may be required to unlock the protein from the cytoplasmic facing conformation (Fig. 3C). The double mutant Tyr 195Phe-Gln 272Ala, however, has a high basal ATPase activity without GSH stimulation, indicating that when neither residue can potentially interact with TM6, the transporter can facilitate undergo the conformational changes associated with ATPase activity.

The interconversion between inward and outward facing conformations of ABC exporters coupled to ATP hydrolysis is generally described within the framework of the alternating access transport mechanism (36). However, an analysis of MsbA structures solved in multiple conformational states emphasized that this interconversion cannot be described in terms of rigid body movements of subunits, but rather significant rearrangements occur at the interfaces between certain TM helices (27). More specifically, the alternating conformations of ABC exporters may be distinguished by the separation of either TM1-2 (outward) or TM4-5 (inward) from the remaining four TM helices within the same subunit (22, 27). Examination of the available structures of ABC exporters identifies four elements that are generally conserved structurally: TM1-2, TM4-5, TM3&6 (Fig. 4A), together with the NBD that is the hallmark of this family. These pairs of TM helices reflect the approximate symmetry in the transmembrane domain noted in the Sav1866 structure (22), where TM1-3 and TM4-6 are related by a twofold axis between TM3&6 perpendicular to the molecular twofold axis. The conformational changes associated with transport involve rearrangements in the relative orientations of these conserved elements (Fig. 4B), mediated by their interactions with the ligand and ultimately coupled to the NBDs through intracellular loop 1 (ICL1) between TM1-2, intracellular loop 2 (ICL2) between TM4-5 and the covalent connection from TM6 to the NBDs (Fig 1). ABC exporters can be considered to have an articulated construction; as this work demonstrates, transported ligands span these structurally conserved elements in the TMD to create adaptable interfaces accommodating conformational rearrangements during the transport cycle. Mutations in residues implicated in mediating such contacts have been identified in CFTR variants, including residues Arg 347 and Asp 993 (37, 38) in TM6 and TM9, respectively. These residues spatially correspond to positions 316 and 157 in TM6 and TM3 on different subunits of *NaAtm1* (based on the TM3&6 structural alignment of *NaAtm1* with the Sav1866-based homology model of CFTR (39)), near the GSH binding site, suggesting that the interactions observed here are of broader relevance for influencing the conformational equilibria essential for proper functioning of ABC exporters.

Supplementary Material

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Acknowledgments

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Fig. 1. Structural representations of *NaAtm1*

A) Ribbon diagram illustrating the dimeric structure of *NaAtm1*, viewed normal to the molecular two-fold axis with the membrane spanning domains and NBDs oriented towards the top and bottom, respectively. The approximate position of the membrane is designated by the gray bilayer, with the periplasmic and cytoplasmic facing surfaces towards the top and bottom, respectively. B) Binding sites for GSSG illustrated in the same orientation as (A), with the ligand depicted as space filling models, and the primary and secondary binding sites represented by green and yellow carbons, respectively. C) Representation of *NaAtm1* emphasizing the secondary structure arrangement, based on the Sav1866 nomenclature (22). ICL denotes intracellular loop.

Substrate	k_{cat} / K_m ($\text{min}^{-1} \text{mM}^{-1}$)	K_m (mM)	k_{cat} (min^{-1})
S-Ag GSH*	2400	0.012 ± 0.001	29 ± 1
S-Hg GSH*	200	0.12 ± 0.04	24 ± 1
S-Dinitrobenzene GSH	120	0.21 ± 0.05	26 ± 2
S-Hexyl GSH	100	0.40 ± 0.12	40 ± 4
S-Bimane GSH	47	1.2 ± 0.2	56 ± 4
GSSG	29	0.97 ± 0.12	28 ± 1
Glycine	3.8	15 ± 1	57 ± 2
γ Glu-Cys	2.8	17 ± 3	47 ± 4
Ophthalmic acid	2.6	10 ± 2	26 ± 2
Cys-Gly	1.7	36 ± 5	62 ± 6
Sodium acetate	0.13	46 ± 35	6.2 ± 3.2
	0.13	10 ± 21	1.3 ± 1.2

$v/[E]_T = k_{cat}[S] / (K_m + [S]) + k_{basal}$ where $k_{basal} = 8.8 \pm 0.8 \text{ min}^{-1}$ in 1 mM ATP
* K_m is reported as per mole of $\text{Ag}^+ / \text{Hg}^{2+}$ basis

Fig. 2. *In vitro* and *in vivo* assays of *NaAtm1* function
A) Kinetic constants for the ATPase activity of selected substrates derived from Michaelis-Menten type analysis, including the basal rate of ATPase activity. B) Amount of GSSG transported per mg of protein accumulated inside vesicles prepared from *E. coli* membranes overexpressing wild type or the E523Q Walker B motif ATPase defective mutant. C) Optical density of cells after 12-h growth in the presence of the indicated AgNO_3 concentrations. *E. coli* strain GG44 (Cu^+/Ag^+ sensitive) was transformed either with an empty pET15 plasmid, or a pET15 plasmid encoding *NaAtm1*. D) Optical density of cells after 12-h growth in the presence of the indicated HgCl_2 concentrations. *E. coli* strain GG48 ($\text{Zn}^{2+}/\text{Cd}^{2+}/\text{Hg}^{2+}$ sensitive) was transformed as in (C). The error bars in Figs. 2B-D represent the standard deviations from three independent measurements.

Fig. 3. Binding sites of glutathione derivatives to NaAtm1

A) The primary binding site of GSSG, with the substrate and interacting side chains depicted as bonds and the polypeptide backbone in ribbons. The substrate carbons are colored light gray, while the protein side chain carbons are green. Hydrogen bonds between the protein and ligands are represented by yellow dashes. B) The secondary binding site for GSSG. C) Interactions between the γ -Glu of GSH and surrounding residues of NaAtm1. The carbon atoms of GSH are shaded light gray; while green carbons denote side chains directly interacting with GSH and blue carbons indicate side chains interacting with (green) residues interacting with GSH. D) The ATPase activity (k_{cat}) of selected mutations in the ligand binding site, with the rate constants in the absence of substrate and in the presence of 10 mM GSH. Error bars represent the standard deviation of 3 independent measurements.

Fig. 4. Structural conservation and the articulated construction of ABC exporters

A) Structural conservation of the individual TM1-2, TM3&6, and TM4-5 elements in the TMDs of ABC exporter structures. The *NaAtm1* structure was used for the reference, with the TM1-2 and TM4-5 superpositions horizontally displaced from TM3&6 by 20 Å to the left and right, respectively. The GSSG ligand in the primary binding site is depicted by a space filling model with the carbons colored green. The underlying symmetry in the TMD, first noted in the Sav1866 structure (22), relates TM1-2-3 and TM4-5-6 by a two-fold rotation axis passing between TM3-6 and normal to the plane of the page. B) Variations in the relative orientations of conserved TMD elements observed in the structures of ABC exporters. The TMDs of different exporters were aligned with *NaAtm1* using only TM3&6 for the superposition; for clarity, only TM3&6 from *NaAtm1*, depicted with black ribbons, is shown. Although the individual TM1-2 and TM4-5 elements are structurally conserved (Fig. 4A), significant changes in their relative positions are evident between these structures (especially TM4-5), reflecting tertiary structure changes associated with the transition between inward and outward facing conformations. The binding site for the GSSG ligand spans between these elements, serving to couple ligand binding to changes in TMD and transporter conformation. The Ca traces are colored purple (Sav1866; PDB 2HYD (22)), blue (TM287/288; PDB 3QF4 (24)), black (*NaAtm1*), green (ABCB10, PDB 4AYT (26)), yellow (mouse Pgp, PDB 3G5U (23)), orange (*C. elegans* Pgp, PDB 4F4C (25)), and red (MsbA, PDB 3B5W (27)), ordered from outward to inward facing conformations.