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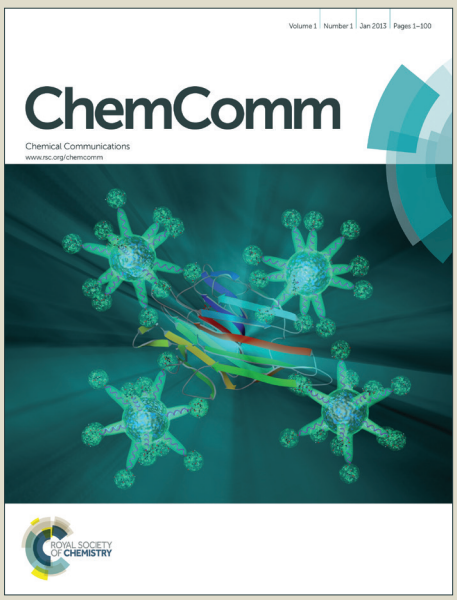

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ARTICLE TYPE

A Structural Model for Glutathione-Complexed Iron-Sulfur Cluster as a Substrate for ABCB7-Type Transporters

Wenbin Qi,[a] Jingwei Li [b] and J. A. Cowan [a,b,*]

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Glutathione-complexed [2Fe-2S] cluster is shown to significantly stimulate the ATPase activity of an ABCB7-type transporter in both solution and proteoliposome-bound forms (K_D ~ 68 μM). The cluster is a likely natural substrate for this transporter, which has been implicated in cytosolic Fe-S cluster protein maturation. A possible substrate-binding site is identified on a new structural model for the active transporter.

Iron-sulfur clusters are essential cofactors in many biological pathways. Several functionally discrete biosynthetic pathways for bacterial iron-sulfur cluster biogenesis have been described (*Isc*, *Nif* and *Suf*) and each has been studied extensively.[1, 2] Eukaryotic cluster assembly involves a pathway based on proteins in the bacterial ISC operon, and it is generally believed that both cytosolic and nuclear iron-sulfur clusters are dependent on mitochondrial iron sulfur cluster assembly.[2, 3] Details of how the mitochondrial and cytosolic iron-sulfur cluster assembly pathways are connected remain unclear, but have been the subject of intense scrutiny with multiple proteins implicated, even if their roles are not unequivocally defined.[4-7] Studies have shown that Atm1p/ABC7 deficiency leads to impaired cytosolic iron-sulfur cluster protein activity and iron accumulation in mitochondria, but there is no impact on mitochondrial iron-sulfur cluster protein activity.[3, 8] In humans, natural mutants of the transporter have been identified in patients affected with X-linked sideroblastic anaemia and cerebellar ataxia,[9] and definition of the substrate and a structural model for the protein are important first steps toward understanding the molecular basis for these disease states

Although the Atm1p/ABC7 membrane spanning protein appears to be the exporter required for cytosolic cluster biosynthesis,[3, 7, 8] the substrate for the transporter is unknown. In this paper we present evidence that a novel glutathione complexed [2Fe-2S] cluster[10,11] is a plausible transporter substrate,[10, 11] and discuss this finding in the context of a new structural model that we have defined for the heretofore structurally uncharacterized ABC7-type transporters. Definition of the pathway for mitochondrial cluster export is a crucial step to understanding the biogenesis and regulation of cellular iron-sulfur cluster cofactors.

Atm1p/ABC7 proteins are ATPase-driven pumps that drive active transport.[3] Previously it has been shown that both reduced and oxidized glutathione stimulate the ATPase activity of Atm1p/ABC7,[12] indicative that the thiol is not a key contributor to the stimulatory mechanism. A role for glutathione in mediating mitochondrial cluster export is supported by the observation that glutathione depletion impairs the maturation of cytosolic iron-sulfur cluster proteins, but has no effect on mitochondrial cluster proteins, consistent with a close genetic relationship between ATM1 and GSH1.[13] It is therefore clear that glutathione is intimately involved in iron-sulfur cluster export.

The involvement of glutathione in both cellular iron chemistry[14] and iron-sulfur cluster biosynthesis has previously been evidenced by the characterization of several glutaredoxin proteins with glutathione-coordinated [2Fe-2S] clusters that mediate cluster transfer chemistry,[15-19] and by the fact that human glutaredoxin can exchange its [2Fe-2S] cluster with the scaffold protein ISU.[20] This glutathione-coordinated iron-sulfur cluster complex is stable under physiological conditions in the presence of physiological concentrations of glutathione, and undergoes cluster exchange with the ISU scaffold protein.[10] Since neither a bare cluster core, nor a protein-bound cluster are likely substrate candidates for this class of exporter (on ligand and size grounds), and given the additional evidence implicating glutathione in cluster export, we viewed such a cluster complex as a viable substrate candidate for the ABC7-type transporter. Herein we present results of investigations that further support the idea that [2Fe-2S](GS)$_4$ is a substrate for mitochondrial cluster export, and identify a possible substrate-binding on a new structural model for the active transporter.

It is generally observed that substrates for ABC transporters stimulate the ATPase activity of the transporter.[21, 22] To test the hypothesis that [2Fe-2S](GS)$_4$ is a substrate for the transporter, yeast Atm1p protein was cloned, expressed and purified (SI). Its activity was confirmed by ATPase assay measurements, yielding standard Michaelis-Menten parameters (K_M ~ 54.6 ± 0.4 μM and k_{cat} ~1.93 ± 0.03 min^{-1}) (Figure S9) in good agreement with other ABC transporters and Atm1p.[23] Varying concentrations of the complex were incubated with the transporter in the presence of physiological glutathione concentrations and the ATPase activity of the transporter was followed (Figures 1 and 2). With 10 mM glutathione, but no cluster complex present, the rate of phosphate formation increases, which is consistent with previous studies that have shown that glutathione can stimulate the ATPase activity of Atm1p.[12] With the same concentration of GSH, cluster complex significantly stimulates the ATPase activity of Atm1p at low μM concentrations (Figure 1 and S8). The dependence of activity on cluster concentration (Figure 2) was fit to a nonessential activation model (equation 1),[24] where v_{max} is the maximum initial ATPase activity of Atm1p in the absence of cluster, [S] is the concentration of substrate Mg-ATP, [A] is the concentration of cluster stimulant, K_D is the binding constant of the cluster to Atm1p, K_M is the binding constant of Mg-ATP to Atm1p, α accounts for the modification of K_M by cluster, and β accounts for v_{max} stimulation by cluster. The data illustrated in Table 1 demonstrates the glutathione cluster complex to serve as a modifier that increases the velocity of Atm1p-catalyzed phosphate formation by 1.9-fold and decreases the K_D 0.6-fold, which sup-

ports the hypothesis that the glutathione iron-sulfur cluster is a likely substrate for this transporter in a manner consistent with previous genetic interaction and knock-out studies.[13] The cluster complex shows saturation binding to the transporter with a measured K_D of 68 μM.

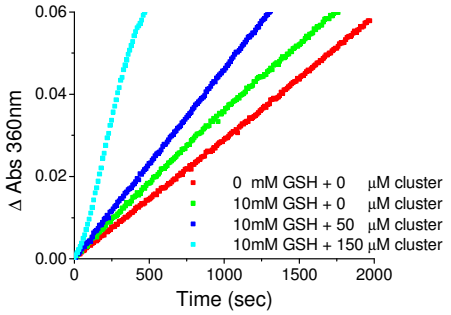

Figure 1. Stimulation of Atm1p ATPase activity by [2Fe-2S](GS)$_4$.

$$v = \frac{v_{max}\frac{[S]}{K_M} + \beta v_{max}\frac{[A][S]}{\alpha K_D K_M}}{1 + \frac{[S]}{K_M} + \frac{[A]}{K_D} + \frac{[A][S]}{\alpha K_D K_M}} \text{ - c[A]} \quad (1)$$

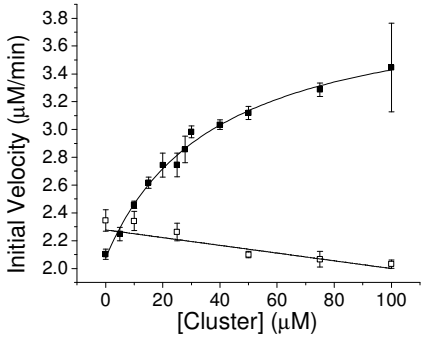

Figure 2. Atm1p ATPase activity is stimulated by [2Fe-2S](GS)$_4$. The data are corrected for magnesium-induced cluster degradation and fit to Eq.1 to yield the fitted parameters listed in Table 1. Solid squares (■), native Atm1p; vacant squares (□), R284E Atm1p.

The stimulation of transporter ATPase activity by glutathione iron-sulfur cluster complex was further studied in a proteoliposome system. The proteoliposome was constructed by reconstituting purified yeast Atm1p protein into liposome made of a mixture of 1:1:1 DOPE, DOPC and DOPG.[25] Similar to the results noted above, [2Fe-2S](GS)$_4$ was found to stimulate the ATPase activity of reconstituted proteoliposome (Figures S7 and S8).

The relative K_D's for [2Fe-2S](GS)$_4$ and glutathione indicate a much higher affinity for the cluster complex (68 μM versus > 689 μM, respectively). Prior observation of very modest levels of stimulation of Atm1p/ABC7 ATPase activity by glutathione are consistent with a glutathione cluster as a natural transporter substrate, with the more modest levels of stimulation reflecting weaker intrinsic binding to the transporter (Table 1), as a result of partial occupation of some of the contact sites on the transport protein occupied by the full tetrameric glutathione complex cluster. Nevertheless, the link between glutathione and iron-sulfur cluster transport remained unclear until the successful synthesis and characterization of the stable glutathione Fe/S cluster complex.[10, 11] In our previous studies, we were able to show that this complex is stable in the presence of physiological glutathione concentration, and that this complex is labile enough to exchange cluster with iron-sulfur cluster scaffold protein,[10, 11] making this a perfect cluster carrier in a cellular environment.

Table 1. Parameters for [2Fe-2S](GS)$_4$ stimulation of the ATPase activity for native and R48E-substituted transporter.

parameter	native	R284E
v_{max} (μM/min)	2.19 ± 0.04	2.50 ± 0.08
S (mM)	1.00 ± 0.01	1.00 ± 0.01
K_M (μM)	54.6 ± 5.3	55.0 ± 16.5
β	1.85± 0.05	0.86 ± 0.02
α	0.55 ± 0.06	0.005 ± 0.004
K_D (μM)	68 ± 2	4610 ± 2280
c (hr^{-1})	0.12 ± 0.02	0.08 ± 0.03

Parameter definitions: v_{max}, ATPase activity in the absence of cluster; S, [Mg-ATP]; K_M: Michaelis-Menten constant for Mg-ATP; β, an activity multiplier reflecting the stimulation of V_{max} by cluster; α, a modifier of K_M reflecting the impact of cluster on Mg-ATP binding; K_D, dissociation constant for cluster; c, rate of cluster degradation. A more modest stimulation of native transporter by glutathione was observed, with β = 1.14 ± 0.01, α = 0.87 ± 0.14, and K_D > 689 ± 215 μM.

Recent crystallographic advances have resulted in determination of the structure of a mitochondrial ABCB10 transporter, which shows the transporter in a functional dimeric state in a closed conformation, and with Mg-ATP bound to classical Walker motifs. This protein shows ~ 30% identity and 50% sequence similarity to the ABCB7 transporter (partial homology shown in Figure S10), and is of value in efforts to understand the structural mechanism of ABCB7 transport. By use of the ABCB10 structure (PDB: 3ZDQ) as a template in SWISS-MODEL, we generated model structures for both yeast Atm1p and human proteins. Electrostatic surface maps of both model structures were calculated using APBS and each showed two positively-charged pockets at the bottom of the transmembrane segment, and close to nucleotide binding domain. These represent possible binding sites for the negatively-charged {[2Fe-2S](GS)$_4$}$^{2-}$ complex (Figure 3).

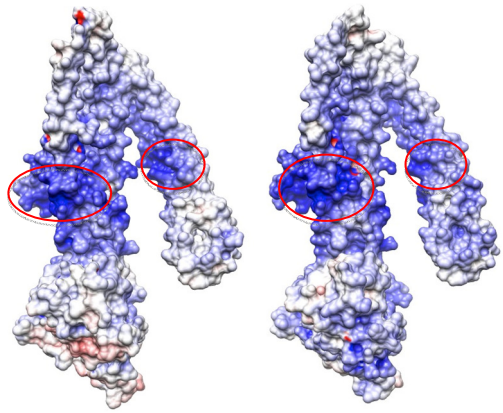

Figure 2. Electrostatic potential map of modeled ABCB7 (left) and Atm1p (right) transporters. The two positively-charged patches are highlighted by red circles.

Two positively-charged patches were noted. One lies between the two transmembrane helix bundles, presumably facing inward

to the channel once the dimeric transporter is formed. This positively-charged patch is formed on one side by a conserved arginine-rich area, Arg313, Arg315, Arg317, Arg319 of the human ABCB7 protein and Arg280, His282, Arg284, Arg285 of yeast Atm1p (Figure S12). On the opposite side, Arg432 and Arg435 of the human ABCB7 protein, and Arg397 and Lys400 of Atm1p complete a positively-charged pocket that is ready to bind the negatively charged iron-sulfur cluster complex (Figure 3). We speculate that in its native dimeric state, these two discrete sites of Atm1p/ABC7 may function as complementary domains to create a positive binding pocket for $[2Fe\text{-}2S](GS)_4$ cluster(s). Significantly, no stimulation of ATPase activity by cluster was observed when Arg284 was substituted with Glu (Figure 2 and Table 1), although full ATPase activity was retained.

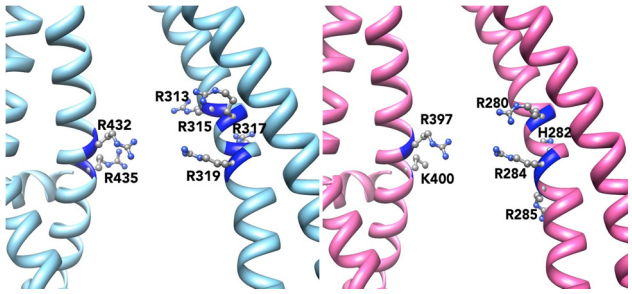

Figure 3. A proposed site for $[2Fe\text{-}2S](GS)_4$ binding in ABCB7 (left) and Atm1p (right). A conserved arginine-rich region and two other positively-charged residues are potentially involved in the formation of this binding site. Substitution of Arg284 with Glu appears to eliminate cluster substrate binding and ATPase stimulation, while retaining base ATPase activity.

In this report, we have shown evidence in support of $[2Fe\text{-}2S](GS)_4$ as a likely iron-sulfur cluster substrate for the Atm1p/ABC7 transporter in both solution and proteoliposome-bound forms, and identify a likely substrate binding site on the transporter. The mitochondrial cluster export pathway can be considered in four steps (Figure S13). First, mitochondrial glutathione abstracts the [2Fe-2S] cluster core from the mitochondrial ISC machinery (most likely ISU),[10, 11] forming a glutathione iron-sulfur cluster complex. Such complexes are then transported through the mitochondrial membrane, driven by ATP hydrolysis. Finally, the exported complexes are delivered to the CIA (cytosolic Iron-sulfur cluster Assembly) machinery, possibly by transfer to the cytosolic ISU. This provides the essential link between mitochondrial cluster biosynthesis and the rest of the cell, as well as providing a test bed for understanding human disease states that stem from natural mutants of this transporter.[9] Future studies will be focused on detailed evaluation of the substrate binding and transport mechanisms, the connection to the CIA pathway, and the activity of disease-causing protein point-substitutions.

[a] *Ohio State Biochemistry Program, The Ohio State University,* [b] *Department of Chemistry and Biochemistry, The Ohio State University.*
* *Corresponding author: cowan@chemistry.ohio-state.edu*

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