

Double and triple thermodynamic mutant cycles reveal the basis for specific MsbA-lipid interactions

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Abstract

Structural and functional studies of the ATP-binding cassette transporter MsbA have revealed two distinct lipopolysaccharide (LPS) binding sites: one located in the central cavity and the other at a membrane-facing, exterior site. Although these binding sites are known to be important for MsbA function, the thermodynamic basis for these specific MsbA-LPS interactions is not well understood. Here, we use native mass spectrometry to determine the thermodynamics of MsbA interacting with the LPS-precursor 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo)₂-lipid A (KDL). The binding of KDL is solely driven by entropy, despite the transporter adopting an inward-facing conformation or trapped in an outward-facing conformation with adenosine 5'-diphosphate and vanadate. An extension of the mutant cycle approach is employed to probe basic residues that interact with KDL. We find the molecular recognition of KDL is driven by a positive coupling entropy (as large as -100 kJ/mol at 298K) that outweighs unfavorable coupling enthalpy. These findings indicate that alterations in solvent reorganization and conformational entropy can contribute significantly to the free energy of protein-lipid association. The results presented herein showcase the advantage of native MS to obtain thermodynamic insight into protein-lipid interactions that would otherwise be intractable using traditional approaches, and this enabling technology will be instrumental in the life sciences and drug discovery.

eLife assessment

This is an **important** biophysical study combining native mass spectrometry with mutant cycles to estimate the thermodynamic components of lipid A binding to the ABC transporter MsbA. **Solid** evidence supports the binding energies for lipid-protein interactions to MsbA using this approach, which could be later applied to other membrane proteins in general.

Introduction

Most Gram-negative bacteria contain outer membrane lipopolysaccharide (LPS) that is crucial for maintaining structural integrity and protection from toxins and antibiotics^{1,2,3}. The ATP-Binding Cassette (ABC) transporter MsbA flips an LPS-precursor, lipooligosaccharide (LOS), from the cytosolic leaflet to the periplasmic leaflet of inner membrane, a process powered by the hydrolysis of adenosine triphosphate (ATP). MsbA functions as a homodimer and each subunit consists of a soluble nucleotide-binding domain (NBD) and a transmembrane domain containing six transmembrane helices⁴. The proposed mechanism of MsbA-mediated LOS transportation involves the binding of LOS to the interior binding site and a conformational change from an inward-facing conformation (IF) to an outward-facing conformation (OF).

Like other ABC transporters, the ATPase activity of MsbA can be stimulated in the presence of different substrates, particularly hexaacylated lipid A species^{5,6,7}. Recent studies have illuminated the location and importance of several LOS binding sites on MsbA^{8,9,11}. The interior binding site is located in the inner cavity, and mutations (R78A, R148A and K299A) engineered to disrupt binding at this site abolish lipid-stimulated ATPase activity and adversely affect cell growth^{8,12}. More recently, the LPS-precursor 3-deoxy-D-manno-oct-2-ulosonic (Kdo)2-lipid A (KDL) was found to bind to an elusive exterior site on MsbA trapped in an OF conformation with adenosine 5'-diphosphate and vanadate^{5,6,7}. Similarly, introducing mutations to disrupt binding at the exterior site also abolishes lipid-induced stimulation of ATPase activity¹⁰.

In 1984, Fersht and colleagues introduced the biochemistry community to the application of double mutant cycles as means to quantify the strength of intramolecular and intermolecular interactions¹³. The method has proven to be highly effective in examining pairwise interactions, as demonstrated by its notable application in determining the spatial orientation of potassium channel residues in relation to high-affinity toxin binding¹⁴. More generally, the technique has been used to measure the strength and coupling for residues in protein-protein complexes, protein-ligand complexes, and stability of secondary structure^{13,21}. In general, mutant cycles analysis involves measuring the changes in Gibbs free energy for the wild-type protein (P), two single point mutations (PX and PY) and the double mutant protein (PXY) for a given process, such as for protein-protein interactions (for review see¹⁵). If residue X and Y are independent of each other, then the Gibbs free energy associated with the double mutant protein will be equal to the sum of changes in Gibbs free energy due to the single mutations relative to the wild-type protein. However, if the Gibbs free energy associated with the structural and functional properties of the double mutant protein differs from the sum of single mutant proteins, then the two residues are energetically coupled or co-operative. The coupling free energy ($\Delta\Delta G_{int}$) is the energy difference between double mutant and two single mutant proteins (see methods). The $\Delta\Delta G_{int}$ values for pairwise interactions in proteins has revealed the contributions of salt bridges (4-20 kJ/mol), aromatic-aromatic interactions (4 kJ/mol), and charge-aromatic interactions (4 kJ/mol) to protein stability^{15,22,23}. Prior work on mutant cycles often employed traditional approaches, but such approaches overlook contributions from conformational changes of the reactants as well as potential changes in the hydration of the complex, including the reacting ligand and the solvent¹⁶.

To demonstrate the utility of the mutant cycle approach, we highlight two well-known examples. First, the high-affinity interaction between barnase, (an extracellular RNase of *Bacillus amyloliquefaciens*) and barstar (inhibitor of barnase) has been extensively studied by double mutant cycles²⁰. For example, pairwise interactions between residues that are less than seven angstrom in distance (based on crystal structures) have been shown to be co-operative. These interactions were shown to be important for stability of the barnase-barstar complex with coupling energies reaching as high as 7 kcal/mol. Another classical example involves the application of mutant cycles to guide docking and spatial arrangement of a high-affinity peptide

inhibitor (scorpion toxin) binding to the Shaker potassium channel.¹⁴ Of the pairwise interactions that underwent mutant cycle analysis, one pair (R24 from toxin and D431 from channel) in particular displayed an extraordinary coupling energy of 17 kJ/mol. This result indicates the two residues interact in the complex. Despite the absence of a structure of toxin-potassium channel complex, results from the mutant cycle analysis provided a strong constraint positioning the toxin relative to the potassium channel pore-forming region. In summary, these studies demonstrate how mutant cycle analysis can be used to determine the energetics of pairwise interactions, which is important for understanding how these molecular interactions contribute to the overall stability of proteins in complex with other molecules, such as ligands and other proteins.

Native mass spectrometry (MS) is well suited to characterize the interactions between protein and other molecules, especially for membrane proteins.^{24–26} The technique is capable of maintaining non-covalent interactions and native-like structure in the gas phase,^{27,28} essential for studying biochemical interactions with small molecules, such as the binding of drugs, lipids, and nucleotides.^{28–35} In combination with a variable temperature nano electrospray ionization device, native MS has determined the thermodynamics for protein-protein and protein-ligand interactions.^{36–41} For example, the molecular interaction between the signaling lipid 4,5-bisphosphate phosphatidylinositol and Kir3.2 is dominated by a large, favorable change in entropy.⁴⁰ Recently, native MS has been combined with mutant cycles analysis to determine the energetic contribution of pairwise inter-protein interactions for a soluble protein complex.^{42,43} Notably, the coupling energies determined by native MS and isothermal calorimetry are in agreement.⁴² Mutant cycle analysis is also being used to study cardiolipin binding to sites on AqpZ with native MS.⁴⁴

Traditional mutant cycles focus on pairwise interactions, such as two interacting residues in a protein complex.⁴⁵ Single- and double-point mutations along with characterizing their impact on protein stability/assembly enable assessment of the energetic contribution for the pairwise interaction. If the two residues are independent (non-co-operative), then the change in free energy will be equal to the sum of the two single mutations. In contrast, if the two residues are dependent on each other, then the coupling energy is a measure of their co-operativity. Although mutant cycles are often applied to protein-protein interactions, here we extend mutant cycle principles to study membrane protein-lipid interactions. It is established that MsbA has two high-affinity binding sites for the LPS-precursor KDL. Here, we examine each site independently followed by simultaneously probing both KDL sites. At present, there is limited availability of synthetic KDL derivatives, limiting this study to focus on residues that interact with KDL, such as basic residues coordinating the conserved phosphoglucosamine (P-GlcN) of KDL. Despite the limitation of commercially available KDL derivatives, the studies below demonstrate how residues energetically contribute to specific binding, providing insight into the driving forces underlying essential membrane protein-lipid interactions. Recently, we reported results using native MS that reveal conformation-dependent lipid binding affinities to MsbA.¹⁰ As these measurements were performed at a single temperature, we set out to perform a more detailed thermodynamic analysis to better understand the molecular driving forces that underpin specific MsbA-lipid interactions. Here, we report binding thermodynamics (ΔH , ΔS , and ΔG) for KDL binding to MsbA in IF and OF conformations. These results reveal the unique thermodynamic contributions of MsbA residues that engage KDL. We also report coupling energetics ($\Delta\Delta G_{\text{int}}$) for pairwise interactions, including, for the first time, the contributions from coupling enthalpy ($\Delta\Delta H_{\text{int}}$) and coupling entropy ($\Delta(-T\Delta S_{\text{int}})$), providing rich molecular insight into specific protein-lipid interactions.

Results

MsbA residues selected for mutant cycles analyses. MsbA is known to bind KDL either in the inner cavity or at the two exterior sites (**Fig. 1**). For both sites, a series of conserved arginine and lysine residues form specific interactions with the headgroup of KDL. To perform mutant cycles analysis, we introduced single mutations into MsbA to target KDL binding to the interior (MsbA^{R78A} and MsbA^{R299A}) and exterior (MsbA^{R188A}, MsbA^{R238A}, and MsbA^{K243A}) sites. More specifically, R78 coordinates one of the characteristic phosphoglucosamine (P-GlcN) substituents of KDL whereas K299 interacts with a carboxylic acid group in the headgroup of KDL. The two P-GlcN constituents of LOS are coordinated by R238 and R188 + K243, respectively. R188 also forms an additional hydrogen bond with the headgroup of KDL. In addition, we prepared double and triple mutants of MsbA for the various residues that were selected for mutagenesis.

Thermodynamics of MsbA-KDL interactions

We performed titrations to determine the equilibrium binding affinity for MsbA-KDL interactions at four different temperatures (288, 293, 298 and 303 K) (**Fig. 2**). These studies used optimized samples of MsbA that do not contain any co-purified LPS (for details see ¹⁰). The transporter was stable at the selected temperatures. For example, binding of KDL to MsbA was enhanced at higher temperatures (**Fig. 2a**), indicating a favorable entropy for the interaction. For a given temperature, the mass spectra from the titration series were deconvoluted and equilibrium dissociation constants (K_D) were determined for MsbA binding up to three KDL molecules (**Fig. 2b**, **Supplementary Fig. 1** and **Supplementary Table 1**). It is important to note that, unlike traditional approaches that struggle to distinguish between free protein from that bound to ligand,⁴⁶ native MS can resolve different ligand bound states, including the free concentration of protein and free concentration of ligand(s), in a single mass spectrum.^{36,47,48–49} Notably, the native MS approach has been cross validated using isothermal calorimetry and surface plasmon resonance.^{36,47,48} Interestingly, van't Hoff analysis showed a non-linear trend for three KDL binding reactions (**Fig. 2c**), indicating that over the selected temperature range, heat capacity is not constant.⁵⁰ The nonlinear form of the van't Hoff equation enabled us to determine the ΔH and change in heat capacity (ΔC_p) at a reference temperature of 298CK (**Fig 2c-d**). In this case, ΔG was calculated directly from K_D values, and entropy (ΔS) was back calculated using both ΔH and ΔG . ΔG values for binding KDL₁₋₂ range from -32.0 ± 0.1 to -35.2 ± 0.1 kJ/mol. The binding reaction has a positive ΔC_p that alters the thermodynamic parameters at different temperatures. At the lowest temperature, KDL binding is driven by favorable enthalpy (-36 ± 12 to -43 ± 7 kJ/mol) with a small entropically penalty ($-T\Delta S$, 2 ± 11 to 12 ± 7 kJ/mol at 288 K). In contrast, KDL binding at higher temperatures displays a large, favorable entropy ($-T\Delta S$, -123 ± 12 to -146 ± 7 kJ/mol at 303 K) that compensates a large enthalpic barrier (86 ± 12 to 112 ± 7 kJ/mol). These results highlight the role of entropy in KDL binding to MsbA that may stem from solvent reorganization.

KDL binding to the interior binding site of MsbA

We next determined the thermodynamics of KDL binding to MsbA containing single and double mutations at the interior binding site (**Fig 2d**). R78 of each subunit interacts with one of the P-GlcN moieties of LPS whereas one of the K299 residues interacts with a carboxylic acid group of LPS molecule in the inner cavity. Therefore, introducing the R78A mutation will impact symmetrically equivalent binding sites. MsbA^{R78A} showed a reduction in binding KDL₁₋₂ with ΔG ranging from -30.2 ± 0.2 to -32.5 ± 0.2 kJ/mol. At 298K, KDL binding is enthalpically and entropically favorable whereas binding of the second KDL is similar to the wild-type protein (**Fig. 2d**). The binding thermodynamics for MsbA^{K299A} is reminiscent of the wild-type protein with a large, favorable change in entropy ($-T\Delta S$, -75 ± 2 to -86 ± 1 kJ/mol at 298 K) and unfavorable enthalpy (43 ± 2 to 53 ± 1 kJ/mol) (**Fig. 2d**). The double mutant MsbA^{R78A,K299A} protein shows a reduction in

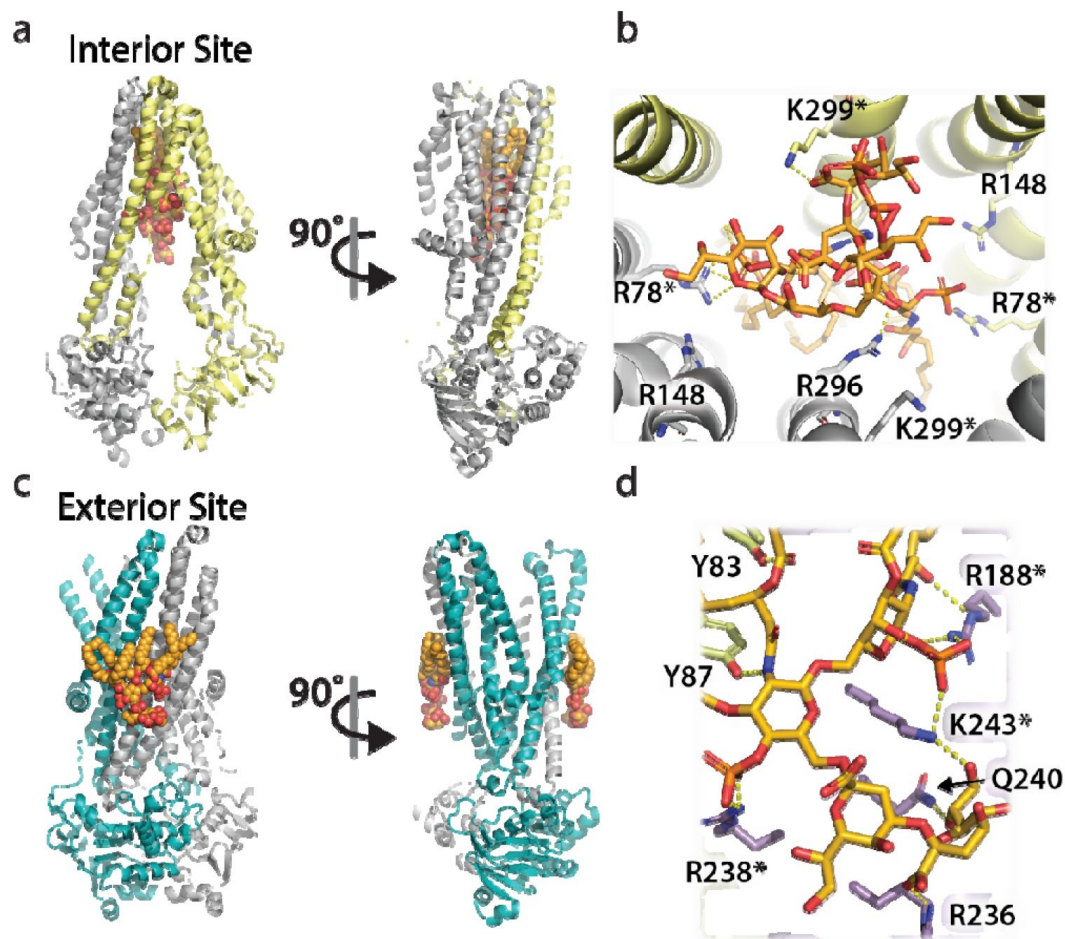


Figure 1.

The two distinct LPS binding sites of MsbA and their molecular interactions.

a) Two views of LPS bound to the interior site or central cavity of MsbA. The protein shown is also bound to the inhibitor G907 (PDB 6BPL).⁹ The protein and lipid are shown in cartoon and stick representation, respectively. b) Molecular details of the residues interacting with LPS at the interior site. Bonds are shown as dashed yellow lines along with residue labels. c) Two views of the KDL molecules bound to the two exterior binding sites of MsbA that are symmetrically related (PDB 8DMM).¹⁰ Shown as described in panel A. d) Molecular view of KDL bound to MsbA and shown as described in panel B. The asterisk denotes residues selected for mutant cycle analysis.

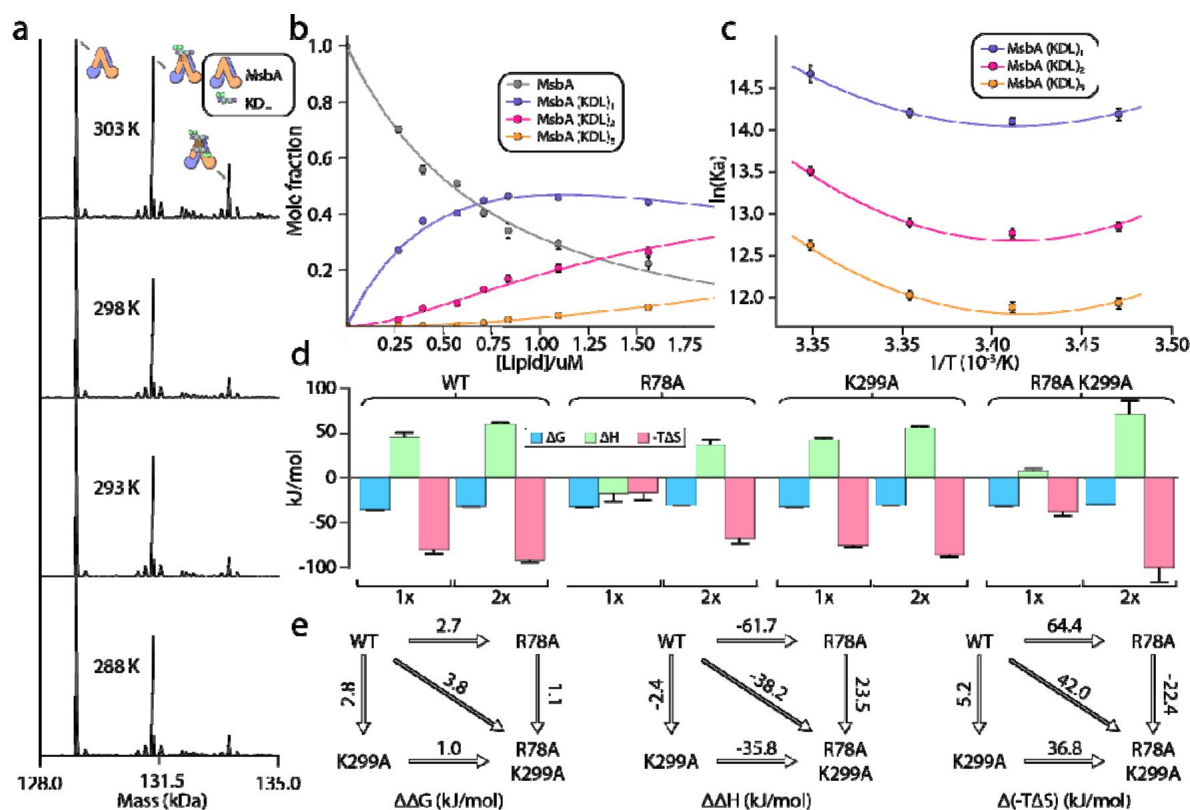


Figure 2.

Thermodynamics of KDL binding at the interior site to wild-type and mutant MsbA.

a) Representative deconvoluted native mass spectra of 0.39 μM wild-type MsbA in C10E5 and in the presence of 0.6 μM KDL recorded at different solution temperatures. b) Plot of mole fraction of MsbA (KDL)₀₋₃ determined from titration of KDL (dots) at 298 K and resulting fit from a sequential ligand binding model (solid line, $R^2 = 0.99$). c) van't Hoff plot for MsbA(KDL)₁₋₃ and resulting fit of a nonlinear van't Hoff equation. d) Thermodynamics for MsbA and mutants (MsbA^{R78A}, MsbA^{K299A} and MsbA^{R78A,K299A}) binding KDL at 298 K. e) Mutant cycles for MsbA and mutants with (from left to right) $\Delta\Delta G$ (mutant minus wild-type), $\Delta\Delta H$ and $\Delta(-T\Delta S)$ values indicated over the respective arrows. Shown are values at 298K. Reported are the average and standard deviation from repeated measurements ($n=13$).

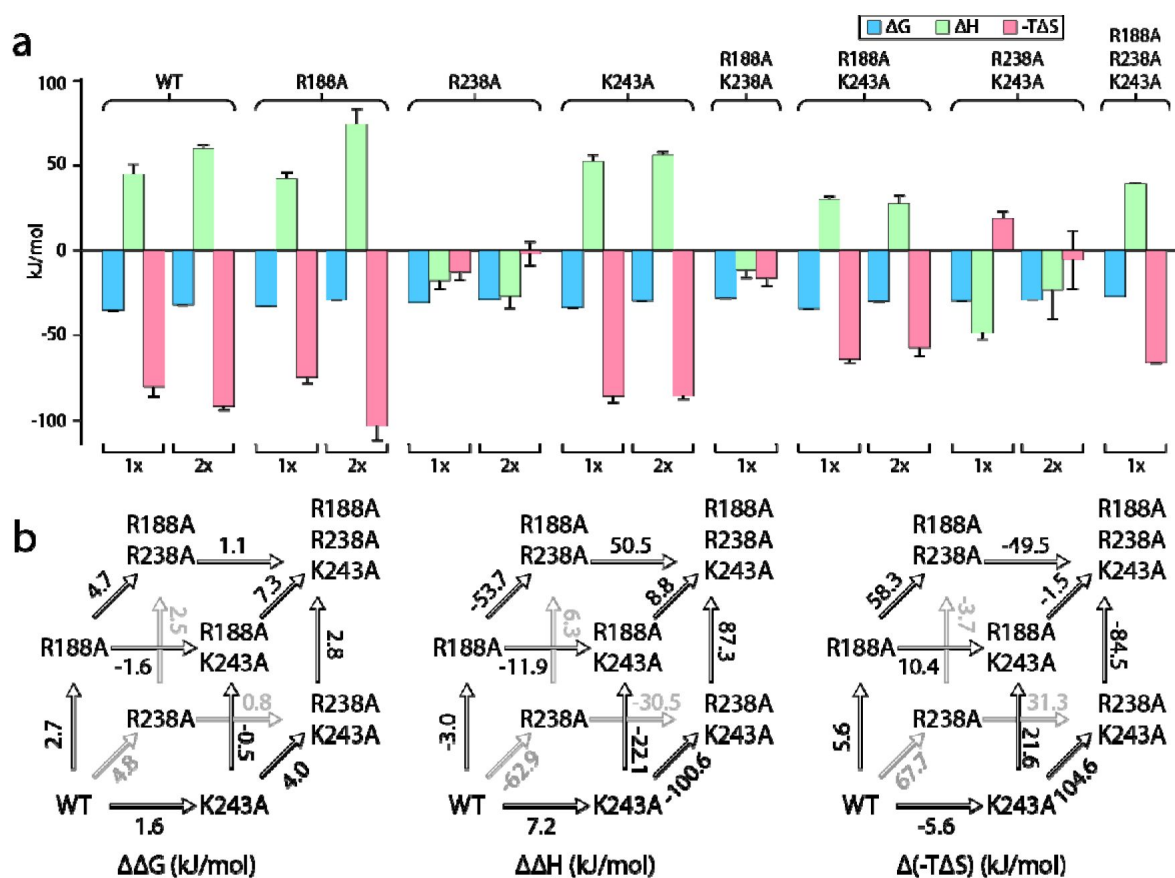
opposing entropic and enthalpic terms leading to an increase in ΔG by ~ 4 kJ/mol relative to wild-type MsbA (**Fig. 2d**). Mutant cycle analysis indicates a coupling energy ($\Delta\Delta G_{\text{int}}$) of 1.7 ± 0.4 kJ/mol that contributes to the stability of KDL-MsbA complex (**Fig. 2e** and **Supplementary Table 3**). More generally, $\Delta\Delta$ with a positive sign means favorable cooperation. Interestingly, the coupling enthalpy ($\Delta\Delta H_{\text{int}}$ of -26 ± 15 kJ/mol) and coupling entropy ($\Delta(-T\Delta S)_{\text{int}}$ of 28 ± 15 kJ/mol at 298K) indicating that these residues contribute to KDL binding through an entropy driven process that overcomes an enthalpic barrier (**Fig. 2e** and **Supplementary Table 3**).

KDL binding to the exterior binding site of MsbA

The recently discovered exterior KDL binding site¹⁰ located on the cytosolic leaflet of inner membrane has not been thoroughly investigated, prompting us to characterize this site by a triple mutant cycle (**Fig 3**). We first investigated R188 and K243, residues that both interact with one of the P-GlcN moieties of LOS. Like mutants targeting the interior LPS binding site, introducing mutants at the exterior site will impact binding at the two exterior sites. Both MsbA^{R188A} and MsbA^{K243A} single mutants marginally weakened the interaction by about 2 kJ/mol (**Fig 3a**). Enthalpy and entropy for KDL binding MsbA^{R188A} and MsbA^{K243A} was largely similar to the wild-type protein (**Fig 3a**). However, the R238A mutation significantly weakened the interaction with KDL, increasing ΔG by nearly 5 kJ/mol compared to the wild-type transporter (**Fig 3b** and **Supplementary Table 3**) and resulted in a distinct thermodynamic pattern with negative enthalpy changes for both the first and second KDL binding events (**Fig 3a**). ΔG for MsbA^{R188A,K243A} was comparable to the K243A single mutant form of the protein (**Fig 3a**). The positive coupling energy of 3.2 ± 0.4 kJ/mol with contributions from a coupling enthalpy of 19 ± 11 kJ/mol and a coupling entropy of -16 ± 12 kJ/mol at 298K (**Fig 3b** and **Supplementary Table 3**). Combining mutation R238A with R188A, MsbA^{R188A,R238A} decreased ΔH by 57 kJ/mol at the cost of increasing $-T\Delta S$ by 64 kJ/mol at 298K (**Fig 3b** and **Supplementary Table 3**). The coupling energy for R188A and R238A is approximately zero as a result of equal coupling enthalpy and entropy of different signs. Compared to the wild-type protein, MsbA^{R238A,K243A} results in an inversion of the thermodynamic signature with binding now being driven by enthalpy. More specifically, this inversion is accompanied by $\Delta\Delta H$ and $\Delta(-T\Delta S)$ of -93 ± 7 kJ/mol and 99 ± 7 kJ/mol at 298K (**Fig 3b** and **Supplementary Table 3**). Again, the coupling enthalpy and entropy (at 298K) of equal magnitude but opposite signs give rise to a coupling energy of zero for R238A and R243A (**Fig 3b** and **Supplementary Table 3**). Introduction of the R188A mutation into MsbA^{R238A,K243A}, results in reversal of the thermodynamic signature to mirror that of MsbA^{R188A,K243A} (**Fig 3a**). The coupling energy, coupling enthalpy, and coupling entropy for R188A, R238A and R243A are 3.4 ± 0.5 kJ/mol, 100 ± 16 kJ/mol, and -97 ± 16 kJ/mol at 298K (**Supplementary Table 4**), respectively. Taken together, these results demonstrate KDL binding to MsbA is sensitive to mutations at both the interior and exterior sites.

Dissecting KDL binding to the interior and exterior site(s) of MsbA

An open question is if the interior and exterior LOS binding sites of MsbA are allosterically coupled? We focused on the R188A and K299A mutants located at the exterior and interior binding sites, respectively. Results for both single mutants were presented above. MsbA containing the R188A and K299A mutations drastically reduced the binding of KDL (**Fig 4a**). The ΔG for MsbA^{R188A,K299A} increased by more than 6 kJ/mol compared to the wild-type protein (**Fig 4b**). This approximately doubles compared to MsbA containing either of the single point mutations. Mutant cycle analysis revealed a negative coupling energy of -1.1 ± 0.4 kJ/mol that partitioned into a coupling enthalpy of -36 ± 13 kJ/mol and coupling entropy of 34 ± 13 kJ/mol at 298K (**Fig. 4b** and **Supplementary Table 3**). In short, mutations at either LOS binding site have a negative impact on binding that is accompanied by a gain in both favorable entropy and unfavorable enthalpy.



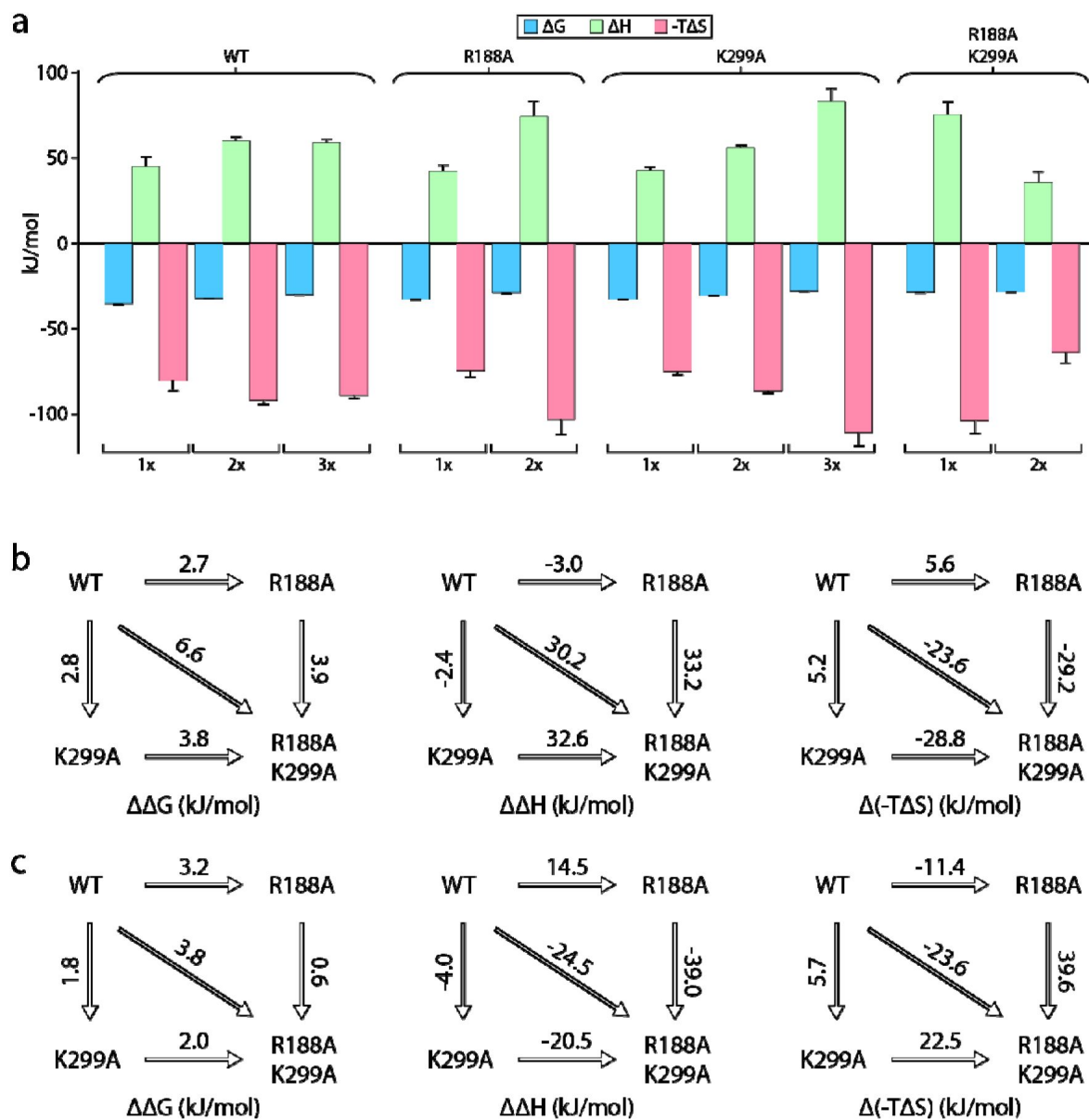


Figure 4.

Mutant cycle of MsbA residues located within the interior and exterior LOS bind sites

. a) Thermodynamic signatures for MsbA and mutants binding KDL at 298 K. b-c) Double mutant cycle analysis for R188 and K299. Shown are results for the first (panel b) and second (panel c) KDL binding to MsbA. Shown from left to right is $\Delta\Delta G$, $\Delta\Delta H$ and $\Delta(-T\Delta S)$ and the values indicated over the respective arrows at 298K. Reported are the average and standard deviation from repeated measurements ($n=13$).

Mutant cycle analysis of KDL binding to vanadate-trapped MsbA

As MsbA, like other ABC transporters, is highly dynamic, we sought to trap the transporter in an OF conformation using ADP and vanadate to interrogate binding at the exterior lipid binding site. We characterized the binding of KDL to vanadate-trapped MsbA and proteins containing single R188A, R238A, and K243A mutations. Here, we focused on the binding of the first and second lipid, since MsbA has two, symmetrically related KDL binding sites in the open, OF conformation. Thermodynamics of MsbA(KDL)₁₋₂ binding is like the non-trapped transporter, wherein entropy (-TΔS ranging from -58 ± 1 to -69 ± 1 kJ/mol at 298K) is more favorable than a positive enthalpic term (ΔH ranging from 22 ± 1 to 35 ± 1 kJ/mol) (**Fig. 5a**). The single mutant proteins (MsbA^{R188A}, MsbA^{R238A}, and MsbA^{K243A}) showed a slight increase in ΔG (at most 5 kJ/mol) (**Fig. 5a**). Notably, we found MsbA^{R238A} and MsbA^{K243A} had about a four-fold increase in ΔH and favorable entropy was about two-fold higher (**Fig. 5a**). Double mutant cycle analysis of the pairwise mutants revealed a positive coupling energy of ~2 kJ/mol for MsbA binding one and two KDLs (**Fig. 5b** and **Supplementary Table 7**). Focusing on the first KDL binding event, the coupling enthalpy and coupling entropy at 298K for R188 and K238 was 89 ± 7 kJ/mol and -87 ± 7 kJ/mol, respectively (**Fig. 5b** and **Supplementary Table 7**). Likewise, R238 and K243 showed 129 ± 11 kJ/mol of coupling enthalpy and -127 ± 11 kJ/mol of coupling entropy at 298K (**Fig. 5b** and **Supplementary Table 7**). However, the R188 and K243 pair revealed a relatively low coupling enthalpy and coupling entropy at 298K of 3.5 ± 7 kJ/mol and 2 ± 7 kJ/mol, respectively (**Fig. 5b** and **Supplementary Table 7**). These results highlight the importance of entropic and enthalpic contributions that underpin specific lipid binding sites.

Discussion

Thermodynamics provide unique insight into the molecular forces that drive specific MsbA-KDL interactions. A recurring thermodynamic strategy for specific KDL-MsbA interactions is a large, favorable entropic term that opposes a positive enthalpic value. The human G-protein-gated inward rectifier potassium channel (Kir3.2) also used a similar thermodynamic strategy to engage phosphoinositides (PIPs).⁴⁰ The large, positive entropy could stem from solvent reorganization of the lipid with a carbohydrate containing headgroup, and desolvation of hydrated binding pockets on the membrane protein. The release of ordered solvent to the bulk solvent would contribute favorably to entropy. These experiments are performed in detergent and reorganization of detergent may also play a role. Previous work has shown soluble protein-ligand interactions can be driven by a large, positive entropy term that outweighs a large, positive enthalpic penalty.⁵¹⁻⁵⁴ In these cases, the reaction is mainly driven by conformational entropy originating in enhanced protein motions. However, it is unclear if the conformational dynamics of MsbA are enhanced when bound to KDL.

Most of the van' t Hoff plots followed non-linear trends, indicating C_p is not constant over the selected temperature range.⁵⁰ In nearly all cases, a positive ΔC was observed that ranged in value from 4 to 12 kJ/mol-K (**Supplementary Table 2, 6**). Solvation of polar groups in aqueous solvent has been ascribed to positive heat capacities whereas the collapse of apolar residues from their solvated state is accompanied by a negative change in heat capacity.^{50,55} Reorganization of the hydrated, polar headgroups of KD is consistent with the positive heat capacity observed here. However, change in heat capacity could also be ascribed to temperature-dependent conformational changes in MsbA and/or KDL. Notably, vanadate-trapped MsbA locked in an open, OF conformation should be less conformationally dynamic than the apo protein, which is known to adopt a number of open, IF conformations where the NBDs are separated by different distances. Similar positive heat capacities were observed for the different conformations, suggesting the dynamics of MsbA marginally contribute to the observed non-linear trends. Notably, the headgroup of KDL is nestled in a hydrophilic, basic patch of MsbA in the open, OF conformation.

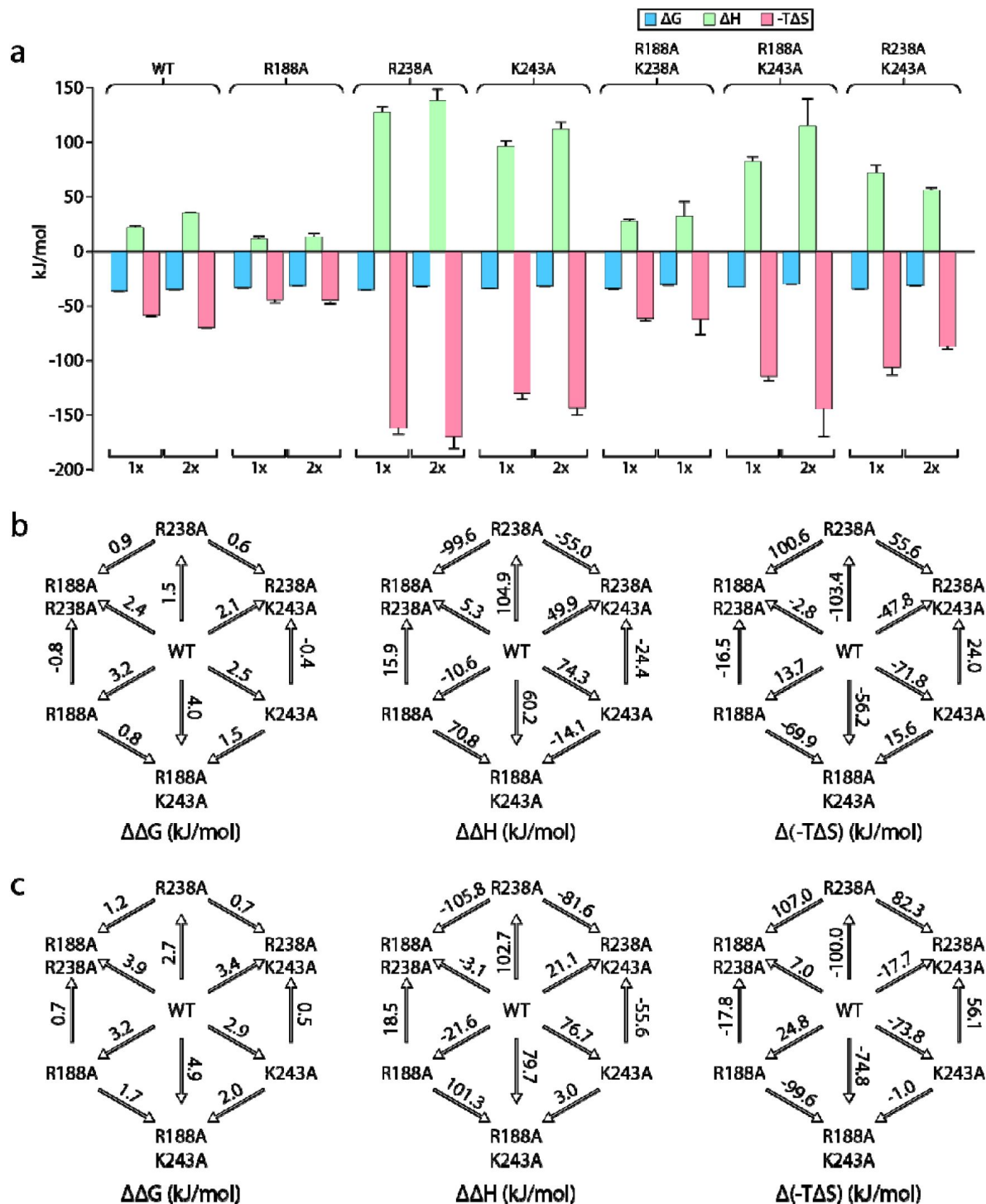


Figure 5.

Double mutant cycles reveal thermodynamic insight into KDL binding vanadate-trapped MsbA.

a) Thermodynamic signatures for MsbA and mutants binding KDL at 298 K. b-c) Double mutant cycle analysis for pairs of R188, R238, and K243 with a total of three combinations. Shown are results for the first (panel b) and second (panel c) KDL binding to MsbA trapped in an open, OF conformation with ADP and vanadate. Within each panel, $\Delta\Delta G$, $\Delta\Delta H$ and $\Delta(-T\Delta S)$ are shown from left to right and their values at 298K indicated over the respective arrows. Reported are the average and standard deviation from repeated measurements ($n=13$).

Similarly, the headgroup of PIP binds a hydrophilic, basic pocket in Kir3.2. These hydrophilic patches will be highly solvated, which will be desolvated upon binding lipids contributing favorably to entropy.

Thermodynamics of MsbA-lipid interactions contrast those observed for a different membrane protein. Phospholipid binding to the bacterial ammonia channel (AmtB) were largely driven by enthalpy and, in most cases, entropy was unfavorable.⁵⁶ Another interesting observation for AmtB-lipid interactions was significant enthalpy-entropy compensation for each sequential lipid binding event. Here, enthalpy-entropy compensation is not as pronounced. This result may reflect the much higher-affinity and specific MsbA-KDL interactions compared to the weaker AmtB-lipid interactions, sometimes referred to as non-annular lipids.²⁴ Moreover, we have focused the titration here to characterize the binding of the first three KDL molecules to MsbA. While we can't rule out that the resolved lipid bound states of MsbA represent binding of lipid to one or multiple site(s) on the transporter, the mole fraction plots are suggestive of binding to distinct sites, i.e., smooth inflections. In a previous study,⁴⁰ we observed abnormal binding curves for some PIPs binding to Kir3.2 that we rationalized by the presence of high-affinity binding and low-affinity binding sites. A revised equilibrium binding model including the two-site model dramatically improved the fits, leading to dissection of at least two lipid binding sites. Further studies are warranted to better understand the binding sites of KDL to MsbA in different conformations.

Results of this study begin to draw a connection between LPS binding at the interior and exterior sites of MsbA. It is presently thought that flipping of LOS occurs at interior MsbA site, and the exterior LOS binding site enables feedforward activation, wherein binding of LOS and precursors thereof stimulates ATPase activity.^{8,10,57,60} It is also thought that binding of LOS and ATP promotes dimerization of the NBDs. Here, we find mutations at either the interior or exterior sites have a direct impact of KDL binding to MsbA, which under these conditions is presumably adopting an open, IF conformation. Of the mutant proteins, MsbA containing single mutations (MsbA^{R188A,K299A}) at both LOS binding sites resulted in the greatest change in ΔG . This result implies that these sites are allosterically coupled and further investigation is warranted to better understand how the exterior LOS binding sites influence MsbA dynamics.

A defining feature of this work is the use of mutant cycles to not only characterize specific membrane protein-lipid interactions but define the coupling energies of specific residue-lipid interactions in terms of enthalpic and entropic contributions. Traditionally, mutant cycles have been used to understand pairwise interactions of residues, such as in protein-protein complexes, in terms of coupling free energy. Here, we extend mutant cycles to understand how pairs of residues contribute to specific MsbA-KDL interactions. Double mutants targeting the interior site reveal a positive coupling energy of nearly 2 kJ/mol for R78 and K299. These stabilize the MsbA-KDL complex largely through nearly 17 kJ/mol of favorable coupling entropy, which outweighs a negative coupling enthalpy. This phenomenon extends to nearly all mutant cycles investigated in this work, even when the transporter is trapped with vanadate. The largest coupling energy is observed from the triple mutant cycle of R188A, R238A and R243A, which again stabilization of the complex was achieved via favorable coupling entropy. While we focused on results at 298K, the coupling energetics among these three residues show 3.4 ± 0.5 kJ/mol. Taken together, mutant cycle analysis reveals that entropy drives high-affinity KDL binding to MsbA and solvent reorganization contributes to KDL binding (**Figure 6**). There are many factors that contribute to the change in entropy of the system, beyond solvation entropy, and deciphering the entropic contributions of the various components warrants additional studies.

While the use of mutant cycles was prominent a few decades ago, native mass spectrometry opens new opportunities to revisit the classical approach, diving deeper into the energetics of non-covalent interactions, such as dissecting energetics in terms of enthalpic and entropic contributions. Native MS coupled with a variable-temperature nanoelectrospray ionization (nESI) apparatus^{41,47} has been used to ascertain equilibrium binding constants and thermodynamic

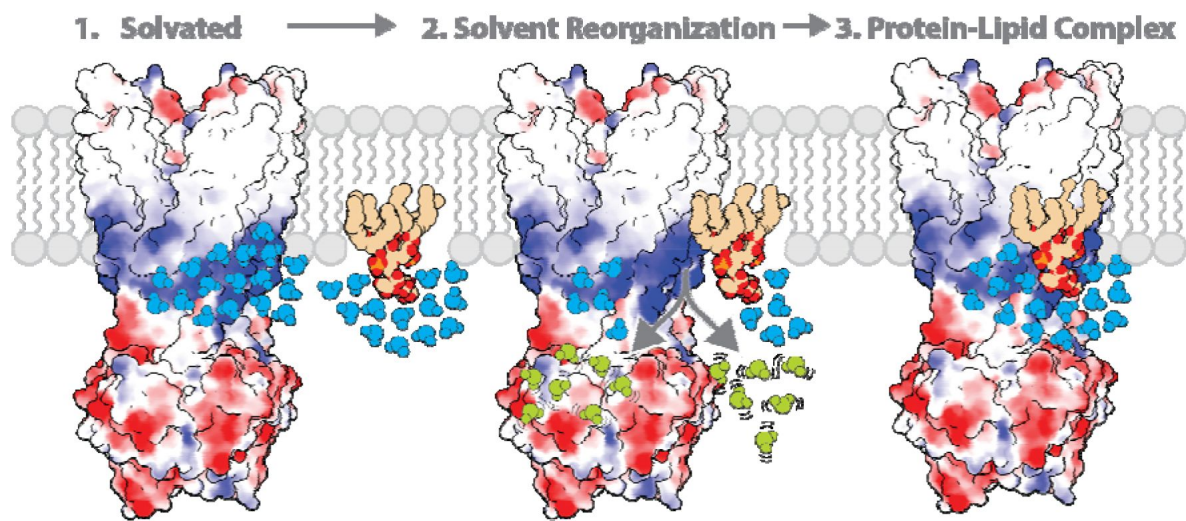


Figure 6.

The role of solvent in contributing to the molecular recognition of membrane protein-lipid complexes.

The lipid headgroup and binding pocket (basic patch illustrated in blue) on the membrane protein are solvated. The ordered solvent (shown in light blue) is then displaced upon lipid binding the membrane protein leading to solvent reorganization. The displacement of ordered solvent (shown in light green) contributes to favorable entropy. This process enables the formation of a high affinity, stable membrane protein-lipid complex.

properties of protein-protein and protein-ligand interactions. The results obtained align closely with those obtained through other biophysical techniques, such as isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR).^{48,56,61} The approach has also uncovered that specific protein-lipid interactions can allosterically modulate other interactions with protein, lipid, and drug molecules.^{33,34,48,49,62,63} More recently, native MS has proved useful in dissecting the thermodynamics of individual nucleotide binding events to GroEL, a 801 kDa tetradecameric chaperonin.³⁹ In contrast to traditional approaches, such as ITC and SPR, native MS can resolve and dissect individual binding events enabling the measurement of binding thermodynamics, which is of paramount importance to understanding the molecular driving forces of non-covalent interactions.

In summary, we demonstrate the utility of native MS to determine the thermodynamic origins of specific KDL-MsbA interactions. Combined with the classical mutant cycle approach,¹³ the thermodynamic contribution of specific interactions with lipids is illuminated. More specifically, MsbA binding KDL is solely driven by entropy, which overcomes an enthalpic penalty. A similar thermodynamic strategy was also observed for Kir3.2-PIP interactions, where entropy plays a central role in the wild-type channel recognizing PIP.⁴⁰ It is tempting to speculate that favorable entropy is a common theme enabling membrane proteins to specifically engage carbohydrate-containing lipids. We envision thermodynamics and mutant cycles will be invaluable in not only better understanding high-affinity lipid binding sites but also in the development of inhibitors, such as those that may target specific protein-lipid binding site(s). In closing, these studies provide deeper insight into the thermodynamic strategies membrane proteins exploit to achieve high-affinity lipid binding site(s).

Methods

MsbA expression constructs

MsbA and mutants were essentially expressed and purified as previously described.¹⁰ In detail, the MsbA gene (from *Escherichia coli* genomic DNA) was amplified by polymerase chain reaction (PCR) using Q5 High-Fidelity DNA Polymerase (New England Biolabs, NEB) and subcloned into a modified pCDF-1b plasmid (Novagen) resulting in expression of MsbA with an N-terminal TEV protease cleavable His6 fusion protein. Primers for generating mutations for MsbA were designed using the online tool NEBaseChanger (NEB) and carried out using the KLD enzyme mix (NEB) as described by the manufacturer. All plasmids were confirmed by DNA sequencing.

Protein expression and purification

MsbA expression plasmids were transformed into *E. coli* (DE3) BL21-AI competent cells (Invitrogen). A single colony was picked and used to inoculate 50 mL LB media to be grown overnight at 37 °C with shaking. The overnight culture was used to inoculate to terrific broth (TB) media and incubated at 37 °C until the OD_{600nm} ≈ 0.6-1.0. After which, the cultures were induced with final concentration of 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and 0.2% (w/v) arabinose. After overnight expression at 25 °C, the cultures were harvested at 4000 x g for 10 minutes and the resulting pellet was resuspended in lysis buffer (20 mM Tris, 300 mM NaCl and pH at 7.4 at room temperature). The resuspended cells were centrifuged, and the pellet was then resuspended in lysis buffer. Cells were lysed by four passages through a Microfluidics M-110P microfluidizer operating at 25,000 psi with reaction chamber emersed in an ice bath. The lysate was clarified by centrifugation at 20,000 x g for 25 minutes and the supernatant was centrifuged at 100,000 x g for 2 hours to pellet membranes. Resuspension buffer (20 mM Tris, 150 mM NaCl, 20% (v/v) glycerol, pH 7.4) was used to homogenize the resulting pellet and 1% (m/v) DDM was added for protein extraction overnight at 4 °C. The extraction was centrifuged at 20,000 x g for 25 minutes and the resulting supernatant was supplemented with 10 mM imidazole and filtered with a 0.45 μm syringe filter prior to purification by immobilized metal affinity chromatography. The

extraction containing solubilized MsbA was loaded onto a column packed with 2.5 mL Ni-NTA resin pre-equilibrated in NHA-DDM buffer (20 mM Tris, 150 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol, pH 7.4 and supplemented with 2x the critical micelle concentration (CMC) of DDM). After the loading, the column was washed with 5 column volumes (CV) of NHA-DDM buffer, 10 CV of NHA-DDM buffer supplemented with additional 2% (w/v) nonyl- β -glucoside (NG), and 5 CV of NHA-DDM buffer. The immobilized protein was eluted with the addition of 2 CV of NHB-DDM buffer (20 mM Tris, 150 mM NaCl, 250 mM imidazole, 10% (v/v) glycerol, 2 x CMC of DDM, pH 7.4). The eluted MsbA was pooled and desalted using HiPrep 26/10 desalting column (GE Healthcare) pre-equilibrated in desalting buffer (NHA-DDM with imidazole omitted). TEV protease (expressed and purified in-house) was added to the desalted MsbA sample and incubated overnight at room temperature. The sample was passed over a pre-equilibrated Ni-NTA column and the flow-through containing the cleaved MsbA protein was collected. The pooled protein was concentrated using a centrifugal concentrator (Millipore, 100 kDa) prior to injection onto a Superdex 200 Increase 10/300 GL (GE Healthcare) column equilibrated with 20 mM Tris, 150 mM NaCl, 10% (v/v) glycerol and 2x CMC C₁₀E₅. Peak fractions containing dimeric MsbA were pooled, flash frozen in liquid nitrogen, and stored at -80 °C prior to use.

Preparation of MsbA for native MS studies

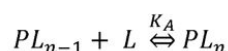
MsbA samples were incubated with 20 μ M copper (II) acetate, to saturate the N-terminal metal binding site,¹⁰ prior to buffer exchange using a centrifugal buffere exchange device (Bio-Spin, Bio-Rad) into 200 mM ammonium acetate supplemented with 2 x CMC of C₁₀E₅. To prepare vanadate-trapped MsbA, ATP and MgCl₂ were added to MsbA at a final concentration of 10mM. After incubation at room temperature for 10 minutes, a freshly boiled vanadate solution (pH 10) was added to reach final concentration of 1 mM followed by incubation at 37 °C for an additional 10 minutes. The sample was then buffer exchanged as described above.

Native Mass Spectrometry

Samples were loaded into gold-coated glass capillaries made in-house⁶⁴ and introduced into at a Thermo Scientific Exactive Plus Orbitrap with Extended Mass Range (EMR) using native electrospray ionization source modified with a variable temperature apparatus.⁴¹ For native mass analysis, the instrument was tuned as follow: source DC offset of 10 V, injection flatapole DC to 8.0 V, inter flatapole lens to 4, bent flatapole DC to 3, transfer multipole DC to 3 and C trap entrance lens to 0, trapping gas pressure to 6.0 with the in-source CID to 65.0 eV and CE to 100, spray voltage to 1.70 kV, capillary temperature to 200 °C, maximum inject time to 200 ms. Mass spectra were acquired with a setting of 17,500 resolution, microscans set to 1 and averaging set to 100.

Determination MsbA-lipid equilibrium binding constants

KDL (Avanti) stock solution was prepared by dissolving lipid powder in water. The concentration of MsbA and KDL were determined by a DC protein assay (BioRad) and phosphorus assay, respectively.^{65,66} MsbA was incubated with varying concentrations of KDL before loading into a glass emitter and mounted on a variable-temperature electrospray ionization (vT-ESI) source.⁴¹ Samples were incubated in the source for two minutes at the desired temperature before data acquisition. All titration data were collected in triplicate. At a given temperature, the mass spectra were deconvoluted using Unidec⁶⁷ and the peak intensities for apo and KDL-bound species were determined and converted to mole fraction. The sequential ligand binding model was applied to determine the mole fraction of each species in measurement:



Where:

$$K_{An} = \frac{[PL_n]}{[PL_{n-1}][L]}$$

To calculate the mole fraction of a particular species:⁵⁶

$$F_{PLn} = \frac{[L]_{free}^n \prod_{j=1}^n K_{Aj}}{1 + \sum_{i=1}^n [L]_{free}^i \prod_{j=1}^i K_{Aj}}$$

For each titrant in the titration, the free concentration of lipid was computed as follows:

$$[L]_{free} = [L]_{total} - [P]_{total} \sum_{i=0}^n i F_{PLi}$$

The sequential ligand binding model was globally fit to the mole fraction data by minimization of pseudo- χ^2 function:

$$\chi^2 = \sum_{j=1}^m \sum_{k=1}^d (F_{i,j,exp} - F_{i,j,calc})^2$$

where n is the number of bound ligands and d is the number of the experimental mole fraction data points.

Van't Hoff analysis⁶⁸ was applied to determine the Gibbs free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) based on the equation:

$$\ln K_A = -\frac{\Delta H}{R} \cdot \frac{1}{T} + \frac{\Delta S}{R}$$

For non-linear trends, the non-linear form of the Van't Hoff equation was applied to determine the thermodynamic parameters:⁶⁹

$$\ln K_A = \frac{\Delta H_{T_0} - T_0 \Delta C_p}{R} \left(\frac{1}{T_0} - \frac{1}{T} \right) + \frac{\Delta C_p}{R} \ln \left(\frac{T}{T_0} \right) + \ln K_0$$

where K_A is the equilibrium association constant, K_0 is the equilibrium association constant at the reference temperature (T_0), ΔH_{T_0} is the standard enthalpy at T_0 , ΔC_p is the change in heat capacity at constant pressure, and R is the universal gas constant.

Mutant cycle analysis

If the two mutated residues are interacting, then the coupling free energy ($\Delta\Delta G_{int}$) will not be 0 and the value may be positive or negative depending upon whether the interactions between mutated residues enhance or weaken the functional property measured.⁷⁰ $\Delta\Delta G_{int}$ can be computed given the change in Gibbs free energy for the wild-type protein (P), two single mutants (PX and PY), and double mutant (PXY) as follows:

$$\Delta\Delta G_{int} = \Delta\Delta G_{PX \rightarrow P} + \Delta\Delta G_{PY \rightarrow P} - \Delta\Delta G_{PXY \rightarrow P}$$

where

$$\Delta\Delta G_{PX \rightarrow P} = \Delta G_{PX} - \Delta G_P, \quad \Delta\Delta G_{PY \rightarrow P} = \Delta G_{PY} - \Delta G_P \quad \text{and} \quad \Delta\Delta G_{PXY \rightarrow P} = \Delta G_{PXY} - \Delta G_P.$$

Analogously, the contributions from coupling enthalpy ($\Delta\Delta H_{int}$) and coupling entropy ($\Delta(-T\Delta S_{int})$) can be computed as follows:

$$\Delta\Delta H_{int} = \Delta\Delta H_{PX \rightarrow P} + \Delta\Delta H_{PY \rightarrow P} - \Delta\Delta H_{PXY \rightarrow P}$$

$$\Delta(-T\Delta S_{int}) = \Delta(-T\Delta S_{PX \rightarrow P}) + \Delta(-T\Delta S_{PY \rightarrow P}) - \Delta(-T\Delta S_{PXY \rightarrow P})$$

where T is temperature in K. As an example,

$$\Delta\Delta H_{PY \rightarrow P} = \Delta H_{PY} - \Delta H_P \quad \text{and} \quad \Delta(-T\Delta G_{PX \rightarrow P}) = T\Delta S_P - T\Delta S_{PX}.$$

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Author Contributions

J.L., M.M., and A.L. designed the research on mutant cycle analysis. J.L. and T.Z. expressed and purified MsbA. J.L. performed mass spectrometry experiments. J.L. and T.Z. carried out the functional assays. J.L., T.Z., M.M., D.C., D.R., and A.L. analyzed the data. J.L. and A.L. wrote the manuscript with input from the other authors.

Competing interests

The authors declare no competing interests.

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Reviewer #1 (Public Review):

Summary:

The preprint by Laganowsky and co-workers describes the use of mutant cycles to dissect the thermodynamic profile of specific lipid recognition by the ABC transporter MsbA. The authors use native mass spectrometry with a variable temperature source to monitor lipid binding to the native protein dimer solubilized in detergent. Analysis of the peak intensities (that is, relative abundance) of 1-3 bound lipids as a function of solution temperature and lipid concentration yields temperature-dependent Kds. The authors use these to then generate van't Hoff plots, from which they calculate the enthalpy and entropy contributions to binding of one, two, and in some cases, three lipids to MsbA. The authors have previously demonstrated that MS can indeed extract thermodynamic contributions to lipid binding. The authors then employ mutant cycles, in which basic residues involved in headgroup binding are mutated to alanine. By comparing the thermodynamic signatures of single and double (and in one instance triple) mutants, they aim to identify cooperativity between the different positions. They furthermore use inward and outward locking conditions which should control access to the different binding sites determined previously. The main conclusion is that lipid binding to MsbA is driven mainly by energetically favorable entropy increase upon binding, which stems from the release of ordered water molecules that normally coordinate the basic residues, which helps to overcome the enthalpic barrier of lipid binding. The authors also report an increase in lipid binding at higher temperatures which they attribute to a non-uniform heat capacity of the protein. Although they find that most residue pairs display some degree of cooperativity, particularly between the inner and outer lipid binding sites, they do not provide a structural interpretation of these results.

Strengths:

The use of double mutant cycles and mass spectrometry to dissect lipid binding is novel and interesting. For example, the observation that mutating a basic residue in the inner and one in the outer binding site abolishes lipid binding to a greater extent than the individual mutations is highly informative even without having to break it down into thermodynamic terms. The method and data reported here opens new avenues for the structure/activity relationship of MsbA. The "mutant cycle" approach is in principle widely applicable to other membrane proteins with complex lipid interactions.

Weaknesses:

The use of double mutant cycles to dissect binding energies is well-established, and has, as the authors point out, been employed in combination with mass spectrometry to study protein-protein interactions. Its application to extract thermodynamic parameters is robust in cases where a single binding event is monitored, e.g. the formation of a complex with well-defined stoichiometry, where dissociation constants can be determined with high confidence. It is, however, complicated significantly by the fact that for MsbA-lipid interactions, we are not looking at a single binding event, but a stochastic distribution of lipids across different sites. Even if the protein is locked in a specific conformation, the observation of a single lipid adduct does not guarantee that the one lipid is always bound to a specific site. The authors discuss this issue in the manuscript. As they point out, one can assume that the most high-affinity sites will be populated first. Hence, the K_d values determined by MS likely describe (mostly) lipid binding to these sites, although this does not seem to hold universally true, as seen for example for the two (in principle equivalent) binding sites in the vanadate-locked protein. In addition, mutation of a binding site (which the authors show reduces lipid binding) may instead allow the lipid to bind to a lower-affinity site elsewhere. In summary, the K_d s are an approximation.

(Minor comment: The protein concentrations used for MS titration experiments should be stated in the methods.)

The authors conclude that solvation entropy is a major factor driving lipid binding (Figure 6). If the increase in entropy upon lipid binding comes from the release of ordered water molecules around the basic residues, we should see a smaller increase in entropy for proteins where several basic residues have been changed to alanine, which is not the case. The authors explain this by stating that other entropic factors likely are at play. Judging from their data, that is certainly correct, but why then focus on solvation entropy in the discussion if its contribution to the total entropy change cannot be determined?

- <https://doi.org/10.7554/eLife.91094.2.sa1>

Reviewer #3 (Public Review):

Summary:

In this paper presented by Liu et al, native MS on the lipid A transporter MsbA was used to obtain thermodynamic insight into protein-lipid interactions. By performing the analyses at different lipid A concentrations and temperatures, dissociation constants for 2-3 lipid A binding sites were determined, as well as enthalpies were calculated using non-linear van't Hoff fitting.

Strengths:

This is an extensive high quality native MS dataset that provides unique opportunities to gain insights into the thermodynamic parameters underlying lipid A binding. In addition, it provides coupling energies between mutations introduced into MsbA, that are implicated in lipid A binding.

Weaknesses:

It remains elusive, which KD values belong to which of the possible lipid A binding sites.

Appraisal:

The authors convincingly addressed the concerns raised by the reviewers.

- <https://doi.org/10.7554/eLife.91094.2.sa0>

Author Response

The following is the authors' response to the original reviews.

We thank the reviewers for their careful, critical, and insightful evaluation of our manuscript.

Public Reviews:**Reviewer #1 (Public Review):***Summary:*

The preprint by Laganowsky and co-workers describes the use of mutant cycles to dissect the thermodynamic profile of specific lipid recognition by the ABC transporter MsbA. The authors use native mass spectrometry with a variable temperature source to monitor lipid binding to the native protein dimer solubilized in detergent. Analysis of the peak intensities (that is, relative abundance) of 1-3 bound lipids as a function of solution temperature and lipid concentration yields temperature-dependent Kds. The authors use these to then generate van't Hoff plots, from which they calculate the enthalpy and entropy contributions to binding of one, two, and in some cases, three lipids to MsbA.

The authors then employ mutant cycles, in which basic residues involved in headgroup binding are mutated to alanine. By comparing the thermodynamic signatures of single and double (and in one instance triple) mutants, they aim to identify cooperativity between the different positions. They furthermore use inward and outward locking conditions which should control access to the different binding sites determined previously.

The main conclusion is that lipid binding to MsbA is driven mainly by energetically favorable entropy increase upon binding, which stems from the release of ordered water molecules that normally coordinate the basic residues, which helps to overcome the enthalpic barrier of lipid binding. The authors also report an increase in lipid binding at higher temperatures which they attribute to a non-uniform heat capacity of the protein. Although they find that most residue pairs display some degree of cooperativity, particularly between the inner and outer lipid binding sites, they do not provide a structural interpretation of these results.

Strengths:

The use of double mutant cycles and mass spectrometry to dissect lipid binding is novel and interesting. For example, the observation that mutating a basic residue in the inner and one in the outer binding site abolishes lipid binding to a greater extent than the individual mutations is highly informative even without having to break it down into thermodynamic terms (see "weaknesses" section). In this sense, the method and data reported here opens new avenues for the structure/activity relationship of MsbA. The "mutant cycle" approach is in principle widely applicable to other membrane proteins with complex lipid interactions.

Weaknesses:

The use of double mutant cycles to dissect binding energies is well-established, and has, as the authors point out, been employed in combination with mass spectrometry to study protein-protein interactions. Its application to extract thermodynamic parameters is robust in cases where a single binding event is monitored, e.g. the formation of a complex with well-defined stoichiometry, where dissociation constants can be determined with high confidence. It is, however, complicated significantly by the fact that for MsbA-lipid interactions, we are not looking at a single binding event, but a stochastic distribution of lipids across different sites. Even if the protein is locked in a specific conformation, the observation of a single lipid adduct does not guarantee that the one lipid is always bound to a specific site. In some of the complexes detected by MS, the lipid is likely bound somewhere else. Lipid binding Kds from mass spectrometry, although helpful in some instances as a proxy for global binding affinities, should therefore be taken with a grain of salt.

We agree with the reviewer in that while we will measure binding of lipid (mass shift) we do not know the binding location(s). Given this issue, we have added to the discussion section on this important point and elaborate more broadly on this problem in the context of membrane protein-lipid interactions. Tackling this issue represents a frontier challenge for the field.

The authors analyze the difference in binding upon mutating binding sites (ddG etc). Here, another complicating factor comes into play, the fact that mutation of a binding site (which the authors show reduces lipid binding) may instead allow the lipid to bind to a lower-affinity site elsewhere. Unfortunately, the authors do not specify the protein concentration, but assuming it is in the single-digit micromolar range, as common for native MS experiments, lipid and protein concentrations are almost equal for most of the data points, resulting in competition between binding sites for free lipids. As a rule of thumb, for Kd measurements, the concentration of the constant component, the protein, should be far below the Kd, to avoid working in the "titration" regime rather than the "binding" regime (see Jarmoskaite et al, eLife 2020). I cannot determine whether this is the case here. The way I understand the double mutant cycle approach, reliable Kd measurements are required to accurately determine dH and TdS, so I would encourage the authors to confirm their Kd values using complementary methods before in-depth interpretations of the thermodynamic components.

The reviewer references an article in eLife by Jarmoskaite and co-workers describing "titration" vs "binding" regimes. Below we paste a snippet from this article:

Author response image 1.

Distinguishing between concentration regimes

The challenge is that distinguishing between the regimes requires the knowledge of the K_D , and consequently it is impossible to know a priori which regime holds. A useful rule of thumb for avoiding the titration regime is to always maintain the concentration of the excess binding partner significantly above that of the trace limiting partner. The reason for this can be gleaned from the equation that describes the fraction of bound RNA for the simple binding scheme of *Figure 3*:

$$\text{Fraction bound} = \frac{[P]_{\text{free}}}{[P]_{\text{free}} + K_D} \quad (4a)$$

Here $[P]_{\text{free}}$ is the unbound protein concentration and K_D is simply the free protein concentration at which half of the RNA is bound. But while *Equation 4a* holds universally, in practice we only know the total concentration of P, $[P]_{\text{total}}$ —how much we added to the solution—not the free concentration ($[P]_{\text{free}}$). Therefore, we want to operate under simplifying conditions where $[P]_{\text{free}} \approx [P]_{\text{total}}$ so that we can substitute $[P]_{\text{total}}$ into *Equation 4a* to give *Equation 4b*:

$$\text{Fraction bound} = \frac{[P]_{\text{total}}}{[P]_{\text{total}} + K_D} \quad (4b)$$

The condition $[P]_{\text{free}} \approx [P]_{\text{total}}$ holds true if P is in large excess of RNA across the entire experiment, meaning that only a small fraction of total protein is used up by binding to RNA. Most importantly, this condition must hold for the protein concentration that gives half-saturation to determine the K_D ; hence the requirement for the binding regime that the concentration of the limiting component must be $\ll K_D$. Nevertheless, simply maintaining an excess of protein over the limiting component may not always be sufficient to maintain a binding regime, given the uncertainty often surrounding concentration measurements and even greater uncertainty surrounding active concentrations.

Equation 4a is an expression for the fraction of protein bound to ligand, which universally holds, i.e., if we know the concentration of molecules at equilibrium (including those unbound or free) then one can obtain the special ratio or equilibrium constant at a given temperature. Jarmoskaite et al. note that in practice (using traditional biophysical approaches) one cannot readily distinguish protein that is free or bound to ligand (see highlighted part above). While this assumption is basis of their eLife assessment, it does NOT apply to native mass spectrometry data. It is important to realize that the mole fraction (or concentration) of apo and each lipid bound states, i.e., [P], [PL], [PL₂], ..., [PL_{n+1}], can readily be obtained directly from the deconvoluted mass spectrum. This is unlike other biophysical methods that are ensemble measurements, which measures the amount of heat or fraction of total ligand bound to protein. Since we can discern each lipid bound state, including the free protein and free ligand concentrations, the equilibrium binding constants can be directly calculated, and the protein and ligand concentration becomes irrelevant. In principle, equilibrium constants for protein-lipid interactions can be calculated from one mass spectrum. To increase transparency, we have updated the results section to highlight the important difference of the native MS approach compared to less robust traditional approaches that are riddled with underlying issues/assumptions.

We appreciated the reviewer's suggestion of using complementary methods to confirm K_D values. In our previous report [1], we determined binding thermodynamics for soluble protein-ligand interactions using native MS, surface plasmon resonance (SPR), and isothermal calorimetry (ITC) and found the techniques yield similar binding constants and thermodynamic parameters. The use of soluble proteins with defined ligand binding studies was rather straightforward to carry out a complementary study. We have also shown consistent findings for native MS and SPR of membrane protein interaction with a soluble, regulatory protein [2]. However, in the case of membrane proteins they can bind the first few

lipids very specifically and, with the addition of more lipid, bind even more lipids that represent rather weak binding. Thus, traditional approaches would report on the ensemble of lipids bound to membranes and specific lipid binding sites (such as inner and outer LPS binding sites in MsbA) are saturable but also additional binding will be observed, i.e., doesn't follow traditional soluble protein-ligand binding studies. In the past we have used a fluorescent-lipid competition binding assay [3] to corroborate native MS results for Kir3.2, which showed a direct correlation. The disadvantage of this complementary approach is using a non-natural, fluorescent-modified lipid. Unfortunately, there is no commercial source for a fluorophore modified KDL.

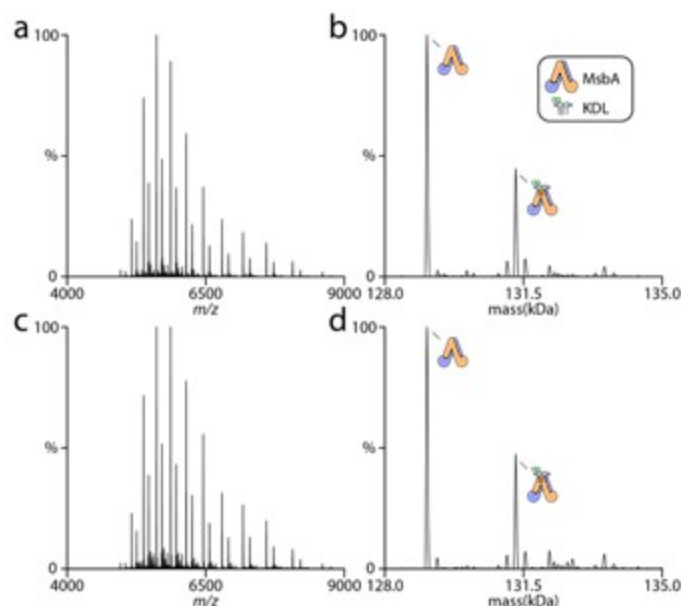
It is somewhat counterintuitive that for many double mutants, and the triple mutant, the entropic component becomes more favorable compared to the WT protein. If the increase in entropy upon lipid binding comes from the release of ordered water molecules around the basic residues (a reasonable assumption) why does this apply even more in proteins where several basic residues have been changed to alanine, which coordinate far fewer water molecules?

There are many factors that contribute to the change in entropy of the system, beyond solvation entropy, and deciphering the entropic contributions of the various components remains a challenging task. We have revised the manuscript to emphasize that solvation is one component of the entropic term and other components are likely at play.

The authors could devote more attention to the fact that they use detergent micelles as a vehicle for lipid binding studies. To a limited extent, detergents compete with lipids for binding, and are present in extreme excess over the lipid. The micelle likely changes its behavior in response to temperature changes. For example, the packing around the protein loosens up upon heating, which may increase the chance for lipids to bind. In this case, the increase in binding at higher temperatures may not be related to a change in heat capacity. This question could be addressed by MD simulations, if it's not already in the literature.

The detergent and its concentration are consistent for all the different MsbA proteins in this study. In fact, we observe linear van't Hoff plots with positive and negative slopes as well as non-linear curves that are convex or concave. The MsbA protein (wt or mutant), trapped or not, all display unique temperature-dependent responses. The reviewers comment of increasing temperature to loosen packing of detergent to promote lipid binding is clearly NOT that simple. If detergent was significantly influencing lipid binding (as suggested by reviewer) then increasing its concentration should impact lipid binding. In a previous study, we found no difference in membrane protein-lipid thermodynamics even when the concentration of detergent was increased five-fold [1]. We repeated similar experiments for MsbA and find the increased detergent concentration does not impact the abundances of lipid bound states. The figure to the right shows MsbA in the presence of lipid in 2x CMC (panel a and b) and 10x CMC (panel c and d). As you will see, no appreciable difference in the lipid bound signal is observed.

Author response image 2.



We applaud the suggestion of MD simulation. However, it is far beyond the scope of this paper and its not clear what will really be learned.

Reviewer #2 (Public Review):

Summary:

This is a solid study that dissects the thermodynamics of lipopolysaccharide (LPS) transporter MsbA and LPS. Native ESI-MS and the novel strategies developed by the authors were employed to quantify the affinities of LPS-MsbA interactions and its temperature dependence. Here, the equilibrium of lipid-protein interactions occurs in the micellar phase. The double-/triple-mutant cycle analysis and van't Hoff analysis allowed a full thermodynamic description of the lipid-protein interactions and the analysis of thermodynamic coupling between LPS binding sites. The most notable result would be that LPS-MsbA interaction is largely driven by entropy involving the negative heat capacity, a signature of the solvent reorganization effect (here authors attribute the solvent effect to "water" reorganization). The entropy driven lipid binding has been previously reported by the same authors for Kir1,2-PIP2 interactions.

Strengths:

- 1. This is overall a very thorough and rigorous study providing the detailed thermodynamic principles of LPS-MsbA interaction.*
- 1. The double and triple-mutant cycle approaches are newly applied to lipid-protein interactions, enabling detailed thermodynamics between LPS binding sites.*
- 1. The entropy-driven protein-lipid interaction is surprising. The binding seems to be mainly mediated by the electrostatic interaction between the positively charged residues on the protein and the negatively charged or polar headgroup of LPS, which could be thought of as "enthalpic" (making of a strong bond relative to that with solvent).*

Weaknesses:

1. This study is a good contribution to the field, but it was difficult to find novel biological insights or methodological novelty from this study.

1a. Thermodynamic analysis of lipid-protein interactions, an example of entropy-driven lipid-protein interactions, and the cooperativity between lipid binding sites have been reported by the author's group. Also, the cooperativity between binding sites in general have been reported from numerous studies of biomolecular interactions.

We appreciate the reviewer for highlighting our previous work. Of course, a single study does not establish a pattern, such as entropy-driven lipid-protein interactions.

While we agree with the reviewer that cooperativity in biomolecular interactions has been established for many soluble protein systems, by no means do we have a detailed understanding of membrane protein-lipid interactions. This work is an important contribution to expanding on classical work on soluble protein systems to more challenging membrane protein systems and their interactions with lipids.

1b. It is not clear how this study provides new insights into the understanding of LPS transport mechanisms. Probably, authors could strengthen the Discussion by providing biological insights-how the residue coupling.

The thermodynamics provides us with a deeper insight into the chemical principles that drive specific membrane protein-lipid interactions. We have revised the discussion to highlight the importance of thermodynamics and the implication of individual residues to KDL binding, and the inner and outer LPS binding sites appear to be coupled, something that is new.

1. One to three LPS molecules bind to MsbA, but it is unclear whether bound KDL occupies inner or outer cavities, or both and how a specific mutation affects the affinity of specific LPS (i.e., to inner or to outer cavities). Based on the known structures, the maximal number of LPS is three. It is possible that the inner and outer cavities have different LPS affinities. Also, there can be multiple one-LPS-bound states, two-LPS-bound states if LPS strictly binds to the binding sites indicated by the structures. This aspect is beyond the scope of this study and difficult to address, but without this information, it seems hard to tell what is going on in the system.

In our response above, we note that lipids will bind to membrane proteins at specific site(s) and weaker sites, often described as non-annular lipids. The revision includes this discussion point.

1. If a single mutation is introduced to the inner cavity, its effect will be "doubled" because the inner cavity is shared by two identical subunits. This effect needs to be clarified in the result section.

Great point. In addition, an outer mutant will also impact not one but both outer binding site(s). The revised manuscript makes note of this point.

1. In the result section, "Mutant cycle analysis of KDL binding to vanadate-trapped MsbA.":

4a. It seems necessary to show the mass spectra for Msb-ADP-vanadate complex as well as its lipid bound forms.

In the original submission, the mass spectra of vanadate trapped MsbA with KDL binding was provided in Supplementary Figures 10 and 11.

4b. The rationale of this section (i.e., what mechanistic insights can be obtained from this study) is unclear. For example, it is not sure what meaningful information can be obtained from a single type (ADP/vanadate) of the bound state regarding the ATP-driven function of MsbA.

MsbA is a dynamic, populates different conformations. Trapping with vanadate locks the transporter in an outwardfacing state with NDB interacting. This provides the opportunity to characterize binding to the exterior site. We revised the manuscript to note this point.

Reviewer #3 (Public Review):

Summary:

*In this paper presented by Liu et al, native MS on the lipid A transporter MsbA was used to obtain thermodynamic insight into protein-lipid interactions. By performing the analyses at different lipid A concentrations and temperatures, dissociation constants for 2-3 lipid A binding sites were determined, as well as enthalpies were calculated using nonlinear van't Hoff fitting. Changes in free Gibb's energies were then calculated based on the determined dissociation constants, and together with the enthalpy values obtained via van' t Hoff analysis, the entropic contribution to lipid binding (ΔS^*T) was indirectly determined.*

Strengths:

This is an extensive high quality native MS dataset that provides unique opportunities to gain insights into the thermodynamic parameters underlying lipid A binding. In addition, it provides coupling energies between mutations introduced into MsbA, that are implicated in lipid A binding.

Weaknesses:

The data all rely on the accuracy of determining KD values for lipid binding to MsbA. For the weaker binding sites, the range of lipid concentrations probed were in fact too low to generate highly accurate data. Another weakness is a lack of clear evidence, which KD values belong to which of the possible lipid A binding sites.

See our detailed response to reviewer 1 regarding Kd determination using native MS compared to other techniques. We chose to focus on the first three lipid binding events and adjusted the concentrations accordingly to titrate these three. As noted above, the Kd values can be determined from one mass spectrum. For rigor, we include different titration points and fit sequential binding model to the data – the fits are shown in supplemental and quite reasonable.

Regarding multiple lipids binding to different site(s), we have been able to distinguish high-affinity vs low-affinity PIP binding to Kir3.2 in a previous study [4]. This was apparent by the mole fraction curves for some lipid bound states not returning back to zero. We agree binding to multiple sites can be an issue. However, other techniques report on the ensemble of binding and, hence, no real useful information is obtained. Native MS enables one step in the right direction by dissecting the different lipid bound states. Future directions will need to

further address this forefront question in the field, which we make point of now in discussion.

Reviewer #1 (Recommendations For The Authors):

Experiments/analysis: In short, there should be a proof of principle experiment that the thermodynamic constants determined by MS are accurate. Once that is done, the authors can add a more engaging structural interpretation of the results from the mutant cycles (which they seem to consciously avoid in the present manuscript?). How are cooperative residues coupled? Why?

See our detailed response to reviewer 1 above.

The manuscript is well-written, but Figures 3-5 are somewhat repetitive and require a lot of time to understand. Schematics of the main findings in each figure would help the uninitiated reader.

We agree the illustrations are complex but there is rich data being shown.

Figure 2 C contains an x-axis label error.

Corrected.

Reviewer #2 (Recommendations For The Authors):

1. Lines 128-129: "Like other mutant cycle studies, we assume the single- and double-mutations do not disrupt binding at specific sites on MsbA."

This statement is obscure and needs to be clarified. Does this mean that the mutations still allow binding of KDL, or the mutations do not disrupt the conformational integrity of the binding sites?

This statement has been removed.

1. Lines 137-139: "More specifically, R78 coordinates one of the characteristic phosphoglucosamine (P-GlcN) substituents of KDL whereas K299 interacts with a carboxylic acid group in the headgroup of KDL."

Two identical subunits form a dimer interface that forms an LPS binding site. Thus, a single mutation on the inner cavity will disrupt two binding sites on LPS. One R78 to P-GlcN and the other to a sugar backbone. Also, one K299 interacts with a carboxylic acid group in the headgroup and the other to an unknown (not clear in the figure).

Also noted above, mutation of the outer site will also impact the two outer sites. We have made note of this caveat.

1. Lines 171-172: "leading to an increase in ΔG by ~ 4 kJ/mol (Fig. 2d)"

Relative to what?

Corrected.

1. Lines 172-173: "Mutant cycle analysis indicates a coupling energy ($\Delta\Delta G_{int}$) of 1.7 (plus minus) 0.4 kJ/mol that contributes to the stability of KDL-MsbA complex."

The sign of DDG (DDH, DDS)_int is a bit confusing. I recommend that authors define the meaning of negative or positive sign of DDG_int (DDH, DDS) at this point. Here, a positive sign means favorable cooperation between the two mutated residues. Sometimes, researchers designate a positive cooperativity as a negative sign.

The literature on mutant cycles does not appear to follow a consensus on the sign. Here, we have revised the manuscript to note positive sign means favorable cooperation and follow the formalism recently described by Horovitz, Sharon, and co-workers [5].

1. Lines 182-185: "Enthalpy and entropy for KDL binding MsbA R188A was largely similar to the wild-type protein (Fig 3a). However, the R243A mutation resulted in an increase in entropy, compensated for by an increase in positive enthalpy (Fig 3a)."

The thermodynamic parameters for R243A mutation change in a similar manner to WT and R188A. It is R238A, not R243A, whose DH-DS interplay shows a distinct pattern from WT. Please, reword this sentence.

The sentence has been revised.

1. Lines 252-253: Solvation of polar groups in aqueous solvent has been ascribed to positive heat capacities whereas negative for apolar solvation.

This statement is not precise. More precisely, the collapse of apolar molecules from their solvated state leads to the negative "change" in heat capacity.

The sentence has been corrected.

1. Line 262-267: "These hydrophilic patches will be highly solvated, which will be desolvated upon binding lipids contributing favorably to entropy. In the case of MsbA, the selected lysine and arginine residues (based alpha carbon position) are separated by about 9 to 18 Å (PDB 8DMM). This distance could result in overlap of solvation shells that collectively contribute to the positive coupling enthalpy observed for MsbA-KDL interactions."

This statement is too speculative without presenting the degree of solvation of the residues targeted for mutation. More quantitative arguments seem to be needed.

We have removed the speculative statement.

Reviewer #3 (Recommendations For The Authors):

In this paper presented by Liu et al, native MS on the lipid A transporter MsbA was used to obtain thermodynamic insight into protein-lipid interactions. By performing the analyses at different lipid A concentrations and temperatures, dissociation constants for 2-3 lipid A binding sites were determined, as well as enthalpies were calculated using nonlinear van't Hoff fitting.

*Changes in free Gibb's energies were then calculated based on the determined dissociation constants, and together with the enthalpy values obtained via van' t Hoff analysis the entropic contribution to lipid binding (ΔS^*T) was indirectly determined.*

Correction – In the case on linear van't Hoff plots, dH and dS were determined directly from the plot. For the nonlinear form of the van't Hoff equation, which does not include an entropy fitting parameter, we back calculated dS using dH and dG at a given temperature.

The authors then included single, double and triple mutants of residues known based on cryo-EM and X-ray structures to interact with Lipid A either in the large inward-facing cavity or at a secondary binding site accessible at the surface of outward-facing MsbA, and determined the thermodynamic parameters of these mutants alone and combined to gain access to coupling energies of pairwise interactions. This method has its roots in studying pair-wise interactions of protein-protein interfaces, generally known as thermodynamic mutant cycle analysis.

Having the main expertise in ABC transporter structure-function, I will judge the paper mostly from the standpoint of what I can learn as a transporter expert from this study and whether the insights are of value for researchers with average biophysical knowledge.

My overall impression of the manuscript is that, while it contains a wealth of experimental data using the innovative and unique method of native mass spectrometry, it is hard to understand what one can learn from this analysis beyond their interesting key finding that entropy plays an important role in lipid binding (but only at certain temperatures). In particular, the lessons learned from the coupling energy analysis of the introduced mutations is hard to grasp/digest for me with regards to what I can learn from these numbers (other than learning that there are such coupling effects).

We agree the thermodynamic data is rich. Often a ddGint of zero is reported as having no coupling/significance but here the value is due to compensating ddH and d-dTS terms. In our view, this work forms the foundation of additional studies to better understand the coupling energetic terms, beyond ddGint.

In some instances, the text/figure legends are a bit unclear or contain some typos; but this part can easily be handled in a revision. The discussion is well written and embeds the main findings in the (still rather limited) literature on thermodynamic analyses of lipid binding of membrane proteins.

Major points

- 1. The authors may have clarified the following point in a previous paper; but at least in this paper, it is unclear to me how they purified MsbA without lipid A. The reason I am asking is that in our experience, if one purifies MsbA expressed from E. coli with standard detergents (e.g. beta-DDM) one will find a perfect density for Lipid A when determining an inward-facing structure by cryo-EM. According to the Methods, MsbA is purified initially in DDM, and rebuffed to C10E5 during size exclusion chromatography. When looking at Fig. 2b, the authors state (or assume?) that if no lipid A is added, MsbA has 0 % lipid A bound.*

We have previously reported details of MsbA sample prep and optimization [6]. The revised manuscript makes note of this previous work and refers the reader to the publication. Yes, we see no appreciable signal for lipid A bound to MsbA (see Fig 2b).

We also note that samples of MsbA prepared using DDM is highly heterogenous, contaminated by a battery of small molecules (that we suspect are co-purified lipids). These contaminants will inadvertently impact biochemical studies.

1. A second topic where further clarification is in my view needed is the question of the conformations that were probed and the lipid binding sites. If I get the experimental rationale correctly, most of the data were determined in the absence of nucleotides, and only a small subset (Fig. 5) of data were determined in the presence of ATP-vanadate. However, structural evidence for the cytosolic lipid A binding site has been only determined for outward-facing MsbA (PDB: 8DMM), but has thus far not been seen in any of the inward-facing cryo-EM structures of MsbA, including recent well-resolved cryo-EM structures showing excellent density for the lipid A bound to the inward-facing cavity (PDB: 7PH2). Further, there is only one lipid A molecule that can be accommodated by the inward-facing cavity, whereas (owing to the symmetry of the homodimer) two lipid A can be bound sideways to outward-facing MsbA. Now, my understanding problem is why one does see up to three lipid A molecules bound to inward-facing apo MsbA, e.g. Fig. 2b and elsewhere. Where are they expected to bind? And what is the evidence supporting these additional binding sites?

See our detailed response to reviewer 1. If we add more lipid, we see more lipid binding to MsbA, like every other membrane protein we have studied. This data clearly indicates that there are more KDL binding site(s) – deciphering the affinity of these site(s) represents a problem on the horizon.

A further question is which lipid A binding sites are present in vanadate-trapped MsbA. Here, there are two identical binding sites (at the surface of each MsbA molecule), and it is therefore surprising to see that the affinities for the first and the second binding site are so different (see e.g. Supplementary Fig. 13).

Great point. A logical explanation (described for other biochemical systems) is the two exterior LPS binding sites display negative cooperativity i.e., binding at one site weakens the affinity at the other site.

Finally, what is the evidence that in vanadate-trapped MsbA, all molecules have closed NBDs and thus assume the outward-facing conformation? It is not uncommon that vanadate trapping leads to NBD closure only in a subfraction of all transporters (hence not in 100 % of them).

Yes, the native mass spectrum shows no appreciable signal for MsbA not trapped with vanadate/ADP. In our previous cryoEM study [6], using the vanadate-trapped transporter, we did not observe particles with NBDs dissociated in space. Regarding samples from other labs, a native mass spectrum could shed light into the population of untrapped protein – however, most studies use SDS-PAGE for quality control of their purified samples. This technology is not sufficient to address underlying biochemical issues.

We do have a new report in preparation describing a new discovery regarding trapping efficiency of MsbA.

1. The key parameter that is underlying the entire thermodynamic analysis of wt and mutant MsbA is the dissociation/association constant, which are used to calculate free Gibb's energy and, via van't Hoff analysis, enthalpy. Entropy is not determined directly, but in fact indirectly from these two numbers both depending on the measurement quality of dissociation/association constant. Now, when looking at the fitted curves as shown in Figure 2b (and in the supplement), determination of the dissociation constant for KDL1 (blue curves) look reasonable and the determined KDs are within the range of measured points. However, for KDL2 (red) and even more so KDL3 (yellow), the determined KD values (Supplementary Table 5), the measured KD values are typically higher than highest KDL conc used in the assay (1.5 μ M). For this reason, and despite the fact that error bars of the fits look reasonably small, I still have doubts about the reliability of these KD values for KDL2 and KDL3.

Hence, the surprisingly strong changes of enthalpy/entropy values for different mutants/temperatures may have their origin in incorrectly determined KD values.

The increase in binding affinity of subsequent lipid binding events is consistent with many reports from our group [1, 2, 4, 6-9] and that of Prof. Robinson [10, 11] on this topic. As noted above, we indeed observe linear van't Hoff plots with positive and negative slopes as well as non-linear curves that are convex or concave. The MsbA protein (wt or mutant), trapped or not, all display unique temperature-dependent responses. If the reviewer suggestion that the Kd values are incorrectly or randomly determined, then none of the binding data should follow thermodynamic van't Hoff equations. This is simply not the case - the error bars and fits are reasonable. Backing up even further, looking the raw native mass spectra (see supplemental figure 1-3 and 10-11) one can see different temperature-dependence of lipid binding.

Minor points

1. Lines 116-131: this section reads as an extended introduction/aims, and does not contain any results.

This section has been moved to introduction.

1. Lines 137-139: suggested to check whether these interactions are also present in recently determined cryo-EM structures determined at fairly high resolution (PDB: 7PH2)

The interactions of MsbA and LPS (bound at the interior site) are comparable for PDB 7PH2 and 6BPL.

1. Lines 144-146: suggested to elude in more detail on the fitting procedure here, as the KD values determined in this way are the foundation of all quantitative assessments.

Details of data analysis and the fitting procedure are provided in methods.

1. Figure legend, Fig. 2: Technically, MsbA was solubilized and purified in DDM and detergent exchange was done on SEC to C10E5.

Corrected.

1. Figure legend, Fig. 4: description in a) on deconvoluted mass spec data is incorrect. Letter below needs to be adjusted accordingly.

Corrected.

1. Figure legend, Fig. 5: suggested to mention in Figure legend title that here we look at ADP-vanadate trapped MsbA.

Corrected.

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