JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
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Determining Membrane Protein–Lipid Binding Thermodynamics Using Native Mass Spectrometry

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Supporting Information

ABSTRACT: Membrane proteins are embedded in the biological membrane where the chemically diverse lipid environment can modulate their structure and function. However, the thermodynamics governing the molecular recognition and interaction of lipids with membrane proteins is poorly understood. Here, we report a method using native mass spectrometry (MS), to determine thermodynamics of individual ligand binding events to proteins. Unlike conventional methods, native MS can resolve individual ligand binding events and, coupled with an apparatus to control the temperature, determine binding thermodynamic parameters, such as for protein–lipid interactions. We validated our approach using three soluble protein–ligand systems (maltose binding protein, lysozyme, and nitrogen regulatory protein) and obtained similar results to those using isothermal titration calorimetry and surface plasmon resonance. We also determined for the first time the thermodynamics of individual lipid binding to the ammonia channel (AmtB), an integral membrane protein from *Escherichia coli*. Remarkably, we observed distinct thermodynamic signatures for the binding of different lipids and entropy–enthalpy compensation for binding lipids of variable chain length. Additionally, using a mutant form of AmtB that abolishes a specific phosphatidylglycerol (PG) binding site, we observed distinct changes in the thermodynamic signatures for binding PG, implying these signatures can identify key residues involved in specific lipid binding and potentially differentiate between specific lipid binding sites.

Understanding the molecular forces that drive noncovalent protein–ligand interactions is essential to the life sciences. The strength of these interactions can be described in terms of the change in Gibbs free energy upon ligand binding (ΔG), which partitions into contributions from enthalpy (ΔH) and entropy ($-T\Delta S$).[1] The binding entropy provides insight into solvent and conformational ordering effects, whereas the enthalpy describes binding modes which may involve hydrogen bonding, electrostatic interactions, and van der Waals forces.[2,3] Thus, binding thermodynamics provides a quantitative description of the binding energetics and as a result used extensively in selecting and optimizing potential drug candidates.[4,5]

Over the past two decades, native and other MS approaches have emerged as powerful techniques to study protein–ligand interactions.[6−11] As a rapid and sensitive technique, native MS is well suited for investigating protein–ligand interactions because it can, in general, preserve noncovalent interactions in the gas-phase and resolve individual binding events.[10−12] In contrast, non-native MS methods have been developed (for review[6,9,13]); however, they rely on chemical perturbation of proteins prior to analysis and suffer from the same limitation as other traditional techniques for thermodynamic analysis, such as isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR). That is, they report on an ensemble of apo and ligand bound states of the protein, whereas native MS can directly identify proteins that are free or bound to ligand(s). Moreover, current methods dealing with thermodynamic analysis have other drawbacks such as requiring immobilization, large amounts of sample and the complication in determining binding stoichiometry. These limitations were inspiration for developing this method.

Herein we report a method to determine the thermodynamics of protein–ligand interactions using native MS. We first describe our setup to control temperature online and then benchmark the method using three soluble protein–ligand systems. We then apply this method to a particularly challenging problem, membrane protein–lipid interactions, and elucidate for the first time the thermodynamics for individual lipid binding events to a membrane protein.

To determine binding thermodynamics using native MS through van't Hoff analysis,[14] we constructed an apparatus to alter and control the temperature of the analyte solution (T_{sample}) and the air surrounding the mass spectrometer source chamber (T_{air}) (Figure 1A and Figure S1) that was inspired by devices developed by others.[15,16] In our apparatus, the equilibrium of T_{sample} is reached within ~40 s after moving the nano-electrospray ionization (nESI) stage into the source chamber with the temperature holding within ±0.3 °C (Figure S1B). A calibration curve was generated for T_{sample} as a function of T_{air} (Figure S1C), which enabled us to set a desired T_{sample}

Received: February 17, 2016
Published: March 25, 2016

DOI: 10.1021/jacs.6b01771
J. Am. Chem. Soc. 2016, 138, 4346–4349

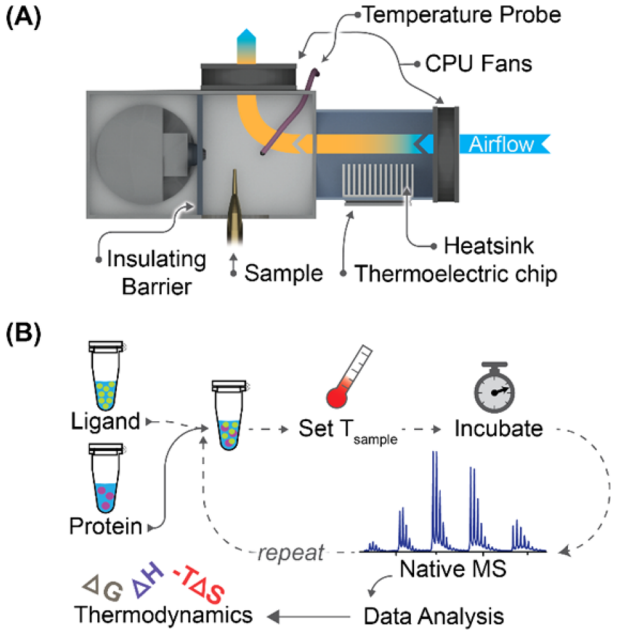

Figure 1. An overview of the method to obtain binding thermodynamics using native MS. (A) Schematic of the apparatus to control sample temperature (T_{sample}) within the source chamber. Shown is the heatsink and thermoelectric chip along with the direction of airflow (yellow and blue arrows) through the use of two central processing unit (CPU) fans, and the temperature probe (T-type thermocouple) to monitor T_{air} to get a desired T_{sample}. (B) The method begins with mixing protein with a given ligand concentration followed by incubation and acquisition of mass spectra at a desired T_{sample}. This procedure is repeated for either a different ligand concentration or temperature. After a series of mass spectra are recorded, the spectra are deconvoluted to obtain the mole fraction of free and bound states and then globally fit to a binding model. The resulting equilibrium association constants are used to determine binding thermodynamics through van't Hoff analysis.

while monitoring T_{air}. This avoids potential cross contamination caused by reinsertion of the thermocouple probe into the next sample. Based on this apparatus, we could easily and reliably place a sample on the instrument, slide the nESI stage into the chamber, adjust T_{sample} to a desired temperature, incubate the sample online for a given time to reach binding equilibrium, and record a native mass spectrum (Figure 1B).

To benchmark our method, we selected three soluble protein−ligand systems. The first one was the nitrogen regulatory protein (GlnK) from *Escherichia coli*, a trimeric protein that binds up to three adenosine diphosphate (ADP) molecules.[17] The other two were maltose binding protein (MBP) from *Escherichia coli* binding either maltose or maltotriose and hen egg white lysozyme binding *N*,*N*′,*N*″-triacetyl-chitotriose (NAG3). The thermodynamic parameters, ΔH, $-T\Delta S$, and ΔG, determined using our native MS method, and including those from other reports,[16−20] yielded similar equilibrium disassociation constant (K_D) and ΔG values to those obtained using ITC and SPR (Table 1, Table S1−S3, and Figure S2−S3). However, the contributions from enthalpy and entropy exhibit some ambiguity between the three biophysical techniques that could likely be due to the diverse experimental conditions. More specifically, in our ITC studies we used 20 to 100 times higher protein concentrations compared to our native MS method which is on the order of 0.1−1 μM and no more than the value of K_D. In fact, we observed heavy precipitation of GlnK in ITC experiments after a few titrations of ADP, rendering the utility of this technique to obtain useful thermodynamic parameters. Taken together, these results demonstrate that our method can obtain binding thermodynamic values comparable to other established biophysical techniques with significantly less material.

To address a challenging and fundamental biological problem, we applied this method to investigate the thermodynamics for lipids interacting with membrane proteins to understand the molecular basis for their recognition. The ammonia channel (AmtB) from *Escherichia coli* was selected as a model integral membrane protein complex, since we have previously characterized its interactions with lipids by quantifying how lipids influence the gas phase unfolding of AmtB.[21,22] However, that is not a direct measurement of binding affinity but rather how the lipids influence the stability of the protein (Figure S4). The studies began by tuning the instrument to preserve the native-like state of membrane protein complexes, which require different settings as compared to soluble proteins (Figure S5). Subsequently, mass spectra series were recorded at different temperatures for AmtB in the tetraethylene glycol monooctyl ether (C_8E_4)[23,24] detergent titrated with cardiolipin (TOCDL, 1,1′,2,2′-tetraoleoyl-cardiolipin) or phospholipids harboring 1-palmitoyl-2-oleoyl (PO, 16:0−18:1) tails but with differing headgroups: phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylserine (PS) (Figure 2, Figures S6−S7, and Tables S4−S5). Focusing on POPA, up to five binding events were observed (Figure 2A) and the mole fraction of each species were determined from deconvoluted mass spectra in the titration series using Unidec software.[25] Applying a sequential ligand-binding model, as done for soluble protein−ligand systems, resulted in poor fits to the experimental data (Figure S8). Considering the physicochemical properties of lipids, we therefore developed a lipid-binding

Table 1. Thermodynamic Parameters for Soluble Protein−Ligand Binding by Native MS, SPR, and ITC

parameter	method	MBP-maltose	MBP-maltotriose	lysozyme-NAG3
ΔH (kJ/mol)	MS	−14.1 ± 0.89	−26.5 ± 1.30	−28.4 ± 1.43
	SPR	–	−23.9 ± 1.21	−43.2 ± 2.49
	ITC	−7.69 ± 0.22	−11.9 ± 0.70	−35.8 ± 0.65
$-T\Delta S$ (298 K, kJ/mol)	MS	−20.8 ± 0.87	−10.5 ± 1.26	−0.01 ± 1.40
	SPR	–	−13.1 ± 1.19	15.6 ± 2.45
	ITC	−25.2	−22.9	7.70
ΔG (298 K, kJ/mol)	MS	−34.9 ± 1.76	−37.0 ± 2.56	−28.4 ± 2.83
	SPR	–	−37.0 ± 2.40	−27.6 ± 4.94
	ITC	−32.9 ± 0.22	−34.8 ± 0.70	−28.1 ± 0.65

DOI: 10.1021/jacs.6b01771
J. Am. Chem. Soc. 2016, 138, 4346−4349

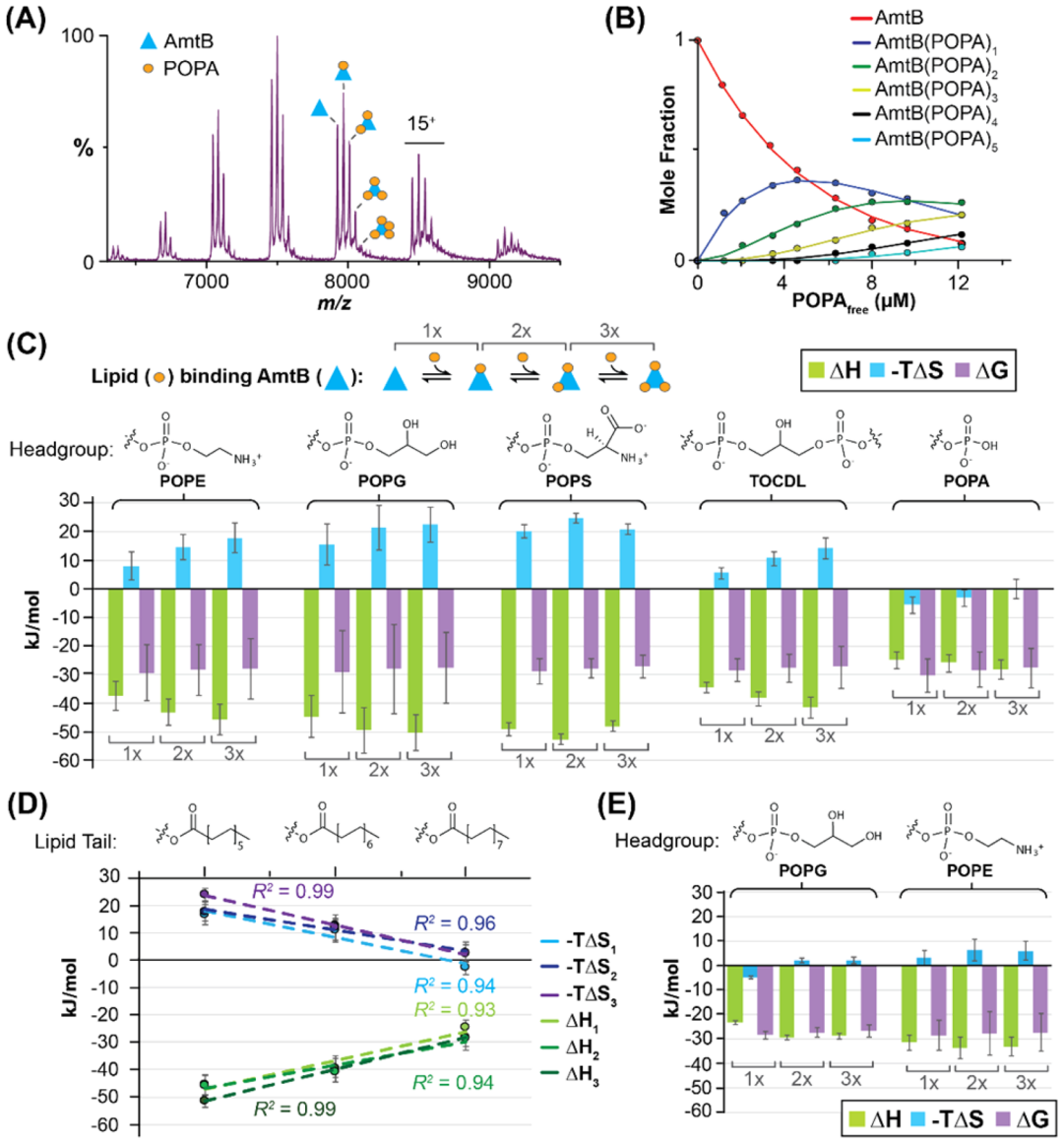

Figure 2. Native MS reveals thermodynamic signatures of individual lipid binding events to the ammonia channel (AmtB) from *Escherichia coli*, an integral membrane protein. (A) Representative mass spectrum in the series of AmtB titrated with phosphatidic acid (PA) having 1-palmitoyl-2-oleoyl (PO, 16:0−18:1) tails collected at T_{sample} of 29 °C. (B) Plots of mole fraction for AmtB and AmtB(POPA)$_{1-5}$ determined from a titration series of POPA (dots) and resulting fit (R^2 = 0.99) from a sequential lipid-binding model (solid lines). (C) Binding thermodynamics for phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), and PA containing PO tails, and 1,1′,2,2′-tetraoleoyl-cardiolipin (TOCDL) to AmtB determined through van't Hoff analysis for binding of the first, second, and third lipid (labeled as 1x−3x). Shown above are the headgroup structures ($p < 0.01$ for ΔH and $-T\Delta S$; $p > 0.9$ for ΔG, one-way anova, $n = 3$). (D) Thermodynamics of AmtB binding PG lipids with increasing acyl chain length: 12 (1,2-dilauroyl), 14 (1,2-dimyristoyl), and 16 (1,2-dipalmitoyl). Trend lines are plotted for binding the first, second, and third lipid ($p < 0.01$ for ΔH and $-T\Delta S$; $p > 0.9$ for ΔG, one-way anova, $n = 3$). (E) Thermodynamics of the AmtB double mutant (N72A/N79A) binding the first, second, and third POPG or POPE molecule. Reported in (C−E) are the mean and standard deviation ($n = 3$).

model taking into account that lipids readily self-associate. This in essence partitions the total lipid added into a fraction "*free*" to bind and a fraction that cannot bind, presumably trapped in lipid aggregates (see Supporting Information Methods for details). Applying the lipid-binding model enabled us to determine the equilibrium association constant (K_A) for each lipid-binding event (Figure 2B).

In a similar fashion to the studies for soluble protein−ligand systems, van't Hoff analyses for five different phospholipids were used to determine their binding thermodynamics (Figure 2C). Interestingly, the five phospholipids studied showed similar ΔG values but significantly different thermodynamic parameters, ΔH and $-T\Delta S$ ($p < 0.01$, one-way anova). POPS and POPG had greater enthalpy compared to the other three lipids, implying that favorable bonds are formed between their headgroups and the protein. Binding of POPG to AmtB is entropically unfavorable, which is in agreement with the crystal structure of AmtB in complex with PG,[21] where a conformational change is observed upon PG binding. A comparable entropic penalty is observed for POPS, suggesting this lipid may bind in a similar fashion. In contrast, POPA$_{1-3}$ binding is entropically and enthalpically favorable, implying a different binding pathway, driven by hydrophobic and van der Waals interactions. Interestingly, an entropic penalty is observed with each additional lipid-binding event, possibly due to structuring the membrane protein (reducing disorder). In addition, for

DOI: 10.1021/jacs.6b01771
J. Am. Chem. Soc. 2016, 138, 4346−4349

each replicate we observe a trend in increasing K_D values for each sequential lipid-binding event (Table S4) that is in line with negative cooperativity, which might be driven by the entropic penalty. Remarkably, these results demonstrate that membrane protein−lipid interactions exhibit different thermodynamic signatures, which are now accessible using this native MS approach.

Next, experiments were conducted to address two fundamental questions[26] regarding lipids: (i) what is the effect of acyl chain length on binding thermodynamics, and (ii) are the binding thermodynamics representative of specific binding modes for different lipids? To address the first question, native MS experiments using PG with varying tail lengths were performed (Figure 2D). Intriguingly, the results revealed an enthalpy−entropy compensation, with the thermodynamic signature of binding becoming more hydrophobic in nature with increasing chain length that is consistent with the physiochemical properties of lipids.[27] The second question was addressed by conducting similar experiments for POPG binding to the double mutant of AmtB ($AmtB^{N72A/N79A}$), engineered to remove a PG-binding site mediated by two asparagine residues.[15] The thermodynamic signature for binding POPG to the AmtB mutant was significantly different ($p < 0.01$, one-way anova) compared to wild-type, exhibiting favorable entropy and reduced enthalpy, which is in accord with reduction in bonds formed as a result of the mutations introduced into the protein (Figure 2E, Tables S6−S7). Moreover, in the AmtB mutant, POPG can be binding either to the same site but a different binding mode or to distinct, different sites. In contrast, thermodynamic signatures undergo subtle changes for POPE binding to the mutant and wild-type AmtB ($p > 0.1$, one-way anova). In short, the thermodynamic signatures for lipid binding become more hydrophobic in nature for longer-chain lipids and these signatures can be used to understand the thermodynamic contributions of residues in binding specific lipids.

In summary, this native MS approach proves powerful for determining binding thermodynamics, with key advantages over other methods such as low sample requirements, resolution of multiple binding equilibria, and amenability to high throughput applications. More specifically, this method enables detailed thermodynamic analysis of individual lipid binding events to membrane proteins and is now set to address key questions in membrane protein biology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b01771.

Methods, supporting figures and tables (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Magnus Höök (Texas A&M Health Science Center), Dr. Eamonn Reading (Kings College, London), and Dr. David Clemmer (Indiana University) for useful discussions. This work was supported by new faculty startup funds from the Texas A&M Health Science Center.

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DOI: 10.1021/jacs.6b01771
J. Am. Chem. Soc. 2016, 138, 4346−4349