



Characterization of Membrane Protein Interactions with Isothermal Titration Calorimetry and Differential Scanning Calorimetry



Application Note

Biological membranes are dynamic assemblies containing lipids and proteins. Membranes serve as selective permeability barriers, and contain transport and translocation systems that regulate the composition of the intracellular medium. Membranes also contain specific receptors for cell signaling. Organelles in eukaryotic cells (e.g. mitochondria) contain internal membranes, and membrane composition varies from organism to organism and from cell to cell.

Cell membranes are made up of lipid bilayers (Figure 1). The outer membrane surface consists of ionic and polar head groups of lipids, which interact with the aqueous solution on both sides of the membrane. In the fluid mosaic model, the inner portion of membranes is composed of hydrocarbon lipid chains. The lipid chains are parallel to one another, and the hydrophobic nonpolar tails contact each other in the middle of the lipid bilayer creating a greasy barrier. This barrier is impermeable to water-soluble molecules but can allow small, non-polar molecules to pass through. (Refer to References 1, 2 and 3 for review.)

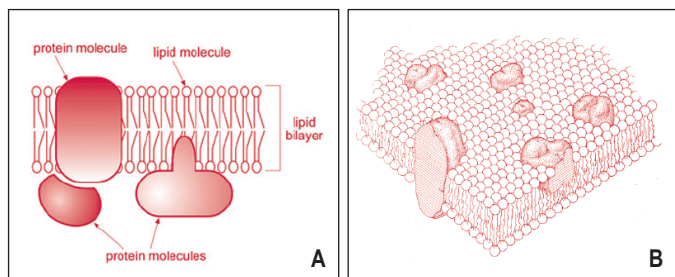


FIGURE 1. Two dimensional (A) and three dimensional (B) depictions of biological membranes. (Figure 1B reproduced from Reference 1, with permission of American Association for the Advancement of Science.)

Although lipids provide the basic structure of biological membranes, proteins perform most of the specific functions, such as cellular transport, catalysis and cell signaling. Membrane proteins have a specific orientation in the lipid bilayer. Some are anchored to the membrane by interactions between positively-charged side chains and negatively-charged lipid head groups, while others are anchored by attachment to a hydrocarbon chain (e.g. myristoyl and palmitoyl), or a lipid like glycosylphosphatidylinositol (GPI). Peripheral membrane proteins have a shallow penetration of the membrane surface and can be released with increased ionic strength.

Transmembrane (or integral) proteins are another category. The hydrophobic region(s) pass through the membrane and interact with the hydrophobic tails of the lipid molecule, while the hydrophilic region(s) are exposed to solution at either side of the membrane. Purification of these proteins requires detergent to disrupt the lipid bilayer.

Many membrane proteins are able to laterally diffuse in the membrane. However, proteins are often immobilized and confined to specific domains of the lipid bilayer.

Human genome sequence homology calculations estimate that 30% of proteins are membrane-associated.⁴ Membrane-associated proteins are of interest to pharmaceutical and biotechnology industries, since almost 50% of the top-selling drugs are targeted to membrane receptors.⁵

It is important to understand the biological processes of cell signaling and transport associated with membrane proteins. This requires the characterization of the interactions between membrane proteins and their ligands. Membrane proteins tend to have hydrophobic regions, and purified proteins may have different binding affinities in aqueous solution compared to membrane environments. Protein-ligand interactions can be studied by *in vivo* assays or with model membrane systems, however most require labeling.

Microcalorimetry is a label-free method that is useful in the characterization of biomolecular interactions of membrane proteins. Isothermal Titration Calorimetry (ITC) directly measures the binding affinity between a protein and its binding partner, and reveals details about the molecular driving forces of biomolecular interactions. ITC can be used to study the binding of a membrane protein with its ligand or inhibitor. Differential Scanning Calorimetry (DSC) provides insights in structure and stability of proteins.

This application review describes recent studies of membrane associated proteins that have utilized ITC and DSC. The membrane proteins discussed in this application note are siderophore transporter protein FhuA, transferrin receptor, zinc transporter protein YiiP, protein kinase, multidrug resistance protein EmrE, and transhydrogenase.

Overview of Microcalorimetry

Isothermal Titration Calorimetry (ITC) is a technique for monitoring binding and is the method of choice for characterizing biomolecular interactions. When two components bind, heat is either generated or absorbed. Measurement of this heat allows accurate determination of binding constants (K_b), reaction stoichiometry (n), enthalpy (ΔH) and entropy (ΔS). In a single experiment, a complete thermodynamic profile of the molecular interaction can be determined.⁶⁻¹⁰ (See Figures 2A, 3, and 7 for examples of ITC data.)

Differential Scanning Calorimetry (DSC) measures the heat changes associated with thermal denaturation of a biomolecule. DSC is used for protein unfolding and stability studies and the thermodynamics associated with unfolding.^{6, 9, 11, 12} (See Figures 2B and 4 for examples of DSC data.)

Microcalorimetry uses native materials and there is no need for labeling, chemical modification or immobilization. In addition, calorimetry is the only method that directly measures the heat change associated with biomolecular interactions, directly resulting in thermodynamic parameters.

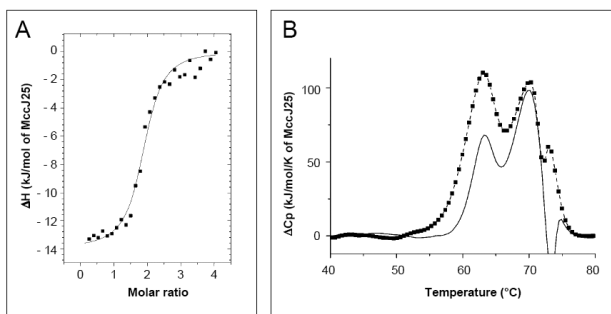


FIGURE 2. ITC and DSC results for *E. coli* FhuA studies. A: Binding isotherm from ITC titration of FhuA and MccJ25. B: Thermograms from DSC scans of FhuA (solid line) or FhuA-MccJ25 complex (dotted line). Experimental methods are described in Reference 16. (Reproduced from Reference 16, with permission of The Biochemical Society.)

Interaction of Microcin J25 and Siderophore Transporter Protein (FhuA) of *Escherichia coli*

Iron is a scarce but essential nutrient, and microorganisms like gram-negative bacteria have developed systems to acquire iron from their host environment. One process involves siderophores, which are synthesized and secreted by bacteria.¹³ Siderophores chelate ferric ions and form soluble iron complexes. Because siderophore-iron complexes are found at low concentrations in the extracellular media, a class of high-affinity siderophore receptors exists within the outer bacterial membrane. These receptors bind specific siderophore-iron complexes and promote their active transport into the periplasm.

E. coli FhuA protein (79 kDa) is a high-affinity receptor for iron chelated to the siderophore ferrichrome.¹³ Along with its primary physiological function of siderophore transport, FhuA also transports the antibiotics albomycin and rifamycin CGP4832, and is a receptor for colicin M and several phages.¹⁴ Iron/ferrichrome transport across the outer membrane requires an energized cytoplasmic membrane, so FhuA function is coupled to the electrochemical gradient of proteins via the membrane-anchored TonB-ExbB-ExbD complex.

Microcins are antimicrobial polypeptides which are secreted by enterobacteria in the gastrointestinal system of animals. These peptides are thought to play a role in regulation of intestinal flora. One example is microcin J25 (MccJ25), secreted by *E. coli* AY25.¹⁵ MccJ25 is known to inhibit transcription by targeting the β' subunit of RNA polymerase, binding to RNA polymerase secondary channel and inhibiting nucleotide triphosphate transport and binding. Other studies also show that MccJ25-resistant bacteria have mutations in *fhuA*, *tonB* or *sbmA* genes, indicating that the FhuA outer membrane protein and the TonB and SbmA inner membrane proteins are involved in MccJ25 uptake. MccJ25 antimicrobial activity was found to be limited to a few species of enterobacteria, which were affected by MccJ25 when present in nanomolar concentration. This high potency and selectivity suggests that FhuA behaves as a receptor for MccJ25, and that MccJ25 prevents interaction between phage and FhuA.

Destoumieux-Garzón et al.¹⁶ used *in vivo* and *in vitro* assays to study the interaction between MccJ25 and FhuA, and to determine the mechanism of molecular recognition of FhuA. *In vivo* assays included antibacterial activity studies of MccJ25, as well as mutant studies. MccJ25 was shown to block T5 phage infections of *E. coli* by inhibiting phage adhesion, suggesting that MccJ25 prevents interaction between phage and its receptor FhuA.

The authors used ITC and size exclusion chromatography (SEC) to demonstrate the direct interaction between MccJ25 and FhuA. ITC and SEC results were the first evidence of the direct interaction between MccJ25 and FhuA. ITC experiments showed there was a binding stoichiometry of 1.9 microcins per FhuA, with a ΔH of -14 kJ mol^{-1} ($-3.3 \text{ kcal mol}^{-1}$), and K_d of $1.2 \text{ }\mu\text{M}$. (Figure 2A).

The authors also used DSC to study the interaction between FhuA and MccJ25 (Figure 2B). Along with *in vivo* assays, these results demonstrated that FhuA is the receptor for MccJ25, and that the ligand-receptor interaction occurs in the absence of other components of the bacterial membrane. DSC and antimicrobial studies demonstrated that MccJ25 bound to the FhuA external loops. Thermolysin disrupts the MccJ25 Val11-Pro16 β -hairpin loop. A thermolysin-cleaved MccJ25 variant did not bind FhuA, and also failed to prevent phage T5 infection in *E. coli*. These results confirmed the hairpin Val11-Pro16 β -hairpin region is required for microcin recognition.

Interaction of Transferrin and Transferrin Receptor of *Neisseria meningitidis*

Meningococcal disease is a worldwide health issue that can lead to death within a few hours, and there is no universal vaccine to control and halt this disease. The proteins that are part of the transferrin receptor of *N. meningitidis* are potential candidates for inclusion in a vaccine. Meningococcae are able to acquire iron ions with a receptor that is specific for human transferrin (htf). The receptor has two types of subunits, TbpA and TbpB (transferrin-binding proteins A and B). TbpB (100 kDa) is an integral membrane protein that may serve as a channel for iron transport across the outer membrane. TbpA is an outer membrane protein that is anchored to the membrane via the lipidated N-terminal part of the protein. There are two different isotypes of TbpB, type I with a molecular weight of approximately 68 kDa, and type II with a molecular weight of 80-90 kDa.

Krell et al.¹⁷ used ITC to characterize the interaction between purified TbpA, TbpB and TbpA+B with iron-loaded (holo) and iron-free (apo) human transferrin (htf). This approach allowed the authors to look at the contributions of each receptor protein to htf binding. The authors considered ITC to be an advantage over other methods because there was no need for labeling or

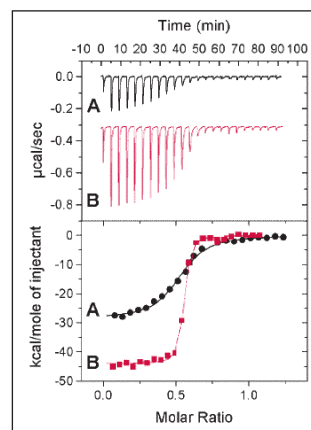


FIGURE 3. ITC results of *N. meningitidis* transferrin protein A (TbpA) binding to iron-free human transferrin (apo-htf) or iron-loaded transferrin (holo-htf).

Top: ITC titrations for binding of holo-htf (A) and apo-htf (B) to TbpA. Bottom: Binding isotherms fit to one set of sites binding model. Experimental methods are described in Reference 17. (Reproduced from Reference 17, with permission of American Society for Biochemistry and Molecular Biology.)

immobilization. The other advantage was that thermodynamic parameters gave valuable insight on the driving forces of the interaction.

ITC studies showed that purified TbpA has a greater affinity for apo-htf than for holo-htf. Binding of apo- and holo-htf to TbpA was enthalpically driven and had an unfavorable entropy change, likely due to loss of conformational freedom upon ligand binding (Figure 3). When TbpA was complexed with TbpB, TbpA preferentially bound the holo form of htf. This observation suggests that in the absence of TbpB, iron acquisition from htf is slowed down by saturation of TbpA with apo-htf. These results correlated to other binding studies. The authors proposed that TbpB shifts the ligand specificity of TbpA towards the holo form of htf, and that another TbpB may increase the local concentration of holo-htf by TbpA.

Binding studies with whole cells showed there were two independent htf binding sites on the receptor. ITC experiments confirmed that the entire receptor (TbpA + TbpB) contained a single high affinity htf-binding site on TbpA (K_d of 0.7 nM), and approximately two lower affinity binding sites on TbpB (K_d of 22 nM). The binding sites appeared to be independent and non-cooperative.

ITC results also showed that TbpB isotypes I and II bound htf with different thermodynamic mechanisms. Binding of htf to isotype I TbpB was enthalpically driven and had an unfavorable entropy change. Binding of htf to isotype II TbpB was driven by favorable entropy and enthalpy changes, and the binding affinity was less than for isotype I TbpB.

The authors also demonstrated the interaction between the N- and C-terminal domains of TbpB by ITC and circular dichroism (CD). ITC was performed at different temperatures to determine the change in heat capacity (ΔC_p) of $-3.1 \text{ kcal mol}^{-1} \text{ deg K}^{-1}$, suggesting that extensive apolar surface area is buried with domain interaction. CD spectroscopy also showed C-terminal and N-terminal interactions changes secondary structure.

Purified N-terminal and C-terminal domains of TbpB were analyzed by DSC (Figure 4, scans C and D). Each domain had two separate transitions, and the C-terminal domain was more thermostable (one of the transitions had a T_m above 80°C) compared to the N-terminal domain. This suggests that each TbpB domain has two subdomains. Stoichiometric mix of N- and C-terminal domains showed three different transitions with one of the N-terminal sub-domains stabilized when it interacts with the C-terminal domain (Figure 4, scan E). DSC data also indicated that C-terminal domain denaturation was 80-85% reversible. The observed thermostability and reversibility of the C-terminal domain of TbpB, along with its immunological properties, makes C-terminal domain of TbpB a potential vaccine antigen for meningococcal disease.

Renauld-Mongénie et al.¹⁸ continued this research and identified the holo-htf binding site on TbpB. The authors used a sequence-based prediction method to identify three ligand-binding domains, two of which were htf binding sites, all located in the N-terminal domain. Based on sequence data, they prepared a series of TbpB point mutations, and evaluated binding using Western blots, ITC and surface plasmon resonance (SPR). Site directed mutations in the htf binding domains resulted in no change to htf binding (mutant R80N), decreased binding affinity (mutant R55N), or no binding at all (mutants R55E and R243N). Circular dichroism studies of the mutant and wild-type TbpB proteins are essentially superimposable, indicating no major conformational changes of protein due to mutations.

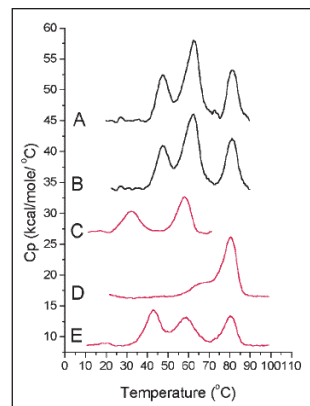


FIGURE 4. Thermograms from DSC scans of *N. meningitidis* transferrin binding protein B (TbpB). A: His6-TbpB purified from insoluble fraction. B: His6-TbpB purified from soluble fraction. C: N-terminal domain of TbpB. D: C-terminal domain of TbpB. E: Stoichiometric mix of purified N-terminal and C-terminal domains of TbpB. Experimental methods are described in reference 17. (Reproduced from Reference 17, with permission of American Society for Biochemistry and Molecular Biology.)

The overall aim of this study was to explore the possibility of TbpB as a viable vaccine candidate against meningococcal disease. One concern was that wild-type TbpB would form a complex with holo-htf after injection, causing conformational alterations to the vaccine antigen. The intention of the point mutation study was to see if the htf binding site could be modified without reducing its capacity to induce antibodies. R243N mutant had strong cross-bactericidal activities compared to wild-type, based on immunization experiments with rabbits, and mutant TbpB R243N could be a potential vaccine candidate.

Zinc Transporter (YiiP) of *Escherichia coli*

Zinc is used by many metalloproteins for catalysis, structural stability and regulation. Cells need to regulate zinc concentration within a narrow physiological range – low zinc levels inhibit cell growth, while high zinc levels are toxic. Cation diffusion facilitators (CDFs) are a family of membrane transporters that are involved in zinc homeostasis. Several mammalian and bacterial CDF proteins have been characterized, and they share a homologous hydrophobic domain with six distinct hydrophobic segments. All CDF proteins identified appear to exclusively transport metal ions. The migration of a metal ion in a transporter involves association of the metal ion with one or more binding sites along a translocation pathway.

To further understand the thermodynamics and mechanism of metal ion binding to CDF proteins, Chao and Fu¹⁹ used ITC to directly measure the heat changes associated with the binding of metal ions to the *E. coli* CDF protein, YiiP. The authors over-expressed YiiP in transformed *E. coli* cells, isolated membrane vesicles, and used detergent buffer to extract YiiP from the membranes. Protein aggregates and trace metal ions were removed by size exclusion HPLC.

ITC data showed that there were at least two sets of independent Zn^{2+} binding sites at pH 7.0, with one set of binding sites having exothermic binding heat (Zn^{2+} Site 1), and second set with endothermic binding heat (Zn^{2+} Site 2). Zn^{2+} Site 1 bound Zn^{2+} about 20-fold tighter than Zn^{2+} Site 2. For Zn^{2+} titrations of YiiP, the presence of saturating amounts of Cd^{2+} or Hg^{2+} resulted in the loss of binding to Zn^{2+} Site 1, suggesting that competitive binding occurs at Zn^{2+} Site 1 by Cd^{2+} or Hg^{2+} . At the same time, in presence of Cd^{2+} or Hg^{2+} , Zn^{2+} still bound to Zn^{2+} Site 2 on YiiP.

The authors also looked at Cd²⁺ and Hg²⁺ binding to YiiP in the absence and presence of other metals. ITC results are summarized in Table 1. ITC showed that there were at least two binding sites for Zn²⁺, Cd²⁺ and Hg²⁺. All three metals bind to a common site (Site 1) in a mutually competitive manner. Site 1 was identified as the exothermic binding site for Zn²⁺, and the higher affinity binding sites for Cd²⁺ and Hg²⁺.

Site	Titrant	Competing metal ion	K ^b (mM ⁻¹)	N	ΔH (kcal/mol)	ΔG (kcal/mol)	TΔS (kcal/mol)
Site 1 (common site)	ZnCl ₂	None	0.33	1.5	-4.5	-7.5	+3.0
		CdCl ₂	Not observed	→			
		HgCl ₂	Not observed	→			
	CdCl ₂	None	8.7	1.2	-6.5	-9.4	+3.0
		HgCl ₂	Not observed	→			
		ZnCl ₂	Not interpretable	→			
	HgCl ₂	None	960	1.0	-22.5	-12.2	-10.3
		CdCl ₂	Not observed	→			
		ZnCl ₂	Not interpretable	→			
Site 2	ZnCl ₂	None	6.3 × 10 ⁻³	0.84	12.2	-5.2	+17.7
		CdCl ₂	Not interpretable	→			
		HgCl ₂	Not interpretable	→			
	CdCl ₂	None	0.30	0.84	-6.1	-7.4	+1.3
		HgCl ₂	0.50	0.77	-7.6	-7.8	+0.12
		ZnCl ₂	Not interpretable	→			
	HgCl ₂	None	1.8	1.3	-4.1	-8.5	+4.4
		CdCl ₂	2.7	1.5	-16.3	-8.7	-7.6
		ZnCl ₂	Not interpretable	→			

TABLE 1. Summary of thermodynamic parameters for metal binding to *E. coli* YiiP. Results were from ITC titrations, and experimental methods are described in Reference 19. (Table adapted from Reference 19, with permission of American Society for Biochemistry and Molecular Biology.)

ITC can also be used to study the linkage between ligand binding and protons.²⁰⁻²² When a binding reaction is performed at the same pH with different buffers, the apparent binding enthalpy (ΔH_{app}) is the sum of binding enthalpy (ΔH_{bind}) and the heat of ionization for the buffer (ΔH_{ion}):

$$\Delta H_{app} = \Delta H_{bind} + n\Delta H_{ion} \quad (1)$$

where n is the number of protons absorbed or released. Chao and Fu¹⁹ measured the ΔH_{app} associated with Cd²⁺ binding to three different buffers at pH 7.0 (Figure 5). The slope corresponded to the release of 1.23 protons for each Cd²⁺ binding to Site 1, consistent with a coupled deprotonation effect upon binding of Cd²⁺.

To confirm the involvement of histidine residues in binding, ITC experiments were performed using YiiP treated with diethyl pyrocarbonate (DEPC). This treatment modifies histidine residues, and resulted in inhibition of Zn²⁺ binding to the common binding site, indicating that binding-deprotonation coupling involved histidine residue(s).

Wei and Fu²³ used ITC to investigate the metal binding to a highly-conserved aspartate in *E. coli* YiiP. ITC provided direct evidence that Zn²⁺ and Cd²⁺ selectively bound to Asp-157, and this amino acid was not a coordination residue for Fe²⁺ and Hg²⁺ binding.

Protein Kinases

Protein kinases C (PKC) are part of a family of serine/threonine kinases that are involved in a broad range of cellular processes. All PKCs have an amino-terminal regulatory domain and a

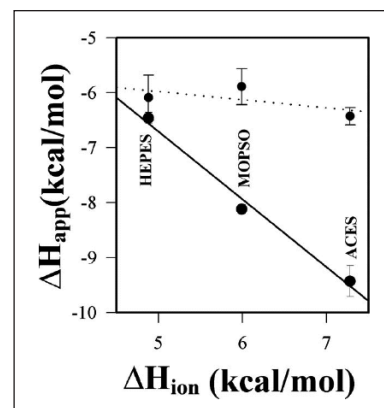


FIGURE 5. Coupling of Cd²⁺ binding to deprotonation of *E. coli* YiiP. ΔH_{app} for Cd²⁺ binding to Site 1 and Site 2 of YiiP at indicated buffer, plotted versus ΔH_{ion} for buffer. Solid line is fit of Site 1 ΔH_{app}, and dotted line is fit of Site 2 ΔH_{app} to ΔH_{ion}. Experimental parameters are described in Reference 19. (Reprinted from Reference 19, with permission of American Society for Biochemistry and Molecular Biology.)

carboxyl-terminal catalytic domain. PKCs are divided into three classes based on structural differences in the regulatory domain: conventional, novel and atypical. Conventional and novel PKCs have two types of membrane-targeting domains in the regulator region (tandem repeat of C1 domains, and a C2 domain). The C1 domain is the interaction site for *sn*-1,2-diacylglycerol (DAG) and phorbol ester. The C2 domain is involved in Ca²⁺-dependent membrane binding. Novel PKCs have a Ca²⁺-independent C2 domain.

Ananthanarayanan et al.²⁴ studied the C1 domains of two conventional PKCs isoforms for affinity of DAG and phorbol ester. The goal of this study was to quantitate binding affinities, and establish any correlation between intrinsic DAG affinity to C1 domains and their contribution to DAG-induced membrane targeting and activation of PKC. The authors cloned C1A and C1B domains from rat PKCα and PKCγ, and over-expressed them in *E. coli*. C1B domain was expressed as a soluble protein, while C1A domain was expressed in inclusion bodies and had to be solubilized in urea and refolded.

ITC and SPR studies demonstrated distinct ligand affinities for C1A and C1B domains of PKCα binding to DAG and phorbol ester. The C1A domain had high affinity for DAG and the C1B domain had high affinity for phorbol ester, demonstrating the role of C1A domain for DAG-induced membrane binding. However, both C1 domains of PKCγ had comparable phorbol ester affinities, as well as DAG affinities.

These binding studies, in conjunction with mutational studies, *in vitro* activity assays and monolayer penetration analysis, confirm that PKCα and PKCγ have distinct membrane binding and activation mechanisms. The authors also determined that C1A and C1B domains are actively involved in DAG-induced membrane binding and activation of PKCγ. Further work is needed to correlate DAG binding to biological function of PKC.

Stahelin et al.²⁵ studied DAG and phorbol ester binding on the C1A and C1B domains of two novel protein kinases PKCδ and PKCε. ITC and SPR results showed that the C1A domain of PKCδ has much higher DAG affinity than C1B domain (similar to PKCα), while the C1A and C1B domains of PKCε have comparably high binding affinities for both DAG and phorbol ester. The authors used ITC, SPR and other *in vitro* assays to demonstrate the role of C1A and C1B domains in DAG-induced membrane binding and activation of PKCε, and the different mechanisms of PKCε and PKCδ.

Multidrug Resistance Protein (EmrE) of *Escherichia coli*

Multidrug transporters are able to recognize a wide variety of toxic compounds. One multidrug transporter, ethidium multidrug resistance protein E (EmrE) is an integral membrane protein which spans the inner membrane of *E. coli* (Figure 6). EmrE is responsible for resistance to a variety of lipophilic cations, such as intercalating dyes and quaternary ammonium compounds (QACs). EmrA is a 12 kDa protein of four transmembrane helices. EmrE uses the energy of the proton gradient across the inner membrane of *E. coli* to transport two protons across the membrane into the cytoplasm, while transporting the drug from cytoplasm into the periplasm. It was previously suggested that EmrE subunits need to be oligomerized for transport to occur.

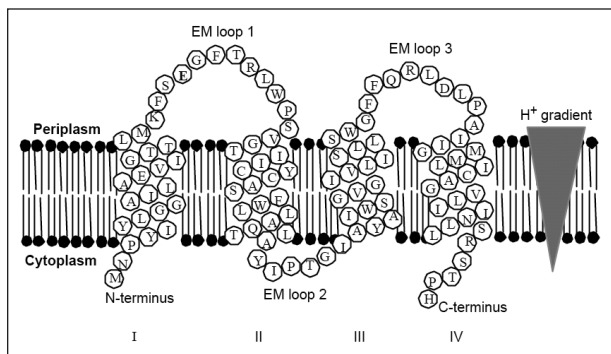


FIGURE 6. Schematic of *E. coli* EmrE in membrane. Extra-membrane (EM) loops and N- and C-termini are noted. Proton gradient is also shown. (Reproduced from Reference 26, with permission of The Biophysical Society.)

Sikora and Turner²⁶ used ITC to study the affinity of different lipophilic cations to EmrA in different membrane mimetics. The authors purified EmrE from *E. coli* membranes using organic solvent extraction and hydrophobic chromatography. Prior to ITC, purified protein was solubilized in buffer containing sodium dodecyl sulfate (SDS), n-dodecyl- β -D-maltoside (DM) or small unilamellar vesicles (SUVs). The five lipophilic drugs were prepared in matched detergent or SUV. Representative ITC results are shown in Figure 7. The K_d values were in the micromolar range, and in the SUV and DM environment, ethidium (Et) and proflavin (Pro) demonstrated tighter binding to EmrE than tetraphenylphosphonium (TPP) and methyl viologen (MV). In the SDS environment, Pro, TPP and MV bound to EmrE with similar affinities. Cetylpyridium (CTPC) binding to EmrE was not detected in any membrane mimetic.

The authors also used ITC to see if there was any detectable binding of the drugs to the three membrane mimetics. This binding was much weaker (in mM range) compared to drug-protein binding. The one exception was binding of CTPC to SUV and SDS, and this had a K_d in the high nanomolar range.

The authors made several conclusions from the ITC data. The SUV and DM membrane environments did not seem to alter the binding of ligands to EmrE, with the exception of TPP binding. TPP binding to EmrE was not observable in SUV by ITC, but could be measured in DM. However, TPP bound to each of the membrane mimetics. Phenyl rings on TPP can result in steric hindrance, so it is less accessible to EmrE binding site than other ligands. Circular dichroism and fluorescence studies of Emr in SDS, SUV and DM show that the protein has similar structure in each of these environments, and EmrE has a more open conformation in SDS than DM and SUVs.²⁷ The more open conformation in SDS could make the drug binding site more available.

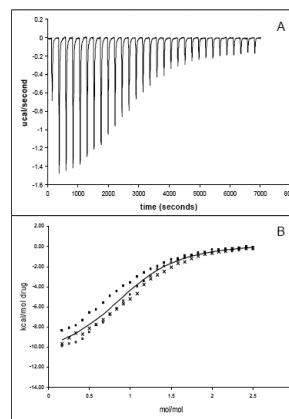


FIGURE 7. ITC titration of *E. coli* EmrE with ethidium.

A: ITC data of titration of ethidium (in SUVs) into solution of EmrE (in SUVs). B: Binding isotherms of three separate ITC titrations (x, ■, □), solid line is least squares fit to three titrations. Experimental methods are described in Reference 26. (Reproduced from Reference 26, with permission of The Biophysical Society.)

ITC could not detect CTPC binding to EmrE, but was able to detect CTPC binding to the three membrane mimetics. CTPC has an acyl chain that could interact more favorably with lipids, and it is possible that partitioning out of the lipid to the EmrE binding site may be energetically unfavorable for CTPC.

The stoichiometry of EmrE binding to each drug was approximately one, suggesting that EmrE oligomerization is not necessary for binding to drug. However, the authors stated that protein oligomerization may still be necessary for drug transport across the membrane.

The suppressor of a GroEL mutation protein E (SugE) is another multidrug resistance protein found in *E. coli*. ITC was used to study binding of QACs to purified SugE in different membrane mimetics, and results to those found with drug binding to EmrE.²⁸

Interaction of Nucleotides and Transhydrogenase of *Escherichia coli*

Transhydrogenase is located in the cytoplasmic membrane of bacteria and the inner membrane of mitochondria. This enzyme couples the redox reaction between nicotinamide nucleotides to the transport of protons across the cell membrane:



where “in” is the cytoplasm and “out” is the periplasm of bacteria (or mitochondrial matrix and cytoplasm respectively, in mammalian cells). Transhydrogenase provides NADPH for biosynthesis as a protective mechanism against oxidation. The enzyme has three components. The dI component binds NAD⁺/NADH, and the dIII component binds NADP⁺/NADPH. These two components are exposed to the bacterial cytosol (or the mitochondrial matrix in animals). The dII component spans the membrane, and is involved in proton transport. Bizouarn et al.²⁹ used intact *E. coli* transhydrogenase in ITC studies to measure binding affinities for nucleotide substrates and products (NAD⁺, NADH, NADP⁺ and NADPH).

E. coli transhydrogenase gene was cloned, protein was over-expressed, purified and prepared in a detergent solution. Binding affinities from ITC are shown in Table 2. K_d for NADH and NAD⁺ binding to intact transhydrogenase was comparable to

Nucleotide in titration	Pre-Bound nucleotide	K _d (μM)	ΔH (kcal/mol)	TΔS (kcal/mol)
NADPH	None	0.87	-20.1	-12.4
NADPH	NADH	1.6	-23.7	-15.8
NADP ⁺	None	16.1	-25.8	-19.3
NADP ⁺	NAD ⁺	214	-21.0	-16.5
NADH	None	50	-14.3	-8.9
NADH	NADPH	>500	Not determined	
NAD ⁺	None	100 to 500	-1 to -2.4	
NADPH at pH 6.5	None	0.68	-19.4	-11.0

TABLE 2. ITC results of nucleotide binding to *E. coli* transhydrogenase. Experimental methods are described in Reference 29. (Reproduced from Reference 29, with permission from Elsevier.)

previously reported binding studies to isolated dI component, but the K_d values of binding of NADP⁺ and NADPH by ITC indicated tighter binding than was previously measured for isolated dIII component.

The authors also studied nucleotide binding during the formation of “dead-end” ternary complexes on the intact transhydrogenase (NADH-E-NADPH and NAD⁺-E-NADP⁺). ITC titration with NADPH in the presence of pre-bound NADH, or with NADH in the presence of pre-bound NADPH, or with NADP⁺ in the presence of pre-bound NAD⁺ all led to a decrease in affinity relative to absence of “pre-bound” nucleotides. The titration of NAD⁺ in presence of NADP⁺ was not carried out since binding of NAD⁺ had low affinity and was difficult to analyze.

These data suggest that when both nucleotides are in the same redox state, there is negative cooperativity between the NAD(H) and the NADP(H) binding sites. This would discourage formation of these catalytically “dead-end” complexes.

The authors described a model of energy transduction between the proton-translocation pathway at dII and the hydride-transfer site at dI/dIII interface. ITC data provided insight into this mechanism, and led to a further understanding of how nucleotide binding energy might be important in linking redox reaction to proton translocation.

Summary

This application note reviewed several studies that used ITC and DSC to characterize the biomolecular interactions involving membrane-associated proteins, such as enzymes, transporters and receptors. ITC and DSC provided information on binding affinity, thermodynamics and stability of the membrane proteins, providing data about the molecular mechanisms that could not be directly measured by other methods, and correlated these data with other biophysical techniques. These results are used to propose binding and transport models for the system under investigation. Using microcalorimetry in the study of proteins associated with membrane structures brings an added dimension to the understanding of the critical structure activity relationships required for complex biomolecular activity.

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