

ITC V –Antibiotic, antifungal, antibacterial and antimicrobial studies

Adao R., Seixas R., Gomes P., Pessoa J. C. and Bastos M. (2008) Membrane structure and interactions of a short Lycotoxin I analogue. *J Pept. Sci* **14**, 528-534.

Abstract: Lycotoxin I and Lycotoxin II are natural anti-microbial peptides that were identified in the venom of the Wolf Spider *Lycosa carolinensis*. These peptides were found to be potent growth inhibitors for bacteria (*Escherichia coli*) and yeast (*Candida glabrata*) at micromolar concentrations. Recently, shortened analogues of LycoI and LycoII have been reported to have decreased haemolytic effects. A shorter Lyco-I analogue studied, LycoI 1-15 (H-IWLTALKFLGKHAAK-NH₂), was active only above 10 microM, but was also the least haemolytic. On the basis of these findings, we became interested in obtaining a deeper insight into the membrane activity of LycoI 1-15, as this peptide may represent the first major step for the future development of selective, i.e. non-haemolytic, Lycotoxin-based antibiotics. The interaction of this peptide with liposomes of different composition was studied by microcalorimetry [differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC)] and CD. The results obtained from the calorimetric and spectroscopic techniques were jointly discussed in an attempt to further understand the interaction of this peptide with model membranes

Alguel Y., Meng C., Teran W., Krell T., Ramos J. L., Gallegos M. T. and Zhang X. (2007) Crystal structures of multidrug binding protein TtgR in complex with antibiotics and plant antimicrobials. *J Mol Biol* **369**, 829-840.

Abstract: Antibiotic resistance is a widely spread phenomenon. One major mechanism that underlies antibiotic resistance in bacteria is the active extrusion of toxic compounds through the membrane-bound efflux pumps that are often regulated at the transcriptional level. TtgR represses the transcription of TtgABC, a key efflux pump in *Pseudomonas putida*, which is highly resistant to antibiotics, solvents and toxic plant secondary products. Previously we showed that TtgR is the only reported repressor that binds to different classes of natural antimicrobial compounds, which are also extruded by the efflux pump. We report here five high-resolution crystal structures of TtgR from the solvent-tolerant strain DOT-T1E, including TtgR in complex with common antibiotics and plant secondary metabolites. We provide structural basis for the unique ligand binding properties of TtgR. We identify two distinct and overlapping ligand binding sites; the first one is broader and consists of mainly hydrophobic residues, whereas the second one is deeper and contains more polar residues including Arg176, a unique residue present in the DOT-T1E strain but not in other *Pseudomonas* strains. Phloretin, a plant antimicrobial, can bind to both binding sites with distinct binding affinities and stoichiometries. Results on ligand binding properties of native and mutant TtgR proteins using isothermal titration calorimetry confirm the binding affinities and stoichiometries, and suggest a potential positive cooperativity between the two binding sites. The importance of Arg176 in phloretin binding was further confirmed by the reduced ability of phloretin in releasing the mutant TtgR from bound DNA compared to the native protein. The results presented here highlight the importance and versatility of regulatory systems in bacterial antibiotic resistance and open up new avenues for novel antimicrobial development.

Andrushchenko V. V., Aarabi M. H., Nguyen L. T., Prenner E. J. and Vogel H. J. (2008) Thermodynamics of the interactions of tryptophan-rich cathelicidin antimicrobial peptides with model and natural membranes. *Biochim Biophys Acta* **1778**, 1004-1014.

Abstract: Tritrpticin and indolicidin are short 13-residue tryptophan-rich antimicrobial peptides that hold potential as future alternatives for antibiotics. Isothermal titration calorimetry (ITC) has been applied as the main tool in this study to investigate the thermodynamics of the interaction of these two cathelicidin peptides as well as five tritrpticin analogs with large unilamellar vesicles (LUVs), representing model and natural anionic membranes. The anionic LUVs were composed of (a) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine/1-palmitoyl-2-oleoyl -sn-glycero-3-phosphoglycerol (POPE/POPG) (7:3) and (b) natural *E. coli* polar lipid extract. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was used to make model zwitterionic membranes. Binding isotherms were obtained to characterize the antimicrobial peptide binding to the LUVs, which then allowed for calculation of the thermodynamic parameters of the interaction. All peptides exhibited substantially stronger binding to anionic POPE/POPG and *E. coli* membrane systems than to the zwitterionic POPC system due to strong electrostatic attractions between the highly positively charged peptides and the negatively charged membrane surface, and results with

tripticin derivatives further revealed the effects of various amino acid substitutions on membrane binding. No significant improvement was observed upon increasing the Tritrp peptide charge from +4 to +5. Replacement of Arg residues with Lys did not substantially change peptide binding to anionic vesicles but moderately decreased the binding to zwitterionic LUVs. Pro to Ala substitutions in tripticin, allowing the peptide to adopt an alpha-helical structure, resulted in a significant increase of the binding to both anionic and zwitterionic vesicles and therefore reduced the selectivity for bacterial and mammalian membranes. In contrast, substitution of Trp with other aromatic amino acids significantly decreased the peptide's ability to bind to anionic LUVs and essentially eliminated binding to zwitterionic LUVs. The ITC results were consistent with the outcome of fluorescence spectroscopy membrane binding and perturbation studies. Overall, our work showed that a natural *E. coli* polar lipid extract as a bacterial membrane model was advantageous compared to the simpler and more widely used POPE/POPG lipid system

Arriaga P., Laynez J., Menendez M., Canada J., and Garcia-Blanco F. (1990) Thermodynamic analysis of the interaction of the antibiotic teicoplanin and its aglycone with cell-wall peptides. *Biochem J* **265**, 69-77.
Abstract: The thermodynamics of the interaction of the glycopeptidic antibiotic teicoplanin and its peptidic moiety with analogues of bacterial cell-wall peptides were studied by means of calorimetric and spectrophotometric techniques. The analysis of the thermodynamic data has allowed us to evaluate the contributions of the different peptide groups to the binding process. The nature of the primary binding forces is also discussed for each interacting group, on the basis of their enthalpic and entropic contribution and in connection with the detailed structural information available for these antibiotics from n.m.r. data. Similar analyses for the case of vancomycin and ristocetin are also reported.

Augusto L. A., Decottignies P., Synguelakis M., Nicaise M., Le Marechal P., and Chaby R. (2003) Histones: a novel class of lipopolysaccharide-binding molecules. *Biochemistry* **42**, 3929-3938.
Abstract: Unlike soluble and membrane forms of lipopolysaccharide (LPS)-binding proteins, intracellular LPS-binding molecules are poorly documented. We looked for such molecules in a murine lung epithelial cell line. Two proteins with LPS-binding activity were isolated and unambiguously identified as histones H2A.1 and H4 by mass spectrometry. Synthetic peptides representing partial structures indicated that the LPS binding site is located in the C-terminal moiety of the histones. Extending the study, we found that histones H1, H2A, H2B, H3, and H4 from calf thymus are all able to bind LPS. Bindings were specific, and affinities, determined by isothermal titration calorimetry, were (except for H4) higher than that of the LPS-binding antibiotic polymyxin B. In the presence of H2A the binding of LPS to the macrophage cell line RAW 264.7, and the LPS-induced production of TNF-alpha and nitric oxide by these cells, were markedly reduced. Histones may thus represent a new class of intracellular and extracellular LPS sensors.

Barbieri C. M. and Pilch D. S. (2006) Complete Thermodynamic Characterization of the Multiple Protonation Equilibria of the Aminoglycoside Antibiotic Paromomycin: A Calorimetric and Natural Abundance ^{15}N NMR Study. *Biophys J* **90**, 1338-1349.
Abstract: The binding of aminoglycoside antibiotics to a broad range of macromolecular targets is coupled to protonation of one or more of the amino groups that typify this class of drugs. Determining how and to what extent this linkage influences the energetics of the aminoglycoside-macromolecule binding reaction requires a detailed understanding of the thermodynamics associated with the protonation equilibria of the aminoglycoside amino groups. In recognition of this need, a calorimetric- and NMR-based approach for obtaining the requisite thermodynamic information is presented using paromomycin as the model aminoglycoside. Temperature- and pH-dependent (^{15}N) NMR studies provide pKa values for the five paromomycin amino groups, as well as the temperature dependence of these pKa values. These studies also indicate that the observed pKa values associated with the free base form of paromomycin are lower in magnitude than the corresponding values associated with the sulfate salt form of the drug. This difference in pKa is due to drug interactions with the sulfate counterions at the high drug concentrations (≥ 812 mM) used in the (^{15}N) NMR studies. ITC studies conducted at drug concentrations ≤ 45 μM reveal that the extent of paromomycin protonation linked to the binding of the drug to its pharmacologically relevant target, the 16 S rRNA A-site, is consistent with the pKa values of the free base and not the sulfate salt form of the drug. Temperature- and pH-dependent isothermal titration calorimetry studies yield exothermic enthalpy changes (ΔH) for protonation of the five paromomycin amino groups, as well as positive heat capacity changes (ΔC_p) for three of the five amino groups. Regarded as a whole, the results presented here represent an important first step toward establishing a thermodynamic database that can be used to

predict how aminoglycoside-macromolecule binding energetics will be influenced by conditions such as temperature, pH, and ionic strength. Such a predictive capability is a critical component of any drug design strategy.

Barcelo F., Capo D., and Portugal J. (2002) Thermodynamic characterization of the multivalent binding of chartreusin to DNA. *Nucleic Acids Res* **30**, 4567-4573.

Abstract: Characterization of the thermodynamics of DNA- drug interactions is a very useful part in rational drug design. Isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC) and UV melting experiments have been used to analyze the multivalent (intercalation plus minor groove) binding of the antitumor antibiotic chartreusin to DNA. Using DNA UV melting studies in the presence of the ligand and the binding enthalpy determined by ITC, we determined that the binding constant for the interaction was $3.6 \times 10^5 \text{ M}^{-1}$ at 20 degrees C, in a solution containing 18 mM Na(+). The DNA-drug interaction was enthalpy driven, with a $\Delta H(b)$ of -7.07 kcal/mol at 20 degrees C. Binding enthalpies were determined by ITC in the 20-35 degrees C range and used to calculate a binding-induced change in heat capacity (ΔC_p) of -391 cal/mol K. We have obtained a detailed thermodynamic profile for the interaction of this multivalent drug, which makes possible a dissection of ΔG_{obs} into the component free energy terms. The hydrophobic transfer of the chartreusin chromophore from the solution to the DNA intercalating site is the main contributor to the free energy of binding.

Barcelo F., Scotta C., Ortiz-Lombardia M., Mendez C., Salas J. A. and Portugal J. (2007) Entropically-driven binding of mithramycin in the minor groove of C/G-rich DNA sequences. *Nucleic Acids Res* **35**, 2215-2226.

Abstract: The antitumour antibiotic mithramycin A (MTA) is a DNA minor-groove binding ligand. It binds to C/G-rich tracts as a dimer that forms in the presence of divalent cations such as Mg(2+). Differential scanning calorimetry, UV thermal denaturation, isothermal titration calorimetry and competition dialysis were used, together with computations of the hydrophobic free energy of binding, to determine the thermodynamic profile of MTA binding to DNA. The results were compared to those obtained in parallel using the structurally related mithramycin SK (MSK). The binding of MTA to salmon testes DNA determined by UV melting studies ($K(\text{obs}) = 1.2 (+/-0.3) \times 10(5) \text{ M}(-1)$) is tighter than that of MSK ($2.9 (+/-1.0) \times 10(4) \text{ M}(-1)$) at 25 degrees C. Competition dialysis studies showed a tighter MTA binding to both salmon testes DNA (42% C + G) and *Micrococcus lysodeikticus* DNA (72% C + G). The thermodynamic analysis of binding data at 25 degrees C shows that the binding of MTA and MSK to DNA is entropically driven, dominated by the hydrophobic transfer of the antibiotics from solution to the DNA-binding site. Direct molecular recognition between MTA or MSK and DNA through hydrogen bonding and van der Waals contacts may also contribute significantly to complex formation.

Bernacchi S., Freisz S., Maechling C., Spiess B., Marquet R., Dumas P. and Ennifar E. (2007) Aminoglycoside binding to the HIV-1 RNA dimerization initiation site: thermodynamics and effect on the kissing-loop to duplex conversion. *Nucleic Acids Res* **35**, 7128-7139.

Abstract: Owing to a striking, and most likely fortuitous, structural and sequence similarity with the bacterial 16 S ribosomal A site, the RNA kissing-loop complex formed by the HIV-1 genomic RNA dimerization initiation site (DIS) specifically binds 4,5-disubstituted 2-deoxystreptamine (2-DOS) aminoglycoside antibiotics. We used chemical probing, molecular modeling, isothermal titration calorimetry (ITC) and UV melting to investigate aminoglycoside binding to the DIS loop-loop complex. We showed that apramycin, an aminoglycoside containing a bicyclic moiety, also binds the DIS, but in a different way than 4,5-disubstituted 2-DOS aminoglycosides. The determination of thermodynamic parameters for various aminoglycosides revealed the role of the different rings in the drug-RNA interaction. Surprisingly, we found that the affinity of lividomycin and neomycin for the DIS ($K(d)$ approximately 30 nM) is significantly higher than that obtained in the same experimental conditions for their natural target, the bacterial A site ($K(d)$ approximately 1.6 microM). In good agreement with their respective affinity, aminoglycoside increase the melting temperature of the loop-loop interaction and also block the conversion from kissing-loop complex to extended duplex. Taken together, our data might be useful for selecting new molecules with improved specificity and affinity toward the HIV-1 DIS RNA.

Boehr D. D., Farley A. R., Wright G. D., and Cox J. R. (2002) Analysis of the pi-pi stacking interactions between the aminoglycoside antibiotic kinase APH(3')-IIIa and its nucleotide ligands. *Chem Biol* **9**, 1209-

1217.

Abstract: A key contact in the active site of an aminoglycoside phosphotransferase enzyme (APH(3')-IIIa) is a pi-pi stacking interaction between Tyr42 and the adenine ring of bound nucleotides. We investigated the prevalence of similar Tyr-adenine contacts and found that many different protein systems employ Tyr residues in the recognition of the adenine ring. The geometry of these stacking interactions suggests that electrostatics play a role in the attraction between these aromatic systems. Kinetic and calorimetric experiments on wild-type and mutant forms of APH(3')-IIIa yielded further experimental evidence of the importance of electrostatics in the adenine binding region and suggested that the stacking interaction contributes approximately 2 kcal/mol of binding energy. This type of information concerning the forces that govern nucleotide binding in APH(3')-IIIa will facilitate inhibitor design strategies that target the nucleotide binding site of APH-type enzymes.

Boland J. S., Davidson P. M., Bruce B., and Weiss J. (2004) Cations reduce antimicrobial efficacy of lysozyme-chelator combinations. *J Food Prot* **67**, 285-294.

Abstract: Reduction of the antimicrobial efficacy of lysozyme-chelator combinations against two *Escherichia coli* O157:H7 strains on addition of mineral salts was studied. The objective of the study was to determine the effect of type and concentration of mono-, di-, and trivalent mineral salts on the antimicrobial effectiveness of lysozyme and various chelators against *E. coli* O157:H7. Seven salts (Al³⁺, Ca²⁺, Fe²⁺, Fe³⁺, K⁺, Mg²⁺, and Na⁺) at 1 to 10 mM were added to aqueous solutions of lysozyme and disodium ethylenediamine tetraacetic acid (EDTA), disodium pyrophosphate (DSPP), or pentasodium tripolyphosphate (PSTPP) at pH 6, 7, or 8 and applied to cultures of *E. coli* O157:H7 strains 932 and H1730. Inhibitory activity of lysozyme chelator combinations against both strains was completely lost after addition of > or = 1 mM Ca²⁺ and Mg²⁺ at pH 7 and 8. At pH 6, antimicrobial activity of lysozyme-EDTA against both strains was retained in the presence of calcium or magnesium cations. DSPP-lysozyme inhibited strain H1730 at pH 6 despite the presence of Mg²⁺. Concentrations above 4 mM Fe²⁺ neutralized activity of all lysozyme-chelator combinations. Reversal of inhibition by lysozyme-chelator complexes by the monovalent Na⁺ and K⁺ ions depended on *E. coli* O157:H7 strain type. Neither monovalent cation reversed inhibition of strain 932. However, Na⁺ and K⁺ reversed lysozyme-chelator inhibition of strain H1730. The addition of > or = 1 mM Fe³⁺ or Al³⁺ was effective in reversing inhibition of both strains by lysozyme and EDTA at pH 6, 7, and 8. Isothermal titration calorimetry was used to determine the amount of ion-specific competitive binding of free cations by EDTA-lysozyme combinations. A mechanistic model for the antimicrobial functionality of chelator-lysozyme combinations is proposed.

Brandenburg K., Jurgens G., Muller M., Fukuoka S., and Koch M. H. (2001) Biophysical characterization of lipopolysaccharide and lipid A inactivation by lactoferrin. *Biol Chem* **382**, 1215-1225.

Abstract: The interaction of bacterial endotoxins (LPS Re and lipid A, the 'endotoxic principle' of LPS) with the endogenous antibiotic lactoferrin (LF) was investigated using various physical techniques and biological assays. By applying Fourier-transform infrared (FTIR) spectroscopy, we find that LF binds to the phosphate group within the lipid A part and induces a rigidification of the acyl chains of LPS. The secondary structure of the protein - as monitored by the amide I band - is, however, not changed. Concomitant with the IR data, scanning calorimetric data indicate a sharpening of the acyl chain phase transition. From titration calorimetric and zeta potential data, saturation of LF binding to LPS was found to lie at a [LF]:[LPS] ratio of 1:3 to 1:5 M from the former and 1:10 M from the latter technique. X-ray scattering data indicate a change of the lipid A aggregate structure from inverted cubic to multilamellar, and with fluorescence (FRET) spectroscopy, LF is shown to intercalate by itself into phospholipid liposomes and may also block the lipopolysaccharide-binding protein (LBP)-induced intercalation of LPS. The LPS-induced cytokine production of human mononuclear cells exhibits a decrease due to LF binding, whereas the coagulation of amoebocyte lysate in the Limulus test exhibited concentration-dependent changes. Based on these results, a model for the mechanisms of endotoxin inactivation by LF is proposed.

Brandenburg K., David A., Howe J., Koch M. H., Andra J., and Garidel P. (2005) Temperature dependence of the binding of endotoxins to the polycationic peptides polymyxin B and its nonapeptide. *Biophys J* **88**, 1845-1858.

Abstract: The interaction between endotoxins - free lipid A and various lipopolysaccharide (LPS) chemotypes with different sugar chain lengths - and the polycationic peptides polymyxin B (PMB) and polymyxin nonapeptide (PMBN) has been investigated by isothermal titration calorimetry (ITC) between 20

and 50 (degrees)C. The results show a strong dependence of the titration curves on the phase state of the endotoxins. In the gel phase (< 30 (degrees)C for LPS and < 45 (degrees)C for lipid A) an endothermic reaction is observed, for which the driving force is an entropically driven endotoxin-polymyxin interaction, due to disruption of the ordered water structure and cation assembly in the lipid A backbone and adjacent molecules. In the liquid crystalline phase (> 35 (degrees)C for LPS and >47 (degrees)C for lipid A) an exothermic reaction takes place, which is mainly due to the strong electrostatic interaction of the polymyxins with the negative charges of the endotoxins, i.e., the entropic change ΔS is much lower than in the gel phase. For endotoxins with short sugar chains (lipid A, LPS Re, LPS Rc) the stoichiometry of the polymyxin binding corresponds to pure charge neutralization, for the compounds with longer sugar chains (LPS Ra, LPS S-form) this is no longer valid. This can be related to the lower susceptibility of the corresponding bacterial strains to antibiotics.

Breukink E., Ganz P., de Kruijff B., and Seelig J. (2000) Binding of Nisin Z to bilayer vesicles as determined with isothermal titration calorimetry. *Biochemistry* **39**, 10247-10254.

Abstract: Nisin Z, a 34-residue lantibiotic, is secreted by some lactic acid bacteria and exerts its antibacterial activity against various Gram-positive bacteria by permeabilizing the cell membrane. It is a cationic amphiphilic peptide with several unusual dehydro residues and thioether-bridged lanthionines. Isothermal titration calorimetry was used to provide a quantitative thermodynamic description for nisin Z adsorption to and penetration into negatively charged and neutral lipid bilayers. The binding of the cationic peptide (electric charge z approximately 3.8) to anionic membranes was found to be dominated by electrostatic forces which could be described with the Gouy-Chapman theory. For biologically relevant conditions with a membrane surface potential of -40 mV, the peptide concentration near the membrane surface increases by about 2-3 orders of magnitude compared to the bulk concentration. The binding step proper, i.e., the transition from the lipid-water interface into the membrane, is almost exclusively driven by the high surface concentration. Binding can be described by a partition equilibrium of the form $X(b) = KC(M) = KC(p,f) \exp(-z(p)\psi(0)F(0)/RT)$, where $C(M)$ is the peptide surface concentration, $C(p,f)$ the bulk concentration, and $\psi(0)$ the membrane surface potential. The intrinsic partition coefficient ($K = 1.8 M^{-1}$) is remarkably small, indicating a correspondingly small hydrophobic energy contribution to the binding process. The electrostatic model was confirmed with nisin Z mutants in which valine-32 was replaced with either lysine (V32K) or glutamate (V32E), increasing or decreasing the electric charge by 1 unit. The extent of peptide binding increased for V32K and decreased for V32E as predicted by the electrostatic theory. In contrast, electrostatic effects were almost negligible for the binding of nisin Z to neutral membranes. However, the binding isotherms were characterized by a distinctly larger intrinsic binding constant $K(0)$ of approximately 540 M^{-1} and an enhanced hydrophobic free energy of binding. The binding of nisin Z to sonicated lipid vesicles is exothermic with a ΔH degrees of ca. -9 and -3.4 kcal/mol for charged and neutral membranes, respectively.

Bringezu F., Wen S., Dante S., Hauss T., Majerowicz M. and Waring A. (2007) The insertion of the antimicrobial peptide dicynthaurin monomer in model membranes: thermodynamics and structural characterization. *Biochemistry* **46**, 5678-5686.

Abstract: This paper is focused on the thermodynamics and the structural investigation of the interaction of the antimicrobial peptide dicynthaurin monomer with model lipid membranes composed of mixtures of 1-palmitoyl-2-oleyl-glycerophosphocholine and -glycerophosphoglycerol. The thermodynamic binding parameters as obtained by isothermal titration calorimetry reveal strong binding toward the lipid model system dominated by large chemical binding constants which exceeds the electrostatic binding effects and thus suggests insertion of the amphipathic alpha-helical peptide into the hydrophobic membrane core. Circular dichroism study shows that the peptide exhibits trans-membrane alpha-helix secondary structure. Neutron diffraction measurements using partially deuterated sequences were successfully applied to determine the orientation of the peptide thus proving insertion into the hydrophobic membrane core. This insertion and the formation of higher order porelike aggregates is assumed to be the most relevant event in microbial membrane perturbation that in vivo finally leads to bacterial cell death on a fast time scale.

Buddha M. R. and Crane B. R. (2005) Structures of tryptophanyl-tRNA synthetase II from *Deinococcus radiodurans* bound to ATP and tryptophan. Insight into subunit cooperativity and domain motions linked to catalysis. *J Biol Chem* **280**, 31965-31973.

Abstract: An auxiliary tryptophanyl tRNA synthetase (drTrpRS II) that interacts with nitric-oxide synthase

in the radiation-resistant bacterium *Deinococcus radiodurans* charges tRNA with tryptophan and 4-nitrotryptophan, a specific nitration product of nitric-oxide synthase. Crystal structures of drTrpRS II, empty of ligands or bound to either Trp or ATP, reveal that drTrpRS II has an overall structure similar to standard bacterial TrpRSs but undergoes smaller amplitude motions of the helical tRNA anti-codon binding (TAB) domain on binding substrates. TAB domain loop conformations that more closely resemble those of human TrpRS than those of *Bacillus stearothermophilus* TrpRS (bsTrpRS) indicate different modes of tRNA recognition by subclasses of bacterial TrpRSs. A compact state of drTrpRS II binds ATP, from which only minimal TAB domain movement is necessary to bring nucleotide in contact with Trp. However, the signature KMSKS loop of class I synthetases does not completely engage the ATP phosphates, and the adenine ring is not well ordered in the absence of Trp. Thus, progression of the KMSKS loop to a high energy conformation that stages acyl-adenylation requires binding of both substrates. In an asymmetric drTrpRS II dimer, the closed subunit binds ATP, whereas the open subunit binds Trp. A crystallographically symmetric dimer binds no ligands. Half-site reactivity for Trp binding is confirmed by thermodynamic measurements and explained by an asymmetric shift of the dimer interface toward the occupied active site. Upon Trp binding, Asp68 propagates structural changes between subunits by switching its hydrogen bonding partner from dimer interface residue Tyr139 to active site residue Arg30. Since TrpRS IIs are resistant to inhibitors of standard TrpRSs, and pathogens contain drTrpRS II homologs, the structure of drTrpRS II provides a framework for the design of potentially useful antibiotics.

Carrasco C., Vezin H., Wilson W. D., Ren J., Chaires J. B., and Bailly C. (2001) DNA binding properties of the indolocarbazole antitumor drug NB-506. *Anticancer Drug Des* **16**, 99-107.

Abstract: Indolocarbazoles derived from the antibiotic rebeccamycin represent an important group of antitumor agents. Several indolocarbazoles are currently undergoing clinical trials. These compounds inhibit topoisomerase I to produce DNA breaks that are responsible for cell death. Unlike classical topoisomerase I poisons like camptothecin, glycosyl indolocarbazoles can form stable complexes with DNA even in the absence of topoisomerase I. At least in part, their mode of action is reminiscent of that of the anthracyclines, which also bind to nucleic acids and interfere with topoisomerase II. The lead synthetic compound in the series is the uncharged drug NB-506, which bears a glucose residue attached to the indolocarbazole chromophore substituted with two hydroxyl groups at positions 1 and 11. Here we report a detailed biophysical study aimed at characterizing the DNA binding properties of NB-506. Molecular modeling was used to compare the conformation and electronic properties of NB-506 and its analogue ED-571 bearing the two hydroxyl groups at positions 2 and 10. Surface plasmon resonance experiments, performed with DNA hairpin oligomers, indicate that NB-506 binds almost equally well to both AT and GC base pairs, and the binding affinity ($K = 10^5 \text{ M}^{-1}$) is similar to that of certain classical intercalators such as amsacrine and bisantrene. Isothermal titration calorimetry experiments show that the binding of NB-506 is enthalpy-driven ($\Delta H = -7.2 \text{ kcal/mol}$). The binding enthalpy measured for NB-506 is similar to that obtained with doxorubicin but the DNA interaction processes for the two drugs differ markedly in terms of entropy and ΔG . The free energy of NB-506 binding to DNA is considerably less favorable than that of doxorubicin. These biophysical data help us to understand further how rebeccamycin-type anticancer drugs interact with DNA.

Cederkvist F. H., Saua S. F., Karlsen V., Sakuda S., Eijnsink V. G. and Sorlie M. (2007) Thermodynamic analysis of allosamidin binding to a family 18 chitinase. *Biochemistry* **46**, 12347-12354.

Abstract: Inhibition of family 18 chitinases is emerging as a target for pest and fungal control as well as asthma and inflammatory therapy. One of the best known inhibitors for these enzymes is allosamidin, a natural product. While interactions of this compound with family 18 chitinases have been studied in much detail by X-ray crystallography and standard enzymology, details of the driving forces behind its tight binding remain unknown. We have studied the thermodynamics of allosamidin binding to chitinase B (ChiB), a family 18 chitinase from *Serratia marcescens*, using isothermal titration calorimetry. At pH 6.0, K_d is $0.16 \pm 0.04 \text{ }\mu\text{M}$, and the binding reaction is entropically driven ($\Delta S_r = 44 \text{ cal/K mol}$) with an enthalpic penalty ($\Delta H_r = 3.8 \pm 0.2 \text{ kcal/mol}$). Dissection of the entropic term shows that a favorable conformational change in the allosamidin-ChiB complex ($\Delta S_{\text{conf}} = 37 \text{ cal/K mol}$) is the main contributor to the reaction. At pH 8.5, K_d decreases to $0.03 \text{ }\mu\text{M}$ and the binding reaction is less entropically favorable ($\Delta S_r = 30 \text{ cal/K mol}$). While the solvation entropy change (ΔS_{solv}) increases from 15 cal/K mol at pH 6.0 to 46 cal/K mol at pH 8.5, ΔS_{conf} becomes small and negative (-8 cal/K mol) because of an enthalpy-entropy compensation. Analyses of proton transfer showed that at pH 6.0

binding of allosamidin requires deprotonation of the Asp142-Glu144 catalytic diad. At pH 8.5, the 142-144 diad is ionized in the native enzyme, relieving the deprotonation penalty of binding and explaining why binding becomes enthalpically favorable ($\Delta H_r = -1.2 \pm 0.2$ kcal/mol).

Chaires J. B., Satyanarayana S., Suh D., Fokt I., Przewloka T., and Priebe W. (1996) Parsing the free energy of anthracycline antibiotic binding to DNA. *Biochemistry* **35**, 2047-2053.

Abstract: The DNA binding free energy of eight anthracycline antibiotics was determined as a function of NaCl concentration. Compounds were chosen for study that differed from the parent compounds, doxorubicin or daunorubicin, at a single chemical substituent. Determination of the salt concentration dependence of the binding constant allowed us to dissect the DNA binding free energy of each compound into its component nonelectrostatic and polyelectrolyte contributions. Comparison of the nonelectrostatic free energy contribution allowed us to evaluate the net energetic contribution of specific functional groups to DNA binding. These quantitative data revealed a surprisingly large and favorable energetic contribution (2 kcal mol⁻¹) of the groove-binding daunosamine moiety and a substantial energetic penalty for alteration of its stereochemistry. The energetic cost of removal of hydroxyl groups at the C-9 and C-14 positions (which structural studies indicate may participate in hydrogen-bonding interactions with the DNA) was approximately 1 kcal mol⁻¹. Replacement of the 3'-amino group with a hydroxyl group led to a loss of 0.7 kcal mol⁻¹ in binding free energy, above and beyond the energetic penalty resulting from the removal of its positive charge from the antibiotic. The results and analysis presented here provide a rigorous and detailed description of structure-DNA affinity relationships among anthracycline antibiotics. The results are of general interest in understanding how total ligand binding free energies are partitioned among substituents and will be useful in the formulation of rules for the rational design of novel DNA binding agents.

Chaires J. B. (1998) Drug--DNA interactions. *Curr Opin Struct Biol* **8**, 314-320.

Abstract: Significant progress has been made over the past few years in studies of drug-DNA interactions. Structure-based design strategies have yielded new DNA-binding agents with clinical promise. The hairpin polyamides represent the result of a design strategy with outstanding potential. One specific molecule of this class has now been proven to inhibit the expression of a specific gene in vivo. A new bisintercalating anthracycline antibiotic binds with high affinity to DNA, and appears to overcome a specific form of multidrug resistance. Progress in fundamental studies of drug binding to DNA continues, with detailed thermodynamic studies providing new insights into the forces that drive complex formation. New tools have been developed in order to characterize both the binding mode and the sequence specificity of drug binding to DNA, tools that will enable the fundamental aspects of these biologically important reactions to be understood in more detail.

Charles I., Xi H. and Arya D. P. (2007) Sequence-specific targeting of RNA with an oligonucleotide-neomycin conjugate. *Bioconjug. Chem* **18**, 160-169.

Abstract: The synthesis of neomycin covalently attached at the C5-position of 2'-deoxyuridine is reported. The synthesis outlined allows for incorporation of an aminoglycoside (neomycin) at any given site in an oligonucleotide (ODN) where a thymidine (or uridine) is present. Incorporation of this modified base into an oligonucleotide, which is complementary to a seven-bases-long alpha-sarcin loop RNA sequence, leads to enhanced duplex hybridization. The increase in T_m for this duplex ($\Delta T_m = 6$ degrees C) suggests a favorable interaction of neomycin within the duplex groove. CD spectroscopy shows that the modified duplex adopts an A-type conformation. ITC measurements indicate the additive effects of ODN and neomycin binding to the RNA target ($K_a = 4.5 \times 10^7$ M⁻¹). The enhanced stability of the hybrid duplex from this neomycin-ODN conjugate originates primarily from the enthalpic contribution of neomycin { $\Delta\Delta H_{obs} = -7.21$ kcal/mol ($\Delta H_{neomycin\ conjugated} - \Delta H_{nonconjugated}$)} binding to the hybrid duplex. The short linker length allows for selective stabilization of the hybrid duplex over the hybrid triplex. The results described here open up new avenues in the design and synthesis of nucleo-aminoglycoside-conjugates (N-Ag-C) where the inclusion of any number of aminoglycoside (neomycin) molecules per oligonucleotide can be accomplished.

Cowan J. A., Ohyama T., Wang D., and Natarajan K. (2000) Recognition of a cognate RNA aptamer by neomycin B: quantitative evaluation of hydrogen bonding and electrostatic interactions. *Nucleic Acids Res* **28**, 2935-2942.

Abstract: Aminoglycosides are an important class of antibiotic that selectively target RNA structural

motifs. Recently we have demonstrated copper derivatives of amino-glycosides to be efficient cleavage agents for cognate RNA motifs. To fully develop their potential as pharmaceutical agents it is necessary to understand both the structural mechanisms used by aminoglycosides to target RNA, and the relative contributions of hydrogen bonding and electrostatic interactions to recognition selectivity. Herein we report results from a calorimetric analysis of a stem-loop 23mer RNA aptamer complexed to the aminoglycoside neomycin B. Key thermodynamic parameters for complex formation have been determined by isothermal titration calorimetry, and from the metal-ion dependence of these binding parameters the relative contributions of electrostatics and hydrogen bonding toward binding affinity have been assessed. The principal mechanism for recognition and binding of neomycin B to the RNA major groove is mediated by hydrogen bonding.

Dathe M., Nikolenko H., Klose J., and Bienert M. (2004) Cyclization increases the antimicrobial activity and selectivity of arginine- and tryptophan-containing hexapeptides. *Biochemistry* **43**, 9140-9150.

Abstract: Arginine- and tryptophan-rich motifs have been identified in antimicrobial peptides with various secondary structures. We synthesized a set of linear hexapeptides derived from the sequence AcRRWRF-NH₂ by substitution of tryptophan (W) by tyrosine (Y) or naphthylalanine (Nal) and by replacement of arginine (R) by lysine (K) to investigate the role of cationic charge and aromatic residues in membrane activity and selectivity. A second set of corresponding head-to-tail cyclic analogues was prepared to analyze the role of conformational constraints. The biological activity of the linear peptides followed the order Nal- >> W- > Y-containing compounds and slightly decreased upon R-K substitution. A pronounced activity-improving and bacterial selectivity-enhancing effect was found upon cyclization of the R- and W-bearing parent peptide, whereas the activity-modifying effect of cyclization of Y- and Nal-containing peptides was low. The analysis of the driving forces of peptide interaction with model membranes showed that the activities correlated with the partition coefficients and the depths of peptide insertion into neutral and negatively charged lipid bilayers. Spectroscopic studies, RP-HPLC, and titration calorimetry implied that the combination of cationic and aromatic amino acid composition and conformational rigidity afforded a membrane-active, amphipathic structure with a highly charged face opposed by a cluster of aromatic side chains. However, threshold values of low and high hydrophobicity seemed to exist beyond which the activity-enhancing effect of cyclization was negligible. The results suggest that cyclization of small peptides of an appropriate amino acid composition may serve as a promising strategy in the design of antimicrobial peptides.

Devi P. G., Pal S., Banerjee R., and Dasgupta D. (2007) Association of antitumor antibiotics, mithramycin and chromomycin, with Zn(II). *J Inorg Biochem* **101**, 127-137.

Abstract: Chromomycin A(3) (CHR) and mithramycin (MTR), members of the aureolic acid anticancer antibiotics, supposedly act by inhibiting transcription via reversible association with DNA. The complex(es) with bivalent cation such as Mg(2+) and Zn(2+) is (are) the DNA-binding ligand(s). In this paper, we report a detailed study of the association of these antibiotics with the biologically important bivalent cation, Zn(2+), because the zinc chelating ability of the antibiotics has therapeutic potential in the treatment of diseases relating to zinc dyshomeostasis. Spectroscopic methods such as absorbance, fluorescence, and circular dichroism and NMR spectroscopy have been used to characterize and understand the mechanism of complex formation. Our data show that both antibiotics form a single complex with Zn(2+) in the mole ratio of 2:1 in terms of antibiotic:Zn(2+) with an apparent binding affinity in the micro molar range. The complex has been characterized as [(D)(2)Zn(2+)] (where 'D' stands for the antibiotic). The kinetics study of the complex formation between the antibiotic(s) and Zn(2+) suggests the following mechanism: Isothermal calorimetric titration has shown that the association is entropy driven, implying the role of water molecules in complex formation. (1)H NMR spectroscopic data of the complex favor a tetrahedral arrangement around the Zn(2+) ion with the antibiotic acting as a bidentate ligand.

Devi P. G., Chakraborty P. K. and Dasgupta D. (2008) Inhibition of a Zn(II)-containing enzyme, alcohol dehydrogenase, by anticancer antibiotics, mithramycin and chromomycin A(3). *J Biol Inorg Chem*. (epublication)

Abstract: One of the major attributes for the biological action of the aureolic acid anticancer antibiotics chromomycin A(3) (CHR) and mithramycin (MTR) is their ability to bind bivalent cations such as Mg(II) and Zn(II) ions and form high affinity 2:1 complexes in terms of the antibiotic and the metal ion, respectively. As most of the cellular Zn(II) ion is found to be associated with proteins, we have examined

the effect of MTR/CHR on the structure and function of a representative structurally well characterized Zn(II) metalloenzyme, alcohol dehydrogenase (ADH) from yeast. MTR and CHR inhibit enzyme activity of ADH with inhibitory constants of micromolar order. Results from size-exclusion column chromatography, dynamic light scattering, and isothermal titration calorimetry have suggested that the mechanism of inhibition of the metalloenzyme by the antibiotics is due to the antibiotic-induced disruption of the enzyme quaternary structure. The nature of the enzyme inhibition, the binding stoichiometry of two antibiotics per monomer, and comparable dissociation constants for the antibiotic and free (or substrate-bound) ADH imply that the association occurs as a consequence of the binding of the antibiotics to Zn(II) ion present at the structural center. Confocal microscopy shows the colocalization of the antibiotic and the metalloenzyme in HepG2 cells, thereby supporting the proposition of physical association between the antibiotic(s) and the enzyme inside the cell

Draker K. A., Northrop D. B., and Wright G. D. (2003) Kinetic mechanism of the GCN5-related chromosomal aminoglycoside acetyltransferase AAC(6')-Ii from *Enterococcus faecium*: evidence of dimer subunit cooperativity. *Biochemistry* **42**, 6565-6574.

Abstract: The aminoglycoside 6'-N-acetyltransferase AAC(6')-Ii from *Enterococcus faecium* is an important microbial resistance determinant and a member of the GCN5-related N-acetyltransferase (GNAT) superfamily. We report here the further characterization of this enzyme in terms of the kinetic mechanism of acetyl transfer and identification of rate-contributing step(s) in catalysis, as well as investigations into the binding of both acetyl-CoA and aminoglycoside substrates to the AAC(6')-Ii dimer. Product and dead-end inhibition studies revealed that AAC(6')-Ii follows an ordered bi-bi ternary complex mechanism with acetyl-CoA binding first followed by antibiotic. Solvent viscosity studies demonstrated that aminoglycoside binding and product release govern the rate of acetyl transfer, as evidenced by changes in both the $k(\text{cat})/K(\text{b})$ for aminoglycoside and $k(\text{cat})$, respectively, with increasing solvent viscosity. Solvent isotope effects were consistent with our viscosity studies that diffusion-controlled processes and not the chemical step were rate-limiting in drug modification. The patterns of partial and mixed inhibition observed during our mechanistic studies were followed up by investigating the possibility of subunit cooperativity in the AAC(6')-Ii dimer. Through the use of AAC-Trp(164) \rightarrow Ala, an active mutant which exists as a monomer in solution, the partial nature of the competitive inhibition observed in wild-type dead-end inhibition studies was alleviated. Isothermal titration calorimetry studies also indicated two nonequivalent antibiotic binding sites for the AAC(6')-Ii dimer but only one binding site for the Trp(164) \rightarrow Ala mutant. Taken together, these results demonstrate subunit cooperativity in the AAC(6')-Ii dimer, with possible relevance to other oligomeric members of the GNAT superfamily.

Du W., Liu W. S., Payne D. J., and Doyle M. L. (2000) Synergistic inhibitor binding to *Streptococcus pneumoniae* 5-enolpyruvylshikimate-3-phosphate synthase with both monovalent cations and substrate. *Biochemistry* **39**, 10140-10146.

Abstract: The inhibitor binding synergy mechanism of the bi-substrate enzyme *Streptococcus pneumoniae* 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) has been investigated with a linkage thermodynamics strategy, involving direct binding experiments of one ligand conducted over a range of concentration of the other. The results demonstrate that binding of the inhibitor glyphosate (GLP) is highly synergistic with both a natural substrate shikimate-3-phosphate (S3P) and activating monovalent cations. The synergy between GLP and S3P binding was determined to be 1600-fold and is in qualitative agreement with previous work on *Escherichia coli* EPSPS. The binding molar ratios of S3P and GLP were measured as 1.0 and 0.7 per EPSPS, respectively. Monovalent cations that have been shown previously to stimulate *S. pneumoniae* EPSPS catalytic activity and its inhibition by GLP were found here to exhibit a similar rank-order with respect to their measured GLP binding synergies (ranging from 0 to $>$ or \approx 3000-fold increase in GLP affinity). The cation specificity and the sub-millimolar concentrations where these effects occur strongly suggest the presence of a specific cation binding site. Analytical ultracentrifugation data ruled out GLP-binding synergy mechanisms that derive from, or are influenced by, changes in oligomerization of *S. pneumoniae* EPSPS. Rather, the data are most consistent with an allosteric mechanism involving changes in tertiary structure. The results provide a quantitative framework for understanding the inhibitor binding synergies in *S. pneumoniae* EPSPS and implicate the presence of a specific cation binding regulatory site. The findings will help to guide rational design of novel antibiotics targeting bacterial EPSPS enzymes.

Epanand R. F., Lehrer R. I., Waring A., Wang W., Maget-Dana R., Lelievre D., and Epanand R. M. (2003) Direct comparison of membrane interactions of model peptides composed of only Leu and Lys residues. *Biopolymers* **71**, 2-16.

Abstract: We compared the properties of two peptides of identical size and amino acid composition, Ac-(LKKL)(5)-NH₂ and Ac-(KL)(10)-NH₂. Both are amphipathic, but only Ac-(LKKL)(5)-NH₂ is a potent promoter of negative curvature. CD studies performed in the presence of lipids confirmed that under these conditions Ac-(LKKL)(5)-NH₂ forms an alpha-helix, and Ac-(KL)(10)-NH₂ adopts a beta structure. We studied their binding affinity by centrifugation and isothermal titration calorimetry techniques. The Ac-(LKKL)(5)-NH₂ bound to zwitterionic and anionic liposomes, while Ac-(KL)(10)-NH₂ interacted mainly with anionic liposomes. Ac-(LKKL)(5)-NH₂ was more lytic than Ac-(KL)(10)-NH₂ for zwitterionic palmitoyloleoylphosphatidylcholine (POPC) liposomes, and for liposomes composed of lipids extracted from either sheep or human erythrocytes (RBC). Both peptides had similar lytic and lipid mixing activities for liposomes containing anionic lipids. Both peptides were highly hemolytic, with Ac-(LKKL)(5)-NH₂ active against sheep RBC and Ac-(KL)(10)-NH₂ more active against human RBC. From their respective minimal effective concentrations (MECs) as antimicrobial agents, we judged Ac-(KL)(10)-NH₂ to be 2 to 5-fold more potent than Ac-(LKKL)(5)-NH₂ in media that contained physiological concentrations of NaCl. Notwithstanding, both peptides had MECs <1 µg/mL for *Escherichia coli* and *Pseudomonas aeruginosa* and <4 µg/mL for *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*. Although selectivity of antimicrobial peptides for bacterial membranes may result, in part, from the preferential display of anionic residues in these membranes, inability to interact with or bind to zwitterionic phospholipids offers no guarantee that the peptide will lack appreciable cytotoxicity for host cells.

Epanand R. F., Mowery B. P., Lee S. E., Stahl S. S., Lehrer R. I., Gellman S. H. and Epanand R. M. (2008) Dual mechanism of bacterial lethality for a cationic sequence-random copolymer that mimics host-defense antimicrobial peptides. *J Mol Biol* **379**, 38-50.

Abstract: Flexible sequence-random polymers containing cationic and lipophilic subunits that act as functional mimics of host-defense peptides have recently been reported. We used bacteria and lipid vesicles to study one such polymer, having an average length of 21 residues, that is active against both Gram-positive and Gram-negative bacteria. At low concentrations, this polymer is able to permeabilize model anionic membranes that mimic the lipid composition of *Escherichia coli*, *Staphylococcus aureus*, or *Bacillus subtilis* but is ineffective against model zwitterionic membranes, which explains its low hemolytic activity. The polymer is capable of binding to negatively charged vesicles, inducing segregation of anionic lipids. The appearance of anionic lipid-rich domains results in formation of phase-boundary defects through which leakage can occur. We had earlier proposed such a mechanism of membrane disruption for another antimicrobial agent. Experiments with the mutant *E. coli* ML-35p indicate that permeabilization is biphasic: at low concentrations, the polymer permeabilizes the outer and inner membranes; at higher polymer concentrations, permeabilization of the outer membrane is progressively diminished, while the inner membrane remains unaffected. Experiments with wild-type *E. coli* K12 show that the polymer blocks passage of solutes into the intermembrane space at high concentrations. Cell membrane integrity in *E. coli* K12 and *S. aureus* exhibits biphasic dependence on polymer concentration. Isothermal titration calorimetry indicates that the polymer associates with the negatively charged lipopolysaccharide of Gram-negative bacteria and with the lipoteichoic acid of Gram-positive bacteria. We propose that this polymer has two mechanisms of antibacterial action, one predominating at low concentrations of polymer and the other predominating at high concentrations.

Faehnle, C.R., Le Coq, J., Liu, X., and Viola, R.E. (2006) Examination of Key Intermediates in the Catalytic Cycle of Aspartate-β-semialdehyde Dehydrogenase from a Gram-positive Infectious Bacteria. *J. Biol. Chem.* **281**, 31031-31040.

Abstract: Aspartate-β-semialdehyde dehydrogenase (ASADH) catalyzes a critical branch point transformation in amino acid bio-synthesis. The products of the aspartate pathway are essential in microorganisms, and this entire pathway is absent in mammals, making this enzyme an attractive target for antibiotic development. The first structure of an ASADH from a Gram-positive bacterium, *Streptococcus pneumoniae*, has now been determined. The overall structure of the apoenzyme has a similar fold to those of the Gram-negative and archaeal ASADHs but contains some interesting structural variations that can be exploited for inhibitor design. Binding of the coenzyme NADP, as well as a truncated nucleotide analogue, into an alternative conformation from that observed in Gram-negative ASADHs causes an enzyme domain

closure that precedes catalysis. The covalent acyl-enzyme intermediate was trapped by soaking the substrate into crystals of the coenzyme complex, and the structure of this elusive intermediate provides detailed insights into the catalytic mechanism.

Fournier I., Barwicz J., and Tancrede P. (1998) The structuring effects of amphotericin B on pure and ergosterol- or cholesterol-containing dipalmitoylphosphatidylcholine bilayers: a differential scanning calorimetry study. *Biochim Biophys Acta* **1373**, 76-86.

Abstract: Amphotericin B (AmB) is the most widely used polyene antibiotic to treat systemic fungal infections which affect an increasing number of immunocompromised patients. It is generally thought that AmB forms pores within the fungi membranes by interacting with ergosterol, the main sterol of fungi. However, it also interacts with the cholesterol contained in mammalian cells, hence its toxicity. In order to have a better understanding of the interactions prevailing between AmB and sterols, differential scanning calorimetry was used to study various mixtures incorporating from 6.5 to 25 mol% of AmB in pure dipalmitoylphosphatidylcholine (DPPC) vesicles and in ergosterol- or cholesterol-containing DPPC vesicles. The sterol concentration was kept constant at 12.5 mol% with respect to the phospholipid. Our results show that three phases co-exist when AmB is dispersed in the pure phospholipid. One corresponds to the phospholipid phase alone. The two others are characterised by a broad transition at temperatures higher than the main transition temperature of the pure phospholipid, corresponding to the drug in interaction with the aliphatic chains of the lipid. The fact that the transition temperatures of these additional components are higher than that of the pure phospholipid suggests that AmB interacts strongly with the aliphatic chains of the lipid, consistent with the idea prevailing in the literature that AmB by itself may form pores in a lipid matrix. When AmB interacts with cholesterol-containing bilayers the thermograms also present three components. Upon increasing the concentration of AmB, though, an important broadening of these components is observed which is explained in terms of destabilisation of the organisation of the aliphatic chains. The situation is strikingly different if ergosterol is present in the lipid matrix. The thermograms remain unmodified as the concentration of AmB is increased and a broad transition, now involving only two components when the thermograms are decomposed, is observed. An analysis of the results shows that various interacting units, e.g. AmB+DPPC and (AmB+ergosterol)+DPPC, are present within the membrane. These units involve the phospholipid and hence contribute to its structuration. The important differences between the thermograms obtained with the ergosterol- as compared to the cholesterol-containing bilayers, in spite of the structural similarity of these two sterols, provides strong evidence for the selectivity of interaction of AmB with ergosterol as compared to cholesterol. It is thus clear that the action of AmB on cholesterol- as compared to ergosterol-containing membranes results from different mechanisms. Finally, UV-visible spectra of AmB in pure as well as sterol-containing DPPC vesicles show the presence of absorption bands that give support to the interpretation derived from the calorimetric data.

Gabriel G. J., Pool J. G., Som A., Dabkowski J. M., Coughlin E. B., Muthukumar M. and Tew G. N. (2008) Interactions between antimicrobial polynorbornenes and phospholipid vesicles monitored by light scattering and microcalorimetry. *Langmuir* **24**, 12489-12495.

Abstract: Antimicrobial polynorbornenes composed of facially amphiphilic monomers have been previously reported to accurately emulate the antimicrobial activity of natural host-defense peptides (HDPs). The lethal mechanism of most HDPs involves binding to the membrane surface of bacteria leading to compromised phospholipid bilayers. In this paper, the interactions between biomimetic vesicle membranes and these cationic antimicrobial polynorbornenes are reported. Vesicle dye-leakage experiments were consistent with previous biological assays and corroborated a mode of action involving membrane disruption. Dynamic light scattering (DLS) showed that these antimicrobial polymers cause extensive aggregation of vesicles without complete bilayer disintegration as observed with surfactants that efficiently solubilize the membrane. Fluorescence microscopy on vesicles and bacterial cells also showed polymer-induced aggregation of both synthetic vesicles and bacterial cells. Isothermal titration calorimetry (ITC) afforded free energy of binding values (ΔG) and polymer to lipid binding ratios, plus revealed that the interaction is entropically favorable ($\Delta S > 0$, $\Delta H > 0$). It was observed that the strength of vesicle binding was similar between the active polymers while the binding stoichiometries were dramatically different

Garçon A., Bermingham A., Lian L. Y., and Derrick J. P. (2004) Kinetic and structural characterization of a product complex of 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase from *Escherichia coli*. *Biochem J* **380**, 867-873.

Abstract: HPPK (6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase) catalyses the transfer of pyrophosphate from ATP to HMDP (6-hydroxymethyl-7,8-dihydropterin), to form AMP and DHPPP (6-hydroxymethyl-7,8-dihydropterin pyrophosphate). This transformation is a key step in the biosynthesis of folic acid, and HPPK is consequently a target for antimicrobial drugs. The substrates are known to bind to HPPK in an ordered manner, with ATP binding first followed by HMDP. In the present study we show by isothermal titration calorimetry that the product, DHPPP, can bind to the HPPK apoenzyme with high affinity (equilibrium dissociation constant, $K_d=0.2 \mu\text{M}$), but without the enhancement of pterin fluorescence that occurs on binding of HMDP. The transient kinetics of the enzyme can be monitored by measuring the change in the fluorescence of the pterin ring using stopped-flow methods. The fluorescence exhibits a pronounced biphasic behaviour: it initially rises and then declines back to its original level. This behaviour is in agreement with a two-state kinetic model, with the first phase of fluorescence increase associated with HMDP binding to the enzyme, and the second phase with a slow event that occurs after the reaction has taken place. The HPPK-DHPPP and HPPK-DHPPP-AMP complexes were examined by NMR, and the binding site for DHPPP partially mapped from changes in chemical shifts identified from two dimensional $^1\text{H}/^{15}\text{N}$ heteronuclear single-quantum coherence spectra. The results demonstrate that DHPPP, in contrast to HMDP, is able to bind to the HPPK apoenzyme and suggest that the pyrophosphate moieties on the ligand play an important role in establishment of a high affinity binding site for the pterin ring.

Gormley N. A., Orphanides G., Meyer A., Cullis P. M., and Maxwell A. (1996) The interaction of coumarin antibiotics with fragments of DNA gyrase B protein. *Biochemistry* **35**, 5083-5092.

Abstract: DNA gyrase is the target of the coumarin group of antibacterial agents. The drugs are known to inhibit the ATPase activity of gyrase and bind to the 24-kDa N-terminal subdomain of gyrase B protein. Supercoiling assays with intact DNA gyrase and ATPase assays with a 43-kDa N-terminal fragment of the B protein suggest that the drugs bind tightly, with K_d values $<10^{-7}$ M. In addition, the ATPase data suggest that 1 coumermycin molecule interacts with 2 molecules of the 43-kDa protein while the other coumarins form a 1:1 complex. This result is confirmed by cross-linking experiments. Rapid gel-filtration experiments show that the binding of ADPNP (5'-adneylyl beta,gamm-imidodiphosphate) and coumarins to the 43-kDa protein is mutually exclusive, consistent with a competitive mode of action for the drugs. Rapid gel-filtration binding experiments using both the 24- and 43-kDa proteins also show that the drugs bind with association rate constants of $>10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, and dissociation rate constants of approximately $3 \times 10^{-3} \text{ s}^{-1}$ and approximately $4 \times 10^{-3} \text{ s}^{-1}$ for the 43- and 24-kDa proteins, respectively. Titration calorimetry shows that the K_d values for coumarins binding to both proteins are approximately 10-8M and that binding is enthalpy driven.

Gottler L. M., Lee H. Y., Shelburne C. E., Ramamoorthy A. and Marsh E. N. (2008) Using fluorous amino acids to modulate the biological activity of an antimicrobial peptide. *Chembiochem* **9**, 370-373

Gottler L. M., de la Salud B. R., Shelburne C. E., Ramamoorthy A. and Marsh E. N. (2008) Using fluorous amino acids to probe the effects of changing hydrophobicity on the physical and biological properties of the beta-hairpin antimicrobial peptide protegrin-1. *Biochemistry* **47**, 9243-9250.

Abstract: Protegrins are potent members of the beta-hairpin-forming class of antimicrobial peptides. Key to their antimicrobial activity is their assembly into oligomeric structures upon binding to the bacterial membrane. To examine the relationship between the physicochemical properties of the peptide and its biological activity, we have synthesized variants of protegrin-1 in which key residues in the hydrophobic core, valine-14 and -16, are changed to leucine and to the extensively fluorinated analogue hexafluoroleucine. These substitutions have the effect of making the peptide progressively more hydrophobic while minimally perturbing the secondary structure. The leucine-containing peptide was significantly more active than wild-type protegrin against several common pathogenic bacterial strains, whereas the hexafluoroleucine-substituted peptide, in contrast, showed significantly diminished activity against several bacterial strains. Isothermal titration calorimetry measurements revealed significant changes in the interaction of the peptides binding to small unilamellar vesicles that mimic the lipid composition of

the bacterial membrane. The binding isotherms for wild-type and leucine-substituted protegrins indicate that electrostatic interactions dominate the membrane-peptide interaction, whereas the isotherm for the hexafluoroleucine-substituted protegrin suggests a diminished electrostatic component to binding. Notably both of these substitutions appear to alter the stoichiometry of the lipid-peptide interaction, suggesting that these substitutions may stabilize oligomerized forms of protegrin that are postulated to be intermediates in the assembly of the beta-barrel membrane pore structure

Guazzaroni M. E., Krell T., Felipe A., Ruiz R., Meng C., Zhang X., Gallegos M. T., and Ramos J. L. (2005) The multidrug efflux regulator TtgV recognizes a wide range of structurally different effectors in solution and complexed with target DNA: evidence from isothermal titration calorimetry. *J Biol Chem* **280**, 20887-20893.

Abstract: TtgV modulates the expression of the *ttgGHI* operon, which encodes an efflux pump that extrudes a wide variety of chemicals including mono- and binuclear aromatic hydrocarbons, aliphatic alcohols, and antibiotics of dissimilar chemical structure. Using a 'lacZ fusion to the *ttgG* promoter, we show that the most efficient *in vivo* inducers were 1-naphthol, 2,3-dihydroxynaphthalene, 4-nitrotoluene, benzonitrile, and indole. The thermodynamic parameters for the binding of different effector molecules to purified TtgV were determined by isothermal titration calorimetry. For the majority of effectors, the interaction was enthalpy-driven and counterbalanced by unfavorable entropy changes. The TtgV-effector dissociation constants were found to vary between 2 and 890 μM . There was a relationship between TtgV affinity for the different effectors and their potential to induce gene expression *in vivo*, indicating that the effector binding constant is a major determinant for efficient efflux pump gene expression. Equilibrium dialysis and isothermal titration calorimetry studies indicated that a TtgV dimer binds one effector molecule. No evidence for the simultaneous binding of multiple effectors to TtgV was obtained. The binding of TtgV to a 63-bp DNA fragment containing its cognate operator was tight and entropy-driven ($K(D) = 2.4 \pm 0.35 \text{ nM}$, $\Delta H = 5.5 \pm 0.04 \text{ kcal/mol}$). The TtgV-DNA complex was shown to bind 1-naphthol with an affinity comparable with the free soluble TtgV protein, $K(D) = 4.8 \pm 0.19$ and $3.0 \pm 0.15 \mu\text{M}$, respectively. The biological relevance of this finding is discussed.

Guerin M. E., Kordulakova J., Schaeffer F., Svetlikova Z., Buschiazzi A., Giganti D., Gicquel B., Mikusova K., Jackson M. and Alzari P. M. (2007) Molecular recognition and interfacial catalysis by the essential phosphatidylinositol mannosyltransferase PimA from mycobacteria. *J Biol Chem* **282**, 20705-20714.

Abstract: Mycobacterial phosphatidylinositol mannosides (PIMs) and metabolically derived cell wall lipoglycans play important roles in host-pathogen interactions, but their biosynthetic pathways are poorly understood. Here we focus on *Mycobacterium smegmatis* PimA, an essential enzyme responsible for the initial mannosylation of phosphatidylinositol. The structure of PimA in complex with GDP-mannose shows the two-domain organization and the catalytic machinery typical of GT-B glycosyltransferases. PimA is an amphitrophic enzyme that binds mono-disperse phosphatidylinositol, but its transferase activity is stimulated by high concentrations of non-substrate anionic surfactants, indicating that the early stages of PIM biosynthesis involve lipid-water interfacial catalysis. Based on structural, calorimetric, and mutagenesis studies, we propose a model wherein PimA attaches to the membrane through its N-terminal domain, and this association leads to enzyme activation. Our results reveal a novel mode of phosphatidylinositol recognition and provide a template for the development of potential antimycobacterial compounds.

Gusman H., Lendenmann U., Grogan J., Troxler R. F., and Oppenheim F. G. (2001) Is salivary histatin 5 a metalloprotein? *Biochim Biophys Acta* **1545**, 86-95.

Abstract: Histatins are small histidine-rich salivary polypeptides which exhibit antimicrobial activity against *Candida albicans*. This antimicrobial activity has been ascribed in part to a high content of basic amino acids. However, unlike most other antimicrobial proteins histatins have a high content of histidine, tyrosine and acidic amino acids known to participate in metal ion coordination. This study was conducted to test whether histatin 5 could bind zinc and copper which are metals present in salivary secretions and whole saliva. Physical binding parameters and spectral properties of zinc- and copper-histatin complexes were investigated in order to obtain direct evidence of these interactions. A spectrophotometric competition assay using the metallochromic indicator murexide showed that histatin 5 dissociates metal indicator complexes containing zinc or copper ions. Absorption spectra of histatin 5 at increasing copper chloride

concentrations resulted in higher absorbance in the 230-280 nm wavelength range and this spectral change was saturated at a peptide:metal molar ratio of approx. 1:1. A corresponding band was observed in the visible range of the spectrum with a maximum and molar extinction coefficient corresponding to that of copper binding to an ATCUN motif. Quantitative assessment of zinc and copper binding to histatin 5 using isothermal titration calorimetry revealed at least one high affinity site for each metal, with binding constants of 1.2×10^5 and $2.6 \times 10^7 \text{ M}^{-1}$, respectively. These results indicate that histatin 5 exhibits metallopeptide-like properties. The precise biological significance of this has not yet been established but histatins may contribute significantly to salivary metal binding capacity.

Haq I. (2002) Thermodynamics of drug-DNA interactions. *Arch Biochem Biophys* **403**, 1-15.

Abstract: Many anticancer, antibiotic, and antiviral drugs exert their primary biological effects by reversibly interacting with nucleic acids. Therefore, these biomolecules represent a major target in drug development strategies designed to produce next generation therapeutics for diseases such as cancer. In order to improve the clinical efficacy of existing drugs and also to design new ones it is necessary to understand the molecular basis of drug-DNA interactions in structural, thermodynamic, and kinetic detail. The past decade has witnessed an increase in the number of rigorous biophysical studies of drug-DNA systems and considerable knowledge has been gained in the energetics of these binding reactions. This is, in part, due to the increased availability of high-sensitivity calorimetric techniques, which have allowed the thermodynamics of drug-DNA interactions to be probed directly and accurately. The focus of this article is to review thermodynamic approaches to examining drug-DNA recognition. Specifically, an overview of a recently developed method of analysis that dissects the binding free energy of these reactions into five component terms is presented. The results of applying this analysis to the DNA binding interactions of both minor groove drugs and intercalators are discussed. The solvent water plays a key role in nucleic acid structure and consequently in the binding of ligands to these biomolecules. Any rational approach to DNA-targeted drug design requires an understanding of how water participates in recognition and binding events. Recent studies examining hydration changes that accompany DNA binding by intercalators will be reviewed. Finally some aspects of cooperativity in drug-DNA interactions are described and the importance of considering cooperative effects when examining these reactions is highlighted.

Harrison J. J., Turner R. J., Joo D. A., Stan M. A., Chan C. S., Allan N. D., Vrionis H. A., Olson M. E. and Ceri H. (2008) Copper and quaternary ammonium cations exert synergistic bactericidal and antibiofilm activity against *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **52**, 2870-2881.

Abstract: Biofilms are slimy aggregates of microbes that are likely responsible for many chronic infections as well as for contamination of clinical and industrial environments. *Pseudomonas aeruginosa* is a prevalent hospital pathogen that is well known for its ability to form biofilms that are recalcitrant to many different antimicrobial treatments. We have devised a high-throughput method for testing combinations of antimicrobials for synergistic activity against biofilms, including those formed by *P. aeruginosa*. This approach was used to look for changes in biofilm susceptibility to various biocides when these agents were combined with metal ions. This process identified that $\text{Cu}(2+)$ works synergistically with quaternary ammonium compounds (QACs; specifically benzalkonium chloride, cetalkonium chloride, cetylpyridinium chloride, myristalkonium chloride, and Polycide) to kill *P. aeruginosa* biofilms. In some cases, adding $\text{Cu}(2+)$ to QACs resulted in a 128-fold decrease in the biofilm minimum bactericidal concentration compared to that for single-agent treatments. In combination, these agents retained broad-spectrum antimicrobial activity that also eradicated biofilms of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica* serovar Cholerasuis, and *Pseudomonas fluorescens*. To investigate the mechanism of action, isothermal titration calorimetry was used to show that $\text{Cu}(2+)$ and QACs do not interact in aqueous solutions, suggesting that each agent exerts microbiological toxicity through independent biochemical routes. Additionally, $\text{Cu}(2+)$ and QACs, both alone and in combination, reduced the activity of nitrate reductases, which are enzymes that are important for normal biofilm growth. Collectively, the results of this study indicate that $\text{Cu}(2+)$ and QACs are effective combinations of antimicrobials that may be used to kill bacterial biofilms

Heerklotz H. and Seelig J. (2001) Detergent-like action of the antibiotic peptide surfactin on lipid membranes. *Biophys J* **81**, 1547-1554.

Abstract: Surfactin is a bacterial lipopeptide with powerful surfactant-like properties. High-sensitivity isothermal titration calorimetry was used to study the self association and membrane partitioning of

surfactin. The critical micellar concentration (CMC), was 7.5 μM , the heat of micellization was endothermic with $\Delta H(w \rightarrow m)(\text{Su}) = +4.0 \text{ kcal/mol}$, and the free energy of micellization $\Delta G(O, w \rightarrow m)(\text{Su}) = -9.3 \text{ kcal/mol}$ (25 degrees C; 100 mM NaCl; 10 mM TRIS, 1 mM EDTA; pH 8.5). The specific heat capacity of micellization was deduced from temperature dependence of $\Delta H(w \rightarrow m)(\text{Su})$ as $\Delta C(w \rightarrow m)(\text{P}) = -250 \pm 10 \text{ cal/(mol.K)}$. The data can be explained by combining the hydrophobicity of the fatty acyl chain with that of the hydrophobic amino acids. The membrane partition equilibrium was studied using small (30 nm) and large (100 nm) unilamellar POPC vesicles. At 25 degrees C, the partition coefficient, K, was $(2.2 \pm 0.2) \times 10^4 \text{ M}^{-1}$ for large vesicles leading to a free energy of $\Delta G(O, w \rightarrow b)(\text{Su}) = -8.3 \text{ kcal/mol}$. The partition enthalpy was again endothermic, with $\Delta H(w \rightarrow b)(\text{Su}) = 9 \pm 1 \text{ kcal/mol}$. The strong preference of surfactin for micelle formation over membrane insertion explains the high membrane-destabilizing activity of the peptide. For surfactin and a variety of non-ionic detergents, the surfactant-to-lipid ratio, inducing membrane solubilization, $R(\text{sat})(b)$, can be predicted by the simple relationship $R(\text{sat})(b)$ approximately $K \cdot \text{CMC}$.

Heerklotz H. and Seelig J. (2007) Leakage and lysis of lipid membranes induced by the lipopeptide surfactin. *Eur Biophys J* **36**, 305-314.

Abstract: Surfactin is a lipopeptide produced by *Bacillus subtilis* which possesses antimicrobial activity. We have studied the leakage and lysis of POPC vesicles induced by surfactin using calcein fluorescence de-quenching, isothermal titration calorimetry and $(31)\text{P}$ solid state NMR. Membrane leakage starts at a surfactin-to-lipid ratio in the membrane, $R(b)$ approximately 0.05, and an aqueous surfactin concentration of $C(S)(w)$ approximately 2 μM . The transient, graded nature of leakage and the apparent coupling with surfactin translocation to the inner leaflet of the vesicles, suggests that this low-concentration effect is due to a bilayer-couple mechanism. Different permeabilization behaviour is found at $R(b)$ approximately 0.15 and attributed to surfactin-rich clusters, which can induce leaks and stabilize them by covering their hydrophobic edges. Membrane lysis or solubilization to micellar structures starts at $R(b)(\text{sat}) = 0.22$ and $C(S)(w) = 9 \mu\text{M}$ and is completed at $R(m)(\text{sol}) = 0.43$ and $C(S)(w) = 11 \mu\text{M}$. The membrane-water partition coefficient of surfactin is obtained as $K = 2 \times 10^4 \text{ M}^{-1}$. These data resolve inconsistencies in the literature and shed light on the variety of effects often referred to as detergent-like effects of antibiotic peptides on membranes. The results are compared with published parameters characterizing the hemolytic and antibacterial activity.

Holdgate G. A., Tunnicliffe A., Ward W. H., Weston S. A., Rosenbrock G., Barth P. T., Taylor I. W., Pauptit R. A., and Timms D. (1997) The entropic penalty of ordered water accounts for weaker binding of the antibiotic novobiocin to a resistant mutant of DNA gyrase: a thermodynamic and crystallographic study. *Biochemistry* **36**, 9663-9673.

Abstract: Novobiocin is an antibiotic which binds to a 24 kDa fragment from the B subunit of DNA gyrase. Naturally occurring resistance arises from mutation of Arg-136 which hydrogen bonds to the coumarin ring of novobiocin. We have applied calorimetry to characterize the binding of novobiocin to wild-type and R136H mutant 24 kDa fragments. Upon mutation, the K_d increases from 32 to 1200 nM at 300 K. The enthalpy of binding is more favorable for the mutant (ΔH degrees shifts from -12.1 to -17.5 kcal/mol), and the entropy of binding is much less favorable ($T\Delta S$ degrees changes from -1.8 to -9.4 kcal/mol). Both of these changes are in the direction opposite to that expected if the loss of the Arg residue reduces hydrogen bonding. The change in heat capacity at constant pressure upon binding (ΔC_p) shifts from -295 to -454 $\text{cal mol}^{-1} \text{K}^{-1}$. We also report the crystal structure, at 2.3 Å resolution, of a complex between the R136H 24 kDa fragment and novobiocin. Although the change in ΔC_p often would be interpreted as reflecting increased burial of hydrophobic surface on binding, this structure reveals a small decrease. Furthermore, an ordered water molecule is sequestered into the volume vacated by removal of the guanidinium group. There are large discrepancies when the measured thermodynamic parameters are compared to those estimated from the structural data using empirical relationships. These differences seem to arise from the effects of sequestering ordered water molecules upon complexation. The water-mediated hydrogen bonds linking novobiocin to the mutant protein make a favorable enthalpic contribution, whereas the immobilization of the water leads to an entropic cost and a reduction in the heat capacity of the system. Such a negative contribution to ΔC_p , ΔH degrees, and $T\Delta S$ degrees appears to be a general property of water molecules that are sequestered when ligands bind to proteins.

Howe J., Andra J., Conde R., Iriarte M., Garidel P., Koch M. H., Gutsmann T., Moriyon I. and Brandenburg K. (2007) Thermodynamic analysis of the lipopolysaccharide-dependent resistance of gram-negative bacteria against polymyxin B. *Biophys J* **92**, 2796-2805.

Abstract: Cationic antimicrobial cationic peptides (CAMP) have been found in recent years to play a decisive role in hosts' defense against microbial infection. They have also been investigated as a new therapeutic tool, necessary in particular due to the increasing resistance of microbiological populations to antibiotics. The structural basis of the activity of CAMPs has only partly been elucidated and may comprise quite different mechanism at the site of the bacterial cell membranes or in their cytoplasm. Polymyxin B (PMB) is a CAMP which is effective in particular against Gram-negative bacteria and has been well studied with the aim to understand its interaction with the outer membrane or isolated membrane components such as lipopolysaccharide (LPS) and to define the mechanism by which the peptides kill bacteria or neutralize LPS. Since PMB resistance of bacteria is a long-known phenomenon and is attributed to structural changes in the LPS moiety of the respective bacteria, we have performed a thermodynamic and biophysical analysis to get insights into the mechanisms of various LPS/PMB interactions in comparison to LPS from sensitive strains. In isothermal titration calorimetric (ITC) experiments considerable differences of PMB binding to sensitive and resistant LPS were found. For sensitive LPS the endothermic enthalpy change in the gel phase of the hydrocarbon chains converts into an exothermic reaction in the liquid crystalline phase. In contrast, for resistant LPS the binding enthalpy change remains endothermic in both phases. As infrared data show, these differences can be explained by steric changes in the headgroup region of the respective LPS.

Howe J., Hammer M., Alexander C., Rossle M., Fournier K., Mach J. P., Waelli T., Gorczynski R. M., Ulmer A. J., Zahringer U., Rietschel E. T. and Brandenburg K. (2007) Biophysical characterization of the interaction of endotoxins with hemoglobins. *Med. Chem* **3**, 13-20.

Abstract: Bacterial endotoxin (lipopolysaccharide, LPS) is the major component of the outer leaflet of the outer membrane in gram-negative bacteria. During severe infections, bacteria may reach the blood circuit of humans, and endotoxins may be released from the bacteria due to cell division or cell death. In particular enterobacterial forms of LPS represent extremely strong activator molecules of the human immune system causing a rapid induction of cytokine production in monocytes and macrophages. Various mammalian blood proteins have been documented to display LPS binding activities mediating normally decreasing effects in the biological activity of LPS. In more recent studies, the essential systemic oxygen transportation protein hemoglobin (Hb) has been shown to amplify LPS-induced cytokine production on immune cells. The mechanism responsible for this effect is poorly understood. Here, we characterize the interaction of hemoglobin with LPS by using biophysical methods. The data presented, revealing the changes of the type and size of supramolecular aggregates of LPS in the presence of Hb, allow a better understanding of the hemoglobin-induced increase in bioactivity of LPS.

Huecas S., Schaffner-Barbero C., Garcia W., Yebenes H., Palacios J. M., Diaz J. F., Menendez M. and Andreu J. M. (2007) The interactions of cell division protein FtsZ with guanine nucleotides. *J Biol Chem* **282**, 37515-37528.

Abstract: Prokaryotic cell division protein FtsZ, an assembling GTPase, directs the formation of the septosome between daughter cells. FtsZ is an attractive target for the development of new antibiotics. Assembly dynamics of FtsZ is regulated by the binding, hydrolysis and exchange of GTP. We have determined the energetics of nucleotide binding to model apo-FtsZ from *Methanococcus jannaschii* and studied the kinetics of mant-nucleotide binding and dissociation from FtsZ polymers, employing calorimetric, fluorescence and stopped-flow methods. FtsZ binds GTP and GDP with K_b values ranging from 20 to 300 μM^{-1} under various conditions. $\text{GTP} \cdot \text{Mg}(2)$ and $\text{GDP} \cdot \text{Mg}(2+)$ bind with slightly reduced affinity. Bound GTP and the coordinated $\text{Mg}(2+)$ ion play a minor structural role in FtsZ monomers, but $\text{Mg}(2+)$ -assisted GTP hydrolysis triggers polymer disassembly. Mant-GTP binds and dissociates quickly from FtsZ monomers, with ~ 10 -fold lower affinity than GTP. Mant-GTP displacement measured by fluorescence anisotropy provides a method to test the binding of any competing molecules to the FtsZ nucleotide site. Mant-GTP is very slowly hydrolyzed and remains exchangeable in FtsZ polymers, but the complex becomes kinetically stabilized, with a 30-fold slower $k(+)$ and ~ 500 -fold slower $k(-)$ than in monomers. Mant-GTP dissociation rate from FtsZ polymers is comparable to GTP hydrolysis turnover and to the reported subunit turnover in *Escherichia coli* FtsZ polymers. Although FtsZ polymers can exchange nucleotide, unlike its eukaryotic structural homologue tubulin, GDP dissociation may be slow

enough for polymer disassembly to take place first, resulting in FtsZ polymers cycling with GTP hydrolysis similarly to microtubules.

Hunter H. N., Jing W., Schibli D. J., Trinh T., Park I. Y., Kim S. C., and Vogel H. J. (2005) The interactions of antimicrobial peptides derived from lysozyme with model membrane systems. *Biochim Biophys Acta* **1668**, 175-189.

Abstract: Two peptides, RAWVAWR-NH₂ and IVSDGNGMNAWVAWR-NH₂, derived from human and chicken lysozyme, respectively, exhibit antimicrobial activity. A comparison between the L-RAWVAWR, D-RAWVAWR, and the longer peptide has been carried out in membrane mimetic conditions to better understand how their interaction with lipid and detergent systems relates to the reported higher activity for the all L-peptide. Using CD and 2D 1H NMR spectroscopy, the structures were studied with DPC and SDS micelles. Fluorescence spectroscopy was used to study peptide interactions with POPC and POPG vesicles and DOPC, DOPE, and DOPG mixed vesicle systems. Membrane-peptide interactions were also probed by ITC and DSC. The ability of fluorescein-labeled RAWVAWR to rapidly enter both *E. coli* and *Staphylococcus aureus* was visualized using confocal microscopy. Reflecting the bactericidal activity, the long peptide interacted very weakly with the lipids. The RAWVAWR-NH₂ peptides preferred lipids with negatively charged headgroups and interacted predominantly in the solvent-lipid interface, causing significant perturbation of membrane mimetics containing PG headgroups. Peptide structures determined by 1H NMR indicated a well-ordered coiled structure for the short peptides and the C-terminus of the longer peptide. Using each technique, the two enantiomers of RAWVAWR-NH₂ interacted in an identical fashion with the lipids, indicating that any difference in activity in vivo is limited to interactions not involving the membrane lipids.

Hurtado-Gomez E., Abian O., Munoz F. J., Hernaiz M. J., Velazquez-Campoy A. and Neira J. L. (2008) Defining the epitope region of a peptide from the *Streptomyces coelicolor* phosphoenolpyruvate:sugar phosphotransferase system able to bind to the enzyme I. *Biophys J* **95**, 1336-1348.

Abstract: The bacterial PEP:sugar PTS consists of a cascade of several proteins involved in the uptake and phosphorylation of carbohydrates, and in signal transduction pathways. Its uniqueness in bacteria makes the PTS a target for new antibacterial drugs. These drugs can be obtained from peptides or protein fragments able to interfere with the first reaction of the protein cascade: the phosphorylation of the HPr by the first enzyme, the so-called enzyme EI. To that end, we designed a peptide, HPr(9-30), spanning residues 9 to 30 of the intact HPr protein, containing the active site histidine (His-15) and the first alpha-helix of HPr of *Streptomyces coelicolor*, HPr(sc). By using fluorescence and circular dichroism, we first determined qualitatively that HPr(sc) and HPr(9-30) did bind to EI(sc), the enzyme EI from *S. coelicolor*. Then, we determined quantitatively the binding affinities of HPr(9-30) and HPr(sc) for EI(sc) by using ITC and STD-NMR. The STD-NMR experiments indicate that the epitope region of HPr(9-30) was formed by residues Leu-14, His-15, Ile-21, and Val-23. The binding reaction between EI(sc) and HPr(sc) is enthalpy driven and in other species is entropy driven; further, the affinity of HPr(sc) for EI(sc) was smaller than in other species. However, the affinity of HPr(9-30) for EI(sc) was only moderately lower than that of EI(sc) for HPr(sc), suggesting that this peptide could be considered a promising hit compound for designing new inhibitors against the PTS

Itoh, Y., Watanabe, J., Fukada, H., Mizuno, R., Kezuka, Y., Takamasa Nonaka, T., and Watanabe, T. (2006) Importance of Trp59 and Trp60 in chitin-binding, hydrolytic, and antifungal activities of *Streptomyces griseus* chitinase C. *Appl Microbiol Biotechnology* **72**, 1176-1184.

Abstract: The chitin-binding domain of *Streptomyces griseus* chitinase C (ChBDChiC) belongs to CBM family 5. Only two exposed aromatic residues, W59 and W60, were observed in ChBDChiC, in contrast to three such residues on CBDCel5 in the same CBM family. To study importance of these residues in binding activity and other functions of ChBDChiC, site-directed mutagenesis was carried out. Single (W59A and W60A) and double (W59A/W60A) mutations abolished the binding activity of ChiC to colloidal chitin and decreased the hydrolytic activity toward not only colloidal chitin but also a soluble high Mr substrate, glycol chitin. Interaction of ChBDChiC with oligosaccharide was eliminated by these mutations. The hydrolytic activity toward oligosaccharide was increased by deletion of ChBD but not affected by these mutations, indicating that ChBD interferes with oligosaccharide hydrolysis but not through its binding activity. The antifungal activity was drastically decreased by all mutations and significant difference was observed between single and double mutants. Taken together with the structural

information, these results suggest that ChBDChiC binds to chitin via a mechanism significantly different from CBDCel5, where two aromatic residues play major role, and contributes to various functions of ChiC. Sequence comparison indicated that ChBDChiC-type CBMs are dominant in CBM family 5.

Jin E., Katritch V., Olson W. K., Kharatisvili M., Abagyan R., and Pilch D. S. (2000) Aminoglycoside binding in the major groove of duplex RNA: the thermodynamic and electrostatic forces that govern recognition. *J Mol Biol* **298**, 95-110.

Abstract: We use a combination of spectroscopic, calorimetric, viscometric and computer modeling techniques to characterize the binding of the aminoglycoside antibiotic, tobramycin, to the polymeric RNA duplex, poly(rI).poly(rC), which exhibits the characteristic A-type conformation that is conserved among natural and synthetic double-helical RNA sequences. Our results reveal the following significant features: (i) CD-detected binding of tobramycin to poly(rI).poly(rC) reveals an apparent site size of four base-pairs per bound drug molecule; (ii) tobramycin binding enhances the thermal stability of the host poly(rI).poly(rC) duplex, the extent of which decreases upon increasing in Na(+) concentration and/or pH conditions; (iii) the enthalpy of tobramycin- poly(rI).poly(rC) complexation increases with increasing pH conditions, an observation consistent with binding-induced protonation of one or more drug amino groups; (iv) the affinity of tobramycin for poly(rI).poly(rC) is sensitive to both pH and Na(+) concentration, with increases in pH and/or Na(+) concentration resulting in a concomitant reduction in binding affinity. The salt dependence of the tobramycin binding affinity reveals that the drug binds to the host RNA duplex as trication. (v) The thermodynamic driving force for tobramycin- poly(rI).poly(rC) complexation depends on pH conditions. Specifically, at pH < or = 6.0, tobramycin binding is entropy driven, but is enthalpy driven at pH > 6.0. (vi) Viscometric data reveal non-intercalative binding properties when tobramycin complexes with poly(rI).poly(rC), consistent with a major groove-directed mode of binding. These data also are consistent with a binding-induced reduction in the apparent molecular length of the host RNA duplex. (vii) Computer modeling studies reveal a tobramycin-poly(rI). poly(rC) complex in which the drug fits snugly at the base of the RNA major groove and is stabilized, at least in part, by an array of hydrogen bonding interactions with both base and backbone atoms of the host RNA. These studies also demonstrate an inability of tobramycin to form a stable low-energy complex with the minor groove of the poly(rI).poly(rC) duplex. In the aggregate, our results suggest that tobramycin-RNA recognition is dictated and controlled by a broad range of factors that include electrostatic interactions, hydrogen bonding interactions, drug protonation reactions, and binding-induced alterations in the structure of the host RNA. These modulatory effects on tobramycin-RNA complexation are discussed in terms of their potential importance for the selective recognition of specific RNA structural motifs, such as asymmetric internal loops or hairpin loop-stem junctions, by aminoglycoside antibiotics and their derivatives.

Jing W., Hunter H. N., Hagel J., and Vogel H. J. (2003) The structure of the antimicrobial peptide Ac-RRWWRF-NH₂ bound to micelles and its interactions with phospholipid bilayers. *J Pept Res* **61**, 219-229.

Abstract: The hexapeptide Ac-RRWWRF-NH₂ has earlier been identified as a potent antimicrobial peptide by screening synthetic combinatorial hexapeptide libraries. In this study, it was found that this peptide had a large influence on the thermotropic phase behavior of model membranes containing the negatively charged headgroup phosphatidylglycerol, a major component of bacterial membranes. In contrast, differential scanning calorimetry showed that it had little effect on model membranes containing the zwitterionic phosphatidylcholine headgroup, the main component of erythrocyte membranes. This behavior is consistent with its biological activity and with its affinity to these membranes as determined by titration calorimetry, implying that peptide-lipid interactions play an important role in this process. The structure of this peptide bound to membrane-mimetic sodium dodecyl sulfate (SDS) and dodecylphosphocholine micelles has been determined using conventional two-dimensional nuclear magnetic resonance methods. It forms a marked amphipathic structure in SDS with its hydrophobic residues on one side of the structure and with the positively charged residues on the other side. This amphipathic structure may allow this peptide to penetrate deeper into the interfacial region of negatively charged membranes, leading to local membrane destabilization. Knowledge about the importance of electrostatic interactions of Arg and the role of Trp residues as a membrane interface anchor will provide insight into the future design of potent antimicrobial peptidomimetics.

Jing W., Demcoe A. R., and Vogel H. J. (2003) Conformation of a bactericidal domain of puroindoline a: structure and mechanism of action of a 13-residue antimicrobial peptide. *J Bacteriol* **185**, 4938-4947.

Abstract: Puroindoline a, a wheat endosperm-specific protein containing a tryptophan-rich domain, was reported to have antimicrobial activities. We found that a 13-residue fragment of puroindoline a (FPVTWRWWKWWKG-NH₂) (puroA) exhibits activity against both gram-positive and gram-negative bacteria. This suggests that puroA may be a bactericidal domain of puroindoline a. PuroA interacted strongly with negatively charged phospholipid vesicles and induced efficient dye release from these vesicles, suggesting that the microbicidal effect of puroA may be due to interactions with bacterial membranes. A variety of biophysical and biochemical methods, including fluorescence spectroscopy and microcalorimetry, were used to examine the mode of action of puroA. These studies showed that puroA is located at the membrane interface, probably due to its high content of Trp residues that have a high propensity to partition into the membrane interface. The penetration of these Trp residues in negatively charged phospholipid vesicles resembling bacterial membranes was more extensive than the penetration in neutral vesicles mimicking eukaryotic membranes. Peptide binding had a significant influence on the phase behavior of the former vesicles. The three-dimensional structure of micelle-bound puroA determined by two-dimensional nuclear magnetic resonance spectroscopy indicated that all the positively charged residues are oriented close to the face of Trp indole rings, forming energetically favorable cation- π interactions. This characteristic, along with its well-defined amphipathic structure upon binding to membrane mimetic systems, allows puroA to insert more deeply into bacterial membranes and disrupt the regular membrane bilayer structure.

Kaul M., Barbieri C. M., and Pilch D. S. (2005) Defining the basis for the specificity of aminoglycoside-rRNA recognition: a comparative study of drug binding to the A sites of Escherichia coli and human rRNA. *J Mol Biol* **346**, 119-134.

Abstract: 2-Deoxystreptomycin (2-DOS) aminoglycoside antibiotics exert their antimicrobial activities by targeting the decoding region A site of the rRNA and inhibiting protein synthesis. A prokaryotic specificity of action is critical to therapeutic utility of 2-DOS aminoglycosides as antibiotics. Here, isothermal titration calorimetry (ITC) and fluorescence studies are presented that provide insight into the molecular basis for this prokaryotic specificity of action. Specifically, the rRNA binding properties of the 2-DOS aminoglycosides paromomycin and G418 (geneticin) are compared, using both human and Escherichia coli rRNA A site model oligonucleotides as drug targets. Paromomycin and G418 differ with respect to their specificities of action, with only paromomycin exhibiting a specificity for prokaryotic versus human ribosomes. G418 binds to both the human and E. coli rRNA A sites with a markedly lower affinity than paromomycin, with the affinities of both drugs for the human rRNA A site being lower than those they exhibit for the E. coli rRNA A site. Paromomycin induces the destacking of the base at position 1492 (by E. coli numbering) upon binding to the E. coli rRNA A site, but not the human rRNA A site. By contrast, the binding of G418 induces the destacking of base 1492 when either rRNA A site serves as the drug target. In the aggregate, these results suggest that binding-induced base destacking at the rRNA A site is a critical factor in determining the prokaryotic specificity of aminoglycoside action, with binding affinity for the A site being of secondary importance.

Kaul M., Barbieri C. M., and Pilch D. S. (2004) Fluorescence-based approach for detecting and characterizing antibiotic-induced conformational changes in ribosomal RNA: comparing aminoglycoside binding to prokaryotic and eukaryotic ribosomal RNA sequences. *J Am Chem Soc* **126**, 3447-3453.

Abstract: Aminoglycoside antibiotics bind specifically to a conserved sequence of the 16S ribosomal RNA (rRNA) A site and interfere with protein synthesis. One model for the mechanism underlying the deleterious effects of aminoglycosides on protein synthesis invokes a drug-induced conformational change in the rRNA that involves the destacking of two adenine residues (A1492 and A1493 in Escherichia coli) at the A site. We describe here a fluorescence-based approach for detecting and characterizing this drug-induced conformational change in the target rRNA. In this approach, we insert the fluorescent base analogue 2-aminopurine in place of A1492 in an E. coli 16S rRNA A-site model oligonucleotide (EcWT) as well as in a mutant form of this oligomer (A1408G) in which A1408 has been replaced with a guanine. The presence of guanine at 1408 instead of adenine represents one of the major sequence differences between prokaryotic and eukaryotic A sites, with the latter A sites being resistant to the deleterious effects of aminoglycosides. Binding of the aminoglycoside paromomycin to the 2AP-substituted forms of EcWT and A1408G induced changes in fluorescence quantum yield consistent with drug-induced base destacking

in EcWT but not A1408G. Isothermal titration calorimetry studies reveal that paromomycin binds to the EcWT duplex with a 31-fold higher affinity than the A1408G duplex, with this differential affinity being enthalpic in origin. In the aggregate, these observations are consistent with both rRNA binding affinity and drug-induced base destacking being important determinants in the prokaryotic specificity of aminoglycosides. Combining fluorescence quantum yield and lifetime data allows for quantification of the extent of drug-induced base destacking, thereby providing a convenient tool for evaluating the relative impacts of both novel and existing A-site targeting ligands on rRNA conformation and potentially for predicting relative antibiotic activities and specificities.

Kaul M. and Pilch D. S. (2002) Thermodynamics of aminoglycoside-rRNA recognition: the binding of neomycin-class aminoglycosides to the A site of 16S rRNA. *Biochemistry* **41**, 7695-7706.

Abstract: We use spectroscopic and calorimetric techniques to characterize the binding of the aminoglycoside antibiotics neomycin, paromomycin, and ribostamycin to a RNA oligonucleotide that models the A-site of Escherichia coli 16S rRNA. Our results reveal the following significant features: (i) Aminoglycoside binding enhances the thermal stability of the A-site RNA duplex, with the extent of this thermal enhancement decreasing with increasing pH and/or Na(+) concentration. (ii) The RNA binding enthalpies of the aminoglycosides become more exothermic (favorable) with increasing pH, an observation consistent with binding-linked protonation of one or more drug amino groups. (iii) Isothermal titration calorimetry (ITC) studies conducted as a function of buffer reveal that aminoglycoside binding to the host RNA is linked to the uptake of protons, with the number of linked protons being dependent on pH. Specifically, increasing the pH results in a corresponding increase in the number of linked protons. (iv) ITC studies conducted at 25 and 37 degrees C reveal that aminoglycoside-RNA complexation is associated with a negative heat capacity change (ΔC_p), the magnitude of which becomes greater with increasing pH. (v) The observed RNA binding affinities of the aminoglycosides decrease with increasing pH and/or Na(+) concentration. In addition, the thermodynamic forces underlying these RNA binding affinities also change as a function of pH. Specifically, with increasing pH, the enthalpic contribution to the observed RNA binding affinity increases, while the corresponding entropic contribution to binding decreases. (vi) The affinities of the aminoglycosides for the host RNA follow the hierarchy neomycin > paromomycin > ribostamycin. The enhanced affinity of neomycin relative to either paromomycin or ribostamycin is primarily, if not entirely, enthalpic in origin. (vii) The salt dependencies of the RNA binding affinities of neomycin and paromomycin are consistent with at least three drug NH(3)(+) groups participating in electrostatic interactions with the host RNA. In the aggregate, our results reveal the impact of specific alterations in aminoglycoside structure on the thermodynamics of binding to an A-site model RNA oligonucleotide. Such systematic comparative studies are critical first steps toward establishing the thermodynamic database required for enhancing our understanding of the molecular forces that dictate and control aminoglycoside recognition of RNA.

Kaul M., Barbieri C. M., Kerrigan J. E., and Pilch D. S. (2003) Coupling of drug protonation to the specific binding of aminoglycosides to the A site of 16 S rRNA: elucidation of the number of drug amino groups involved and their identities. *J Mol Biol* **326**, 1373-1387.

Abstract: 2-Deoxystreptamine (2-DOS) aminoglycoside antibiotics bind specifically to the central region of the 16S rRNA A site and interfere with protein synthesis. Recently, we have shown that the binding of 2-DOS aminoglycosides to an A site model RNA oligonucleotide is linked to the protonation of drug amino groups. Here, we extend these studies to define the number of amino groups involved as well as their identities. Specifically, we use pH-dependent ¹⁵N NMR spectroscopy to determine the pKa values of the amino groups in neomycin B, paromomycin I, and lividomycin A sulfate, with the resulting pKa values ranging from 6.92 to 9.51. For each drug, the 3-amino group was associated with the lowest pKa, with this value being 6.92 in neomycin B, 7.07 in paromomycin I, and 7.24 in lividomycin A. In addition, we use buffer-dependent isothermal titration calorimetry (ITC) to determine the number of protons linked to the complexation of the three drugs with the A site model RNA oligomer at pH 5.5, 8.8, or 9.0. At pH 5.5, the binding of the three drugs to the host RNA is independent of drug protonation effects. By contrast, at pH 9.0, the RNA binding of paromomycin I and neomycin B is coupled to the uptake of 3.25 and 3.80 protons, respectively, with the RNA binding of lividomycin A at pH 8.8 being coupled to the uptake of 3.25 protons. A comparison of these values with the protonation states of the drugs predicted by our NMR-derived pKa values allows us to identify the specific drug amino groups whose protonation is linked to complexation with the host RNA. These determinations reveal that the binding of lividomycin A to the host

RNA is coupled to the protonation of all five of its amino groups, with the RNA binding of paromomycin I and neomycin B being linked to the protonation of four and at least five amino groups, respectively. For paromomycin I, the protonation reactions involve the 1-, 3-, 2'-, and 2'''-amino groups, while, for neomycin B, the binding-linked protonation reactions involve at least the 1-, 3-, 2', 6'-, and 2'''-amino groups. Our results clearly identify drug protonation reactions as important thermodynamic participants in the specific binding of 2-DOS aminoglycosides to the A site of 16S rRNA.

Kaul M., Barbieri C. M., Srinivasan A. R. and Pilch D. S. (2007) Molecular determinants of antibiotic recognition and resistance by aminoglycoside phosphotransferase (3')-IIIa: a calorimetric and mutational analysis. *J Mol Biol* **369**, 142-156.

Abstract: The growing threat from the emergence of multidrug resistant pathogens highlights a critical need to expand our currently available arsenal of broad-spectrum antibiotics. In this connection, new antibiotics must be developed that exhibit the abilities to circumvent known resistance pathways. An important step toward achieving this goal is to define the key molecular interactions that govern antibiotic resistance. Here, we use site-specific mutagenesis, coupled with calorimetric, NMR, and enzymological techniques, to define the key interactions that govern the binding of the aminoglycoside antibiotics neomycin and kanamycin B to APH(3')-IIIa (an antibiotic phosphorylating enzyme that confers resistance). Our mutational analyses identify the D261, E262, and C-terminal F264 residues of the enzyme as being critical for recognition of the two drugs as well as for the manifestation of the resistance phenotype. In addition, the E160 residue is more important for recognition of kanamycin B than neomycin, with mutation of this residue partially restoring sensitivity to kanamycin B but not to neomycin. By contrast, the D193 residue partially restores sensitivity to neomycin but not to kanamycin B, with the origins of this differential effect being due to the importance of D193 for catalyzing the phosphorylation of neomycin. These collective mutational results, coupled with (15)N NMR-derived pK(a) and calorimetrically derived binding-linked drug protonation data, identify the 1-, 3-, and 2'-amino groups of both neomycin and kanamycin B as being critical functionalities for binding to APH(3')-IIIa. These drug amino functionalities represent potential sites of modification in the design of next-generation compounds that can overcome APH(3')-IIIa-induced resistance.

Keeble A. H. and Kleanthous C. (2005) The kinetic basis for dual recognition in colicin endonuclease-immunity protein complexes. *J Mol Biol* **352**, 656-671.

Abstract: The antibacterial activity of E colicin endonucleases (DNases) is counteracted by the binding of immunity proteins; the affinities of cognate and non-cognate complexes differing by up to ten orders of magnitude. Here, we address the mechanism of complex formation using a combination of protein engineering, pre-steady-state kinetics and isothermal titration calorimetry, in order to understand the underlying basis for specificity. Contrary to previous work, we show that a pre-equilibrium mechanism does not explain the binding kinetics. Instead, the data are best explained by a modified induced-fit mechanism where cognate and non-cognate complexes alike form a non-specific, conformationally dynamic encounter complex, most likely centred on conserved interactions at the interface. The dynamics appear to be an intrinsic property of the encounter complex where the proteins move relative to one another, thereby sampling different conformations rather than being "induced" by binding. This allows optimal alignment of interface specificity sites, without producing energetically costly conformational changes, essential for high-affinity binding. Importantly, specificity is achieved without slowing the rate of association, an important requirement for rapid inhibition of the colicin in the producing bacterial cell. A rigid-body rotation model is also consistent with the observation that specificity contacts in colicin-immunity protein complexes can involve different regions of the interface. Such a kinetic discrimination mechanism explains the ability of DNase-specific immunity proteins to display dual recognition specificity, wherein they are broadly cross-reactive yet are highly specific, achieving femtomolar binding affinities in complexes with their cognate DNases.

Klocek G. and Seelig J. (2008) Melittin interaction with sulfated cell surface sugars. *Biochemistry* **47**, 2841-2849.

Abstract: Melittin is a 26-residue cationic peptide with cytolytic and antimicrobial properties. Studies on the action mechanism of melittin have focused almost exclusively on the membrane-perturbing properties of this peptide, investigating in detail the melittin-lipid interaction. Here, we report physical-chemical studies on an alternative mechanism by which melittin could interact with the cell membrane. As the outer

surface of many cells is decorated with anionic (sulfated) glycosaminoglycans (GAGs), a strong Coulombic interaction between the two oppositely charged molecules can be envisaged. Indeed, the present study using isothermal titration calorimetry reveals a high affinity of melittin for several GAGs, that is, heparan sulfate (HS), dermatan sulfate, and heparin. The microscopic binding constant of melittin for HS is 2.4×10^5 M⁻¹, the reaction enthalpy is $\Delta H_{\text{melittin}}(0) = -1.50$ kcal/mol, and the peptide-to-HS stoichiometry is approximately 11 at 10 mM Tris, 100 mM NaCl at pH 7.4 and 28 degrees C. $\Delta H_{\text{melittin}}(0)$ is characterized by a molar heat capacity of $\Delta C_P(0) = -227$ cal mol⁻¹ K⁻¹. The large negative heat capacity change indicates that hydrophobic interactions must also be involved in the binding of melittin to HS. Circular dichroism spectroscopy demonstrates that the binding of the peptide to HS induces a conformational change to a predominantly alpha-helical structure. A model for the melittin-HS complex is presented. Melittin binding was compared with that of magainin 2 and nisin Z to HS. Magainin 2 is known for its antimicrobial properties, but it does not cause lysis of the eukaryotic cells. Nisin Z shows activity against various Gram-positive bacteria. Isothermal titration calorimetry demonstrates that magainin 2 and nisin Z do not bind to HS (5-50 degrees C, 10 mM Tris, and 100 mM NaCl at pH 7.4)

Kovari J., Barabas O., Varga B., Bekesi A., Tolgyesi F., Fidy J., Nagy J. and Vertessy B. G. (2008) Methylene substitution at the alpha-beta bridging position within the phosphate chain of dUDP profoundly perturbs ligand accommodation into the dUTPase active site. *Proteins* **71**, 308-319.

Abstract: dUTP pyrophosphatase, a preventive DNA repair enzyme, contributes to maintain the appropriate cellular dUTP/dTTP ratio by catalyzing dUTP hydrolysis. dUTPase is essential for viability in bacteria and eukaryotes alike. Identification of species-specific antagonists of bacterial dUTPases is expected to contribute to the development of novel antimicrobial agents. As a first general step, design of dUTPase inhibitors should be based on modifications of the substrate dUTP phosphate chain, as modifications in either base or sugar moieties strongly impair ligand binding. Based on structural differences between bacterial and human dUTPases, derivatization of dUTP-analogous compounds will be required as a second step to invoke species-specific character. Studies performed with dUTP analogues also offer insights into substrate binding characteristics of this important and structurally peculiar enzyme. In this study, alpha,beta-methylene-dUDP was synthesized and its complex with dUTPase was characterized. Enzymatic phosphorylation of this substrate analogue by pyruvate kinase was not possible in contrast to the successful enzymatic phosphorylation of alpha,beta-imino-dUDP. One explanation for this finding is that the different bond angles and the presence of the methylene group may preclude formation of a catalytically competent complex with the kinase. Crystal structure of E. coli dUTPase:alpha,beta-methylene-dUDP and E. coli dUTPase:dUDP:Mn complexes were determined and analyzed in comparison with previous data. Results show that the "trans" alpha-phosphate conformation of alpha,beta-methylene-dUDP differs from the catalytically competent "gauche" alpha-phosphate conformation of the imino analogue and the oxo substrate, manifested in the shifted position of the alpha-phosphorus by more than 3 Å. The three-dimensional structures determined in this work show that the binding of the methylene analogue with the alpha-phosphorus in the "gauche" conformation would result in steric clash of the methylene group with the protein atoms. In addition, the metal ion cofactor was not bound in the crystal of the complex with the methylene analogue while it was clearly visible as coordinated to dUDP, arguing that the altered phosphate chain conformation also perturbs metal ion complexation. Isothermal calorimetry titrations indicate that the binding affinity of alpha,beta-methylene-dUDP toward dUTPase is drastically decreased when compared with that of dUDP. In conclusion, the present data suggest that while alpha,beta-methylene-dUDP seems to be practically nonhydrolyzable, it is not a strong binding inhibitor of dUTPase probably due to the altered binding mode of the phosphate chain. Results indicate that in some cases methylene analogues may not faithfully reflect the competent substrate ligand properties, especially if the methylene hydrogens are in steric conflict with the protein

Lahiri S., Devi P. G., Majumder P., Das S. and Dasgupta D. (2008) Self-association of the anionic form of the DNA-binding anticancer drug mithramycin. *J Phys. Chem B* **112**, 3251-3258.

Abstract: The aqueous-phase self-association of mithramycin (MTR), an aureolic acid anticancer antibiotic, has been studied using different spectroscopic techniques such as absorption, fluorescence, circular dichroism, and ¹H nuclear magnetic resonance spectroscopy. Results from these studies indicate self-association of the anionic antibiotic at pH 8.0 over a concentration range from micromolar to millimolar. These results could be ascribed to the following steps of self-association: M + M \rightleftharpoons M₂, M₂ + M \rightleftharpoons M₃, and M₃ + M \rightleftharpoons M₄,

where M, M2, M3, and M4 represent the monomer, dimer, trimer, and tetramer of mithramycin, respectively. Dynamic light scattering and isothermal titration calorimetry studies also support aggregation. In contrast, an insignificant extent of self-association is found for the neutral drug (at pH 3.5) and the [(MTR)₂Mg²⁺] complex (at pH 8.0). Analysis of 2D NMR spectra of 1 mM MTR suggests that the sugar moieties play a role in the self-association process. Self-association of the drug might occur either via hydrophobic interaction of the sugar residues among themselves or water-mediated hydrogen bond formation between sugar residue(s). On the other hand, absence of a significant upfield shift of the aromatic protons from 100 μM to 1 mM MTR suggests against the possibility of stacking interactions between the aromatic rings as a stabilizing force for the formation of the dimer and higher oligomers

Lee M., Shea R. G., Hartley J. A., Lown J. W., Kissinger K., Dabrowiak J. C., Vesnaver G., Breslauer K. J., and Pon R. T. (1989) Molecular recognition between oligopeptides and nucleic acids. Sequence specific binding of (4S)-(+)- and (4R)-(-)-dihydrokikumycin B to DNA deduced from 1H NMR, footprinting studies and thermodynamic data. *J Mol Recognit* **2**, 6-17.

Abstract: The sequence specific binding of the antibiotic (4S)-(+)-dihydrokikumycin B and its (4R)-(-) enantiomer, [(S)-1 and (R)-1, respectively] to DNA were characterized by DNase I and MPE footprinting, calorimetry, UV spectroscopy, circular dichroism, and 1H NMR studies. Footprinting analyses showed that both enantiomers [(S)-1 and (R)-1] bind to AT-rich regions of DNA. 1H NMR studies (ligand induced chemical shift changes and NOE differences) of the dihydrokikumycins with d-[CGCAATTGCG]₂ show unambiguously that the N to C termini of the ligands are bound to 5'-A5T6T7-3' reading from left to right. From quantitative 1D-NOE studies, the AH2(5)-ligand H7 distance of complex A [(S)-1 plus decamer (which is bound more strongly)] and complex B [(R)-1 and decamer] are estimated to be 3.8 +/- 0.3 Å and 4.9 +/- 0.4 Å, respectively. This difference in binding properties is reflected in the thermodynamic profiles of the two enantiomeric ligands determined by a combination of spectroscopic and calorimetric techniques. The binding free energies (ΔG degrees) of (S)-1 and (R)-1 to poly d(AT).poly d(AT) at 25 degrees C are -31.8 and -29.3 kJ mol⁻¹, respectively while the corresponding binding enthalpies (ΔH degrees) are -11.3 and -0.8 kJ mol⁻¹. These data permit the construction of models for the binding of the enantiomeric dihydrokikumycins to DNA and account for the more efficient binding of the natural (S) isomer to DNA.

Lynch S. R. and Puglisi J. D. (2001) Structural origins of aminoglycoside specificity for prokaryotic ribosomes. *J Mol Biol* **306**, 1037-1058.

Abstract: Aminoglycoside antibiotics, including paromomycin, neomycin and gentamicin, target a region of highly conserved nucleotides in the decoding region aminoacyl-tRNA site (A site) of 16 S rRNA on the 30 S subunit. Change of a single nucleotide, A1408 to G, reduces the affinity of many aminoglycosides for the ribosome; G1408 distinguishes between prokaryotic and eukaryotic ribosomes. The structures of a prokaryotic decoding region A-site oligonucleotide free in solution and bound to the aminoglycosides paromomycin and gentamicin C1a were determined previously. Here, the structure of a eukaryotic decoding region A-site oligonucleotide bound to paromomycin has been determined using NMR spectroscopy and compared to the prokaryotic A-site-paromomycin structure. A conformational change in three adenosine residues of an internal loop, critical for high-affinity antibiotic binding, was observed in the prokaryotic RNA-paromomycin complex in comparison to its free form. This conformational change is not observed in the eukaryotic RNA-paromomycin complex, disrupting the binding pocket for ring I of the antibiotic. The lack of the conformational change supports footprinting and titration calorimetry data that demonstrate approximately 25-50-fold weaker binding of paromomycin to the eukaryotic decoding-site oligonucleotide. Neomycin, which is much less active against Escherichia coli ribosomes with an A1408G mutation, binds non-specifically to the oligonucleotide. These results suggest that eukaryotic ribosomal RNA has a shallow binding pocket for aminoglycosides, which accommodates only certain antibiotics.

Machaidze G., Ziegler A., and Seelig J. (2002) Specific binding of Ro 09-0198 (cinnamycin) to phosphatidylethanolamine: a thermodynamic analysis. *Biochemistry* **41**, 1965-1971.

Abstract: Ro 09-0198 (cinnamycin) is a tetracyclic peptide antibiotic that is used to monitor the transbilayer movement of phosphatidylethanolamine (PE) in biological membranes during cell division and apoptosis. The molecule is one of the very rare examples where a small peptide binds specifically to a particular lipid. In model membranes and biological membranes containing phosphatidylethanolamine, Ro 09-0198 forms a 1:1 complex with this lipid. We have measured the thermodynamic parameters of complex formation with high sensitivity isothermal titration calorimetry and have investigated the structural

consequences with deuterium and phosphorus solid-state NMR. Complex formation is characterized by a large binding constant, K_0 , of 10^7 to 10^8 M^{-1} , depending on the experimental conditions. The reaction enthalpy, ΔH degrees, varies between zero at 10 degrees C to strongly exothermic -10 kcal/mol at 50 degrees C. For large vesicles with a diameter of approximately 100 nm, ΔH degrees decreases linearly with temperature and the molar heat capacity of complex formation can be evaluated as = -245 cal/mol, indicating a hydrophobic binding mechanism. The free energy of binding is ΔG degrees = -10.5 kcal/mol and shows only little temperature dependence. The constancy of ΔG degrees together with the distinct temperature-dependence of ΔH degrees provide evidence for an entropy-enthalpy compensation mechanism: at 10 degrees C, complex formation is completely entropy-driven, at 50 degrees C it is enthalpy-driven. Varying the PE fatty acid chain-length between 6 and 18 carbon atoms produces similar binding constants and ΔH degrees values. Addition of Ro 09-0198 to PE containing bilayers eliminates the typical bilayer structure and produces ²H- and ³¹P-NMR spectra characteristic of slow isotropic tumbling. This reorganization of the lipid matrix is not limited to PE but also includes other lipids.

Machaidze G. and Seelig J. (2003) Specific binding of cinnamycin (Ro 09-0198) to phosphatidylethanolamine. Comparison between micellar and membrane environments. *Biochemistry* **42**, 12570-12576.

Abstract: Cinnamycin (Ro 09-0198) is a tetracyclic peptide antibiotic that binds specifically to phosphatidylethanolamine (PE). Formation of a complex with phosphatidylethanolamine follows a 1:1 stoichiometry. Using high-sensitivity isothermal titration calorimetry (ITC), we have measured the thermodynamic parameters of complex formation for two different PE environments, namely, PE dissolved either in octyl glucoside (OG) micelles or in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer membrane. We have compared diacyl-PE with lyso-PE and have varied the carbon chain length from 6 to 18. Binding requires both a PE headgroup and at least one fatty acyl chain. The optimum chain length for complex formation (n) is eight. Longer chains do not enhance the binding affinity; for shorter chains, the interaction is weakened. The cinnamycin-PE complex has a binding constant K_0 of approximately 10^7 - 10^8 M^{-1} in the POPC membrane and only approximately 10^6 M^{-1} in the octyl glucoside micelle. The difference can be attributed to the nonspecific hydrophobic interaction of cinnamycin with the lipid membrane. Complex formation is enthalpy-driven in OG micelles, whereas enthalpy and entropy make equal contributions in bilayer membranes. However, for the optimum chain length (n) of eight, the binding reaction is also completely enthalpy-driven for the bilayer membrane.

Marotte K., Sabin C., Preville C., Moume-Pymbock M., Wimmerova M., Mitchell E. P., Imberty A. and Roy R. (2007) X-ray Structures and Thermodynamics of the Interaction of PA-IIL from *Pseudomonas aeruginosa* with Disaccharide Derivatives. *ChemMedChem*. **2**, 1328-1338.

Abstract: *Pseudomonas aeruginosa* is an opportunistic bacterium showing increasing resistance to antibiotics and consequently represents elevated threatening problems in hospital environments, particularly for cystic fibrosis patients. The use of glycomimetics as an anti-adhesive strategy against microorganisms may complement the use of antibiotics. PA-IIL lectin (LecB) from *P. aeruginosa* constitutes an appealing target for antibacterial agents, as it has been proposed to play a key role in binding to airway epithelia and/or to be involved in biofilm formation. The lectin has an unusually high affinity for L-fucose and related oligosaccharides. In the work presented herein, the disaccharide alphaFuc1-4GlcNAc is used as a scaffold toward the synthesis of a series of glycomimetic derivatives. Microcalorimetry and structural studies indicate that several of the derivatives are potent inhibitors of the lectin, with affinity in the same range as the best known natural ligand, Lewis a, and could represent interesting leads for the development of future antibacterial compounds.

Marotte K., Preville C., Sabin C., Moume-Pymbock M., Imberty A. and Roy R. (2007) Synthesis and binding properties of divalent and trivalent clusters of the Lewis a disaccharide moiety to *Pseudomonas aeruginosa* lectin PA-IIL. *Org. Biomol. Chem* **5**, 2953-2961.

Abstract: The synthesis of oligomeric glycomimetics has been performed for targeting the *Pseudomonas aeruginosa* PA-IIL lectin, which is of therapeutic interest for anti-adhesive treatment. The disaccharide alpha-L-Fucp-(1-->4)-beta-D-GlcNAc, which is a high-affinity ligand of the lectin, has been coupled to dimeric and trimeric linkers with various lengths and geometries. A series of linear dimers displayed an efficient clustering effect and a very strong affinity, with a lower dissociation constant of 90 nM. The trimeric compound was less efficient in inhibition assays but displayed high affinity in solution. Titration

microcalorimetry and molecular modeling allowed in-depth analysis and rationalization of the binding data. These glycoclusters could act by crosslinking the lectins present on the surface of bacteria and therefore interfere with host recognition or biofilm formation.

Martin N. I., Hu H., Moake M. M., Churey J. J., Whittall R., Worobo R. W., and Vederas J. C. (2003) Isolation, structural characterization, and properties of mattacin (polymyxin M), a cyclic peptide antibiotic produced by *Paenibacillus kobensis* M. *J Biol Chem* **278**, 13124-13132.

Abstract: Mattacin is a nonribosomally synthesized, decapeptide antibiotic produced by *Paenibacillus kobensis* M. The producing strain was isolated from a soil/manure sample and identified using 16 S rRNA sequence homology along with chemical and morphological characterization. An efficient production and isolation procedure was developed to afford pure mattacin. Structure elucidation using a combination of chemical degradation, multidimensional NMR studies (COSY, HMBC, HMQC, ROESY), and mass spectrometric (MALDI MS/MS) analyses showed that mattacin is identical to polymyxin M, an uncommon antibiotic reported previously in certain *Bacillus* species by Russian investigators. Mattacin (polymyxin M) is cyclic and possesses an amide linkage between the C-terminal threonine and the side chain amino group of the diaminobutyric acid residue at position 4. It contains an (S)-6-methyloctanoic acid moiety attached as an amide at the N-terminal amino group, one D-leucine, six L-alpha,gamma-diaminobutyric acid, and three L-threonine residues. Transfer NOE experiments on the conformational preferences of mattacin when bound to lipid A and microcalorimetry studies on binding to lipopolysaccharide showed that its behavior was very similar to that observed in previous studies of polymyxin B (a commercial antibiotic), suggesting an identical mechanism of action. It was capable of inhibiting the growth of a wide variety of Gram-positive and Gram-negative bacteria, including several human and plant pathogens with activity comparable with purified polymyxin B. The biosynthesis of mattacin was also examined briefly using transpositional mutagenesis by which 10 production mutants were obtained, revealing a set of genes involved in production.

Marynka K., Rotem S., Portnaya I., Cogan U. and Mor A. (2007) In vitro discriminative antipseudomonal properties resulting from acyl substitution of N-terminal sequence of dermaseptin s4 derivatives. *Chem Biol* **14**, 75-85.

Abstract: Truncation and acylation were combined to investigate the broad-spectrum bactericidal and hemolytic peptide S4(1-15). Substitution of up to seven residues with dodecanoic acid (C(12)) gradually led to specific antipseudomonal activity: out of 40 bacterial strains tested in vitro, C(12)-S4(8-15) displayed similar minimal inhibitory concentrations (MICs) as S4(1-15) against *Pseudomonas aeruginosa* sp. (identical MIC(90)) but was practically inactive against most other bacteria or erythrocytes. Surface plasmon resonance and isothermal titration calorimetry experiments revealed the binding properties of S4(1-15) to be consistent with its nonselective activities, while discriminative activities of C(12)-S4(8-15) correlated with high binding affinity to a membrane containing pseudomonal lipopolysaccharides and with lower affinities to membranes containing nonpseudomonal lipopolysaccharides or cholesterol. Various mechanistic studies failed to detect significant differences in secondary structure, bactericidal kinetics, or ability to perturb the cytoplasmic membrane, pointing to a similar mode of action.

Milhaud J., Lancelin J. M., Michels B., and Blume A. (1996) Association of polyene antibiotics with sterol-free lipid membranes: I. Hydrophobic binding of filipin to dimyristoylphosphatidylcholine bilayers. *Biochim Biophys Acta* **1278**, 223-232.

Abstract: The interaction of filipin III with multilamellar vesicles (MLV) of dimyristoylphosphatidylcholine (DMPC) was studied by four complementary methods leading to the following results: (1) The modifications of the filipin dichroic spectrum, by adding preformed fluid DMPC MLV, provide evidence of a saturable association with the stoichiometry DMPC/filipin = 4.2 +/- 0.5, constant between 24 and 35 degrees Celsius. (2) Thermograms obtained by differential scanning calorimetry (DSC) on mixtures where filipin is incorporated during the formation of MLV exhibit a high-temperature tail the more marked the higher the filipin content and some structures at temperatures which depend on this content. The corresponding evolution with the temperature of the CD spectra reveals that the characteristic bound filipin spectrum appears at the temperature at which a structure emerges. (3) Titration calorimetry measurements reveal that the association process is exothermic in the temperature range of the DSC endotherms in agreement with the filipin-induced ordering of the lipid chains, previously established by 2H-NMR in the same temperature range (Milhaud et al.(1989) *Eur. Biophys. J.* 17, 151-158). A

discussion of the relevancy of this exothermicity to the hydrophobic effect is developed by referring to the paper by Wimley and White ((1993) *Biochemistry* 32, 6307-6312).

Miller J. R., Herberg J. T., Tomilo M., McCroskey M. C. and Feilmeier B. J. (2007) Streptococcus pneumoniae gyrase ATPase: development and validation of an assay for inhibitor discovery and characterization. *Anal Biochem* **365**, 132-143.

Abstract: The rise in bacterial resistance to antibiotics demonstrates the medical need for new antibacterial agents. One approach to this problem is to identify new antibacterials that act through validated drug targets such as bacterial DNA gyrase. DNA gyrase uses the energy of ATP hydrolysis to introduce negative supercoils into plasmid and chromosomal DNA and is essential for DNA replication. Inhibition of the ATPase activity of DNA gyrase is the mechanism by which coumarin-class antibiotics such as novobiocin inhibit bacterial growth. Although ATPase inhibitors exhibit potent antibacterial activity against gram-positive pathogens, no gyrase ATPase activity from a gram-positive organism is described in the literature. To address this, we developed and optimized an enzyme-coupled phosphate assay and used this assay to characterize the ATPase kinetics of Streptococcus pneumoniae gyrase. The S. pneumoniae enzyme exhibits cooperativity with ATP and requires organic potassium salts. We also studied inhibition of the enzyme by novobiocin. Apparent inhibition constants for novobiocin increased linearly with ATP concentration, indicative of an ATP-competitive mechanism. Similar binding affinities were measured by isothermal titration calorimetry. These results reveal unique features of the S. pneumoniae DNA gyrase ATPase and demonstrate the utility of the assay for screening and kinetic characterization of ATPase inhibitors.

Morgunova E., Meining W., Illarionov B., Haase I., Jin G., Bacher A., Cushman M., Fischer M., and Ladenstein R. (2005) Crystal structure of lumazine synthase from Mycobacterium tuberculosis as a target for rational drug design: binding mode of a new class of purinetrione inhibitors. *Biochemistry* **44**, 2746-2758.

Abstract: The enzymes involved in the biosynthesis of riboflavin represent attractive targets for the development of drugs against bacterial pathogens, because the inhibitors of these enzymes are not likely to interfere with enzymes of the mammalian metabolism. Lumazine synthase catalyzes the penultimate step in the riboflavin biosynthesis pathway. A number of substituted purinetrione compounds represent a new class of highly specific inhibitors of lumazine synthase from Mycobacterium tuberculosis. To develop potent antibiotics for the treatment of tuberculosis, we have determined the structure of lumazine synthase from M. tuberculosis in complex with two purinetrione inhibitors and have studied binding via isothermal titration calorimetry. The structures were determined by molecular replacement using lumazine synthase from Saccharomyces cerevisiae as a search model and refined at 2 and 2.3 Å resolution. The R-factors were 14.7 and 17.4%, respectively, and the R_(free) values were 19.3 and 26.3%, respectively. The enzyme was found to be a pentamer consisting of five subunits related by 5-fold local symmetry. The comparison of the active site architecture with the active site of previously determined lumazine synthase structures reveals a largely conserved topology with the exception of residues Gln141 and Glu136, which participate in different charge-charge interactions in the core space of the active site. The impact of structural changes in the active site on the altered binding and catalytic properties of the enzyme is discussed. Isothermal titration calorimetry measurements indicate highly specific binding of the purinetrione inhibitors to the M. tuberculosis enzyme with dissociation constants in micromolar range.

Morgunova E., Illarionov B., Sambaiah T., Haase I., Bacher A., Cushman M., Fischer M., and Ladenstein R. (2006) Structural and thermodynamic insights into the binding mode of five novel inhibitors of lumazine synthase from Mycobacterium tuberculosis. *FEBS J* **273**, 4790-4804.

Abstract: Recently published genomic investigations of the human pathogen Mycobacterium tuberculosis have revealed that genes coding the proteins involved in riboflavin biosynthesis are essential for the growth of the organism. Because the enzymes involved in cofactor biosynthesis pathways are not present in humans, they appear to be promising candidates for the development of therapeutic drugs. The substituted purinetrione compounds have demonstrated high affinity and specificity to lumazine synthase, which catalyzes the penultimate step of riboflavin biosynthesis in bacteria and plants. The structure of M. tuberculosis lumazine synthase in complex with five different inhibitor compounds is presented, together with studies of the binding reactions by isothermal titration calorimetry. The inhibitors showed the association constants in the micromolar range. The analysis of the structures demonstrated the specific features of the binding of different inhibitors. The comparison of the structures and binding modes of five

different inhibitors allows us to propose the ribityl-purinetri- and ribityl-phosphate compounds with C4-C5 alkylphosphate chains as most promising leads for further development of therapeutic drugs against *M. tuberculosis*.

Morgunova E., Saller S., Haase I., Cushman M., Bacher A., Fischer M. and Ladenstein R. (2007) Lumazine synthase from *Candida albicans* as an anti-fungal target enzyme: structural and biochemical basis for drug design. *J Biol Chem* **282**, 17231-17241.

Abstract: Lumazine synthase is an enzyme involved in riboflavin biosynthesis in many plants and microorganisms, including numerous human pathogens. The fact that the enzymes of the riboflavin biosynthesis pathway are not present in the human or animal host makes them potential targets for anti-infective agents. The crystal structure of lumazine synthase from *Candida albicans* was solved by molecular replacement and refined at 2.5-Å resolution. The results of crystallographic investigations and sedimentation equilibrium experiments clearly indicated the presence of pentameric assemblies of the enzyme either in crystals or in solution. Isothermal titration calorimetry measurements of the binding reactions of four different inhibitors revealed high affinity for all four compounds with binding constants in the micromolar range. Structural comparison with previously determined structures of the enzyme-ligand complexes of other orthologues allowed modeling of the binding of four different inhibitors into the active site of lumazine synthase from *Candida albicans*.

Mueller-Dieckmann, C., Kernstock, S., Lisurek, M., von Kries, J.P., Haag, F., Weiss, M.S., and Koch-Nolte, F. (2006) The structure of human ADP-ribosylhydrolase 3 (ARH3) provides insights into the reversibility of protein ADP-ribosylation. *PNAS USA* **103**, 15026-15031.

Abstract: Posttranslational modifications are used by cells from all kingdoms of life to control enzymatic activity and to regulate protein function. For many cellular processes, including DNA repair, spindle function, and apoptosis, reversible mono- and polyADP-ribosylation constitutes a very important regulatory mechanism. Moreover, many pathogenic bacteria secrete toxins which ADP-ribosylate human proteins, causing diseases such as whooping cough, cholera, and diphtheria. Whereas the 3D structures of numerous ADP-ribosylating toxins and related mammalian enzymes have been elucidated, virtually nothing is known about the structure of protein de-ADP-ribosylating enzymes. Here, we report the 3D structure of human ADP-ribosylhydrolase 3 (hARH3). The molecular architecture of hARH3 constitutes the archetype of an all- α -helical protein fold and provides insights into the reversibility of protein ADP-ribosylation. Two magnesium ions flanked by highly conserved amino acids pinpoint the active-site crevice. Recombinant hARH3 binds free ADP-ribose with micromolar affinity and efficiently de-ADP-ribosylates poly- but not mono-ADP-ribosylated proteins. Docking experiments indicate a possible binding mode for ADP-ribose polymers and suggest a reaction mechanism. Our results underscore the importance of endogenous ADP-ribosylation cycles and provide a basis for structure-based design of ADP-ribosylhydrolase inhibitors.

Nomura K. and Corzo G. (2006) The effect of binding of spider-derived antimicrobial peptides, oxyopinins, on lipid membranes. *Biochim Biophys Acta* **1758**, 1475-1482.

Abstract: Oxyopinins (Oxki1 and Oxki2) are antimicrobial peptides isolated from the crude venom of the wolf spider *Oxyopes kitabensis*. The effect of oxyopinins on lipid bilayers was investigated using high-sensitivity titration calorimetry and ^{31}P solid-state NMR spectroscopy. High-sensitivity titration calorimetry experiments showed that the binding of oxyopinins was exothermic, and the binding enthalpies (ΔH) to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) small unilamellar vesicles (SUVs) were -18.1 kcal/mol and -15.0 kcal/mol for Oxki1 and Oxki2, respectively, and peptide partition coefficient ($K(p)$) was found to be $3.9 \times 10^3 \text{ M}^{-1}$. ^{31}P NMR spectra of 1,2-dielaidoyl-sn-glycero-3-phosphoethanolamine (DEPE) membranes in the presence of oxyopinins indicated that they induced a positive curvature in lipid bilayers. The induced positive curvature was stronger in the presence of Oxki2 than in the presence of Oxki1. ^{31}P NMR spectra of phosphatidylcholine (PC) membranes in the presence of Oxki2 showed that Oxki2 produced micellization of membranes at low peptide concentrations, but unsaturated PC membranes or acidic phospholipids prevented micellization from occurring. Furthermore, ^{31}P NMR spectra using membrane lipids from *E. coli* suggested that Oxki1 was more disruptive to bacterial membranes than Oxki2. These results strongly correlate to the known biological activity of the oxyopinins.

Ozen C., Malek J. M., and Serpersu E. H. (2006) Dissection of aminoglycoside-enzyme interactions: a calorimetric and NMR study of neomycin B binding to the aminoglycoside phosphotransferase(3')-IIIa. *J Am Chem Soc* **128**, 15248-15254.

Abstract: In this work, for the first time, we report pKa values of the amino functions in a target-bound aminoglycoside antibiotic, which permitted dissection of the thermodynamic properties of an enzyme-aminoglycoside complex. Uniformly enriched ^{15}N -neomycin was isolated from cultures of *Streptomyces fradiae* and used to study its binding to the aminoglycoside phosphotransferase(3')-IIIa (APH) by ^{15}N NMR spectroscopy. ^{15}N NMR studies showed that binding of neomycin to APH causes upshifts of approximately 1 pKa unit for the amines N2' and N2' " while N6' experienced a 0.3 pKa unit shift. The pKa of N6' " remained unaltered, and resonances of N1 and N3 showed significant broadening upon binding to the enzyme. The binding-linked protonation and pH dependence of the association constant (K_b) for the enzyme-aminoglycoside complex was determined by isothermal titration calorimetry. The enthalpy of binding became more favorable (negative) with increasing pH. At high pH, binding-linked protonation was attributable mostly to the amino functions of neomycin; however, at neutral pH, functional groups of the enzyme, possibly remote from the active site, also underwent protonation/deprotonation upon formation of the binary enzyme-neomycin complex. The K_b for the enzyme-neomycin complex showed a complicated dependence on pH, indicating that multiple interactions may affect the affinity of the ligand to the enzyme and altered conditions, such as pH, may favor one or another. This work highlights the importance of determining thermodynamic parameters of aminoglycoside-target interactions under different conditions before making attributions to specific sites and their effects on these global parameters.

Pilch D. S., Kaul M., Barbieri C. M., and Kerrigan J. E. (2003) Thermodynamics of aminoglycoside-rRNA recognition. *Biopolymers* **70**, 58-79.

Abstract: 2-Deoxystreptamine (2-DOS) aminoglycosides are a family of structurally related broad-spectrum antibiotics that are used widely in the treatment of infections caused by aerobic Gram-negative bacilli. Their antibiotic activities are ascribed to their abilities to bind a highly conserved A site in the 16 S rRNA of the 30 S ribosomal subunit and interfere with protein synthesis. The abilities of the 2-DOS aminoglycosides to recognize a specific subdomain of a large RNA molecule make these compounds archetypical models for RNA-targeting drugs. This article presents a series of calorimetric, spectroscopic, osmotic stress, and computational studies designed to evaluate the thermodynamics (ΔG , ΔH , ΔS , ΔC_p) of aminoglycoside-rRNA interactions, as well as the hydration changes that accompany these interactions. In conjunction with the current structural database, the results of these studies provide important insights into the molecular forces that dictate and control the rRNA binding affinities and specificities of the aminoglycosides. Significantly, identification of these molecular driving forces [which include binding-linked drug protonation reactions, polyelectrolyte contributions from counterion release, conformational changes, hydration effects, and molecular interactions (e.g., hydrogen bonds and van der Waals interactions)], as well as the relative magnitudes of their contributions to the binding free energy, could not be achieved by consideration of structural data alone, highlighting the importance of acquiring both thermodynamic and structural information for developing a complete understanding of the drug-RNA binding process. The results presented here begin to establish a database that can be used to predict, over a range of conditions, the relative affinity of a given aminoglycoside or aminoglycoside mimetic for a targeted RNA site vs binding to potential competing secondary sites. This type of predictive capability is essential for establishment of a rational design approach to the development of new RNA-targeted drugs.

Pilch D.S., Kaul M., and Barbieri C. M. (2005) Ribosomal RNA Recognition by Aminoglycoside Antibiotics. *Top Curr Chem* **253**, 179-204.

Abstract: 2-Deoxystreptamine (2-DOS) aminoglycosides are a family of structurally related broad-spectrum antibiotics that are used widely in the clinic. Their antibiotic activities are ascribed to their abilities to bind a highly conserved sequence (termed the A site) in the 16S rRNA of the 30S ribosomal subunit and interfere with protein synthesis. The aminoglycosides represent a paradigm for both drug-RNA and drug-ribosome interactions, and information gleaned from their study has relevance with regard to other RNA- and ribosome-directed drugs of acute clinical importance. This contribution provides an integrated overview of structural and thermodynamic studies of the rRNA binding of aminoglycosides. The results of these studies have enhanced our understanding of the molecular forces that govern aminoglycoside recognition of the rRNA A site, and underlie the mechanism and specificity of action of

these drugs. Such knowledge provides the type of predictive capabilities that are essential for the development of a rational basis for future drug design strategies.

Rezansoff A. J., Hunter H. N., Jing W., Park I. Y., Kim S. C., and Vogel H. J. (2005) Interactions of the antimicrobial peptide Ac-FRWHR-NH(2) with model membrane systems and bacterial cells. *J Pept Res* **65**, 491-501.

Abstract: The acetylated and amidated hexapeptide FRWWHR (combi-2), previously identified by combinatorial chemistry methods, shows strong antimicrobial activity. The binding of the peptide to 1-palmitoyl-2-oleoyl-sn-glycero-3-[(phospho-rac-(1-glycerol))] (POPG) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles was studied using fluorescence spectroscopy and isothermal titration calorimetry (ITC). Differential scanning calorimetry (DSC) with dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) multilamellar vesicles was performed to determine changes in the lipid phase behaviour upon binding the peptide. Two-dimensional proton nuclear magnetic resonance (NMR) spectroscopy, to solve the bound peptide structure, was performed in the presence of dodecylphosphatidylcholine (DPC) and sodium dodecyl sulphate (SDS) micelles. The fluorescence, ITC and DSC studies indicate that the peptide interacts preferentially with lipid vesicles containing negatively charged head groups. Conformational information determined using NMR indicate that the combi-2 peptide adopts a coiled amphipathic conformation when bound to SDS and DPC micelles. Leakage assays indicate that the peptide is not very efficient at causing leakage from calcein-filled large unilamellar vesicles comprised of POPG/POPC (1 : 1). The rapid passage of either the fluorescent-tagged peptides combi-2 or the previously studied peptide Ac-RRWRF-NH(2) (combi-1) into *Escherichia coli* and *Staphylococcus aureus* suggests that instead of membrane disruption, the main bactericidal site of action of these peptides might be located inside bacteria.

Ozen C. and Serpersu E. H. (2004) Thermodynamics of aminoglycoside binding to aminoglycoside-3'-phosphotransferase IIIa studied by isothermal titration calorimetry. *Biochemistry* **43**, 14667-14675.

Abstract: The aminoglycoside-3'-phosphotransferase IIIa [APH(3')-IIIa] phosphorylates aminoglycoside antibiotics and renders them ineffective against bacteria. APH(3')-IIIa is the most promiscuous aminoglycoside phosphotransferase enzyme, and it modifies more than 10 different aminoglycoside antibiotics. A wealth of information exists about the enzyme; however, thermodynamic properties of enzyme-aminoglycoside complexes are still not known. This study describes the determination of the thermodynamic parameters of the binary enzyme-aminoglycoside and the ternary enzyme-metal-ATP-aminoglycoside complexes of structurally related aminoglycosides using isothermal titration calorimetry. Formation of the binary enzyme-aminoglycoside complexes is enthalpically driven and exhibits a strongly disfavored entropic contribution. Formation of the ternary enzyme-metal-ATP-aminoglycoside complexes yields much smaller negative ΔH values and more favorable entropic contributions. The presence of metal-ATP generally increases the affinity of aminoglycosides to the enzyme. This is consistent with the kinetic mechanism of the enzyme in which ordered binding of substrates occurs. However, the observed ΔH values neither correlate with kinetic parameters k_{cat} , K_m , and k_{cat}/K_m nor correlate with the molecular size of the substrates. Comparison of the thermodynamic properties of the complexes formed by structurally similar aminoglycosides indicated that the 2'- and the 6'-amino groups of the substrates are involved in binding to the enzyme. Thermodynamic properties of the complexes formed by aminoglycosides differing only at the 3'-hydroxyl group suggested that the absence of this group does not alter the thermodynamic parameters of the ternary APH(3')-IIIa-metal-ATP-aminoglycoside complex. Our results also indicate that protonation of ligand and protein ionizable groups is coupled to the complex formation between aminoglycosides and APH(3')-IIIa. Comparison of ΔH values for different aminoglycoside-enzyme complexes indicates that enzyme and substrates undergo significant conformational changes in complex formation.

Ozen C., Malek J. M. and Serpersu E. H. (2007) Dissection of Aminoglycoside-Enzyme Interactions: A Calorimetric and NMR Study of Noemycin B Binding to the Aminoglycoside Phosphotransferase(3')-IIIa [J. Am. Chem. Soc. 2006, 128, 15248-15254]. *J Am. Chem Soc.* **129**, 11872. (no abstract)

Ozen C., Norris A. L., Land M. L., Tjioe E. and Serpersu E. H. (2008) Detection of Specific Solvent Rearrangement Regions of an Enzyme: NMR and ITC Studies with Aminoglycoside Phosphotransferase(3')-IIIa. *Biochemistry* **47**, 40-49.

Abstract: This work describes differential effects of solvent in complexes of the aminoglycoside phosphotransferase(3')-IIIa (APH) with different aminoglycosides and the detection of change in solvent structure at specific sites away from substrates. Binding of kanamycins to APH occurs with a larger negative ΔH in H₂O relative to D₂O ($\Delta\Delta H(\text{H}_2\text{O}-\text{D}_2\text{O}) < 0$), while the reverse is true for neomycins. Unusually large negative ΔC_p values were observed for binding of aminoglycosides to APH. ΔC_p for the APH-neomycin complex was $-1.6 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{deg}^{-1}$. A break at 30 degrees C was observed in the APH-kanamycin complex yielding ΔC_p values of $-0.7 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{deg}^{-1}$ and $-3.8 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{deg}^{-1}$ below and above 30 degrees C, respectively. Neither the change in accessible surface area (ΔASA) nor contributions from heats of ionization were sufficient to explain the large negative ΔC_p values. Most significantly, ¹⁵N-¹H HSQC experiments showed that temperature-dependent shifts of the backbone amide protons of Leu 88, Ser 91, Cys 98, and Leu143 revealed a break at 30 degrees C only in the APH-kanamycin complex in spectra collected between 21 degrees C and 38 degrees C. These amino acids represent solvent reorganization sites that experience a change in solvent structure in their immediate environment as structurally different ligands bind to the enzyme. These residues were away from the substrate binding site and distributed in three hydrophobic patches in APH. Overall, our results show that a large number of factors affect ΔC_p and binding of structurally different ligand groups cause different solvent structure in the active site as well as differentially affecting specific sites away from the ligand binding site.

Paetzel M., Goodall J. J., Kania M., Dalbey R. E., and Page M. G. (2004) Crystallographic and biophysical analysis of a bacterial signal peptidase in complex with a lipopeptide-based inhibitor. *J Biol Chem* **279**, 30781-30790.

Abstract: We report here the crystallographic and biophysical analysis of a soluble, catalytically active fragment of the Escherichia coli type I signal peptidase (SPase Delta2-75) in complex with arylomycin A2. The 2.5-Å resolution structure revealed that the inhibitor is positioned with its COOH-terminal carboxylate oxygen (O45) within hydrogen bonding distance of all the functional groups in the catalytic center of the enzyme (Ser90 O-gamma, Lys145 N-zeta, and Ser88 O-gamma) and that it makes beta-sheet type interactions with the beta-strands that line each side of the binding site. Ligand binding studies, calorimetry, fluorescence spectroscopy, and stopped-flow kinetics were also used to analyze the binding mode of this unique non-covalently bound inhibitor. The crystal structure was solved in the space group P4(3)2(1)2. A detailed comparison is made to the previously published acyl-enzyme inhibitor complex structure (space group: P2(1)2(1)2) and the apo-enzyme structure (space group: P4(1)2(1)2). Together this work provides insights into the binding of pre-protein substrates to signal peptidase and will prove helpful in the development of novel antibiotics.

Revuelta J., Vacas T., Torrado M., Corzana F., Gonzalez C., Jimenez-Barbero J., Menendez M., Bastida A. and Asensio J. L. (2008) NMR-based analysis of aminoglycoside recognition by the resistance enzyme ANT(4'): the pattern of OH/NH3(+) substitution determines the preferred antibiotic binding mode and is critical for drug inactivation. *J Am. Chem Soc.* **130**, 5086-5103.

Abstract: The most significant mechanism of bacterial resistance to aminoglycosides is the enzymatic inactivation of the drug. Herein, we analyze several key aspects of the aminoglycoside recognition by the resistance enzyme ANT(4') from Staphylococcus aureus, employing NMR complemented with site-directed mutagenesis experiments and measurements of the enzymatic activity on newly synthesized kanamycin derivatives. From a methodological perspective, this analysis provides the first example reported for the use of transferred NOE (trNOE) experiments in the analysis of complex molecular recognition processes, characterized by the existence of simultaneous binding events of the ligand to different regions of a protein receptor. The obtained results show that, in favorable cases, these overlapping binding processes can be isolated employing site-directed mutagenesis and then independently analyzed. From a molecular recognition perspective, this work conclusively shows that the enzyme ANT(4') displays a wide tolerance to conformational variations in the drug. Thus, according to the NMR data, kanamycin-A I/II linkage exhibits an unusual anti-Psi orientation in the ternary complex, which is in qualitative agreement with the previously reported crystallographic complex. In contrast, closely related, kanamycin-B is recognized by the enzyme in the syn-type arrangement for both glycosidic bonds. This observation together with the enzymatic activity displayed by ANT(4') against several synthetic kanamycin derivatives strongly suggests that the spatial distribution of positive charges within the aminoglycoside scaffold is the key feature that governs its preferred binding mode to the protein catalytic region and also the regioselectivity of the

adenylation reaction. In contrast, the global shape of the antibiotic does not seem to be a critical factor. This feature represents a qualitative difference between the target A-site RNA and the resistance enzyme ANT(4') as aminoglycoside receptors

Roe S. M., Prodromou C., O'Brien R., Ladbury J. E., Piper P. W., and Pearl L. H. (1999) Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J Med Chem* **42**, 260-266.

Abstract: The cellular activity of several regulatory and signal transduction proteins, which depend on the Hsp90 molecular chaperone for folding, is markedly decreased by geldanamycin and by radicicol (monorden). We now show that these unrelated compounds both bind to the N-terminal ATP/ADP-binding domain of Hsp90, with radicicol displaying nanomolar affinity, and both inhibit the inherent ATPase activity of Hsp90 which is essential for its function in vivo. Crystal structure determinations of Hsp90 N-terminal domain complexes with geldanamycin and radicicol identify key aspects of their nucleotide mimicry and suggest a rational basis for the design of novel antichaperone drugs.

Samland A. K., Amrhein N., and Macheroux P. (1999) Lysine 22 in UDP-N-acetylglucosamine enolpyruvyl transferase from *Enterobacter cloacae* is crucial for enzymatic activity and the formation of covalent adducts with the substrate phosphoenolpyruvate and the antibiotic fosfomycin. *Biochemistry* **38**, 13162-13169.

Abstract: UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) catalyzes the first committed step in the biosynthesis of the bacterial cell wall component peptidoglycan. The enzyme is the target of the antibiotic fosfomycin. A lysine residue (K22), strictly conserved in MurAs and the structurally and mechanistically related 5-enolpyruvylshikimate 3-phosphate synthases (EPSPS), is located near the active center of the enzyme. This residue is thought to be involved directly in the binding of the substrate phosphoenolpyruvate (PEP) and also to participate in the conformational change leading to the formation of the catalytically competent enzyme complex. Using site-directed mutagenesis, we have replaced this lysine with arginine (K22R), valine (K22V), and glutamate (K22E). These mutant proteins were expressed, purified, and characterized in comparison to wild-type MurA and a previously described inactive C115S mutant protein. It was found that all three K22 mutant proteins had less than 0.5% of the wild-type activity. Using isothermal titration calorimetry, it could be shown that the binding parameters for the UDP-sugar nucleotide substrate are not affected by the mutations, except for the K22E mutant protein. Similarly, binding of PEP was found to be unaffected in the K22 mutant proteins as demonstrated by tryptophan fluorescence quench titrations. On the other hand, the level of formation of a covalent adduct with either PEP or fosfomycin with the thiol group of cysteine 115 was diminished. The propensity to form an adduct with PEP decreased in the following order: wild type >> K22R > K22V > K22E. A comparable effect was found on the formation of the inhibitory covalent adduct of MurA and the antibiotic fosfomycin. These results are discussed in terms of an involvement of lysine 22 in a conformational change of MurA.

Samland A. K., Jelesarov I., Kuhn R., Amrhein N., and Macheroux P. (2001) Thermodynamic characterization of ligand-induced conformational changes in UDP-N-acetylglucosamine enolpyruvyl transferase. *Biochemistry* **40**, 9950-9956.

Abstract: The binding of UDP-N-acetylglucosamine (UDP-NAG) to the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) was studied in the absence and presence of the antibiotic fosfomycin by isothermal titration calorimetry. Fosfomycin binds covalently to MurA in the presence of UDP-NAG and also in its absence as demonstrated by MALDI mass spectrometry. The covalent attachment of fosfomycin affects the thermodynamic parameters of UDP-NAG binding significantly: In the absence of fosfomycin the binding of UDP-NAG is enthalpically driven ($\Delta H = -35.5 \text{ kJ mol}^{-1}$ at 15 degrees C) and opposed by an unfavorable entropy change ($\Delta S = -25 \text{ J mol}^{-1} \text{ K}^{-1}$). In the presence of covalently attached fosfomycin the binding of UDP-NAG is entropically driven ($\Delta S = 187 \text{ J mol}^{-1} \text{ K}^{-1}$ at 15 degrees C) and associated with unfavorable changes in enthalpy ($\Delta H = 28.8 \text{ kJ mol}^{-1}$). Heat capacities for UDP-NAG binding in the absence or presence of fosfomycin were -1.87 and $-2.74 \text{ kJ mol}^{-1} \text{ K}^{-1}$, respectively, indicating that most (approximately 70%) of the conformational changes take place upon formation of the UDP-NAG-MurA binary complex. The major contribution to the heat capacity of ligand binding is thought to be due to changes in the solvent-accessible surface area. However, associated conformational changes, if any, also contribute to the experimentally measured magnitude of the heat capacity. The changes in

solvent-accessible surface area were calculated from available 3D structures, yielding a ΔC_p of $-1.3 \text{ kJ mol}^{-1} \text{ K}^{-1}$; i.e., the experimentally determined heat capacity exceeds the calculated one. This implies that other thermodynamic factors exert a large influence on the heat capacity of protein-ligand interactions.

Samland A. K., Jelesarov I., Kuhn R., Amrhein N., and Macheroux P. (2001) Thermodynamic characterization of ligand-induced conformational changes in UDP-N-acetylglucosamine enolpyruvyl transferase. *Biochemistry* **40**, 9950-9956.

Abstract: The binding of UDP-N-acetylglucosamine (UDP-NAG) to the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) was studied in the absence and presence of the antibiotic fosfomycin by isothermal titration calorimetry. Fosfomycin binds covalently to MurA in the presence of UDP-NAG and also in its absence as demonstrated by MALDI mass spectrometry. The covalent attachment of fosfomycin affects the thermodynamic parameters of UDP-NAG binding significantly: In the absence of fosfomycin the binding of UDP-NAG is enthalpically driven ($\Delta H = -35.5 \text{ kJ mol}^{-1}$ at 15 degrees C) and opposed by an unfavorable entropy change ($\Delta S = -25 \text{ J mol}^{-1} \text{ K}^{-1}$). In the presence of covalently attached fosfomycin the binding of UDP-NAG is entropically driven ($\Delta S = 187 \text{ J mol}^{-1} \text{ K}^{-1}$ at 15 degrees C) and associated with unfavorable changes in enthalpy ($\Delta H = 28.8 \text{ kJ mol}^{-1}$). Heat capacities for UDP-NAG binding in the absence or presence of fosfomycin were -1.87 and $-2.74 \text{ kJ mol}^{-1} \text{ K}^{-1}$, respectively, indicating that most (approximately 70%) of the conformational changes take place upon formation of the UDP-NAG-MurA binary complex. The major contribution to the heat capacity of ligand binding is thought to be due to changes in the solvent-accessible surface area. However, associated conformational changes, if any, also contribute to the experimentally measured magnitude of the heat capacity. The changes in solvent-accessible surface area were calculated from available 3D structures, yielding a ΔC_p of $-1.3 \text{ kJ mol}^{-1} \text{ K}^{-1}$; i.e., the experimentally determined heat capacity exceeds the calculated one. This implies that other thermodynamic factors exert a large influence on the heat capacity of protein-ligand interactions.

Savic M., Ilic-Tomic T., Macmaster R., Vasiljevic B. and Conn G. L. (2008) Critical residues for cofactor binding and catalytic activity in the aminoglycoside resistance methyltransferase Sgm. *J Bacteriol.* **190**, 5855-5861.

Abstract: The 16S rRNA methyltransferase Sgm from "Micromonospora zionensis" confers resistance to aminoglycoside antibiotics by specific modification of the 30S ribosomal A site. Sgm is a member of the FmrO family, distant relatives of the S-adenosyl-L-methionine (SAM)-dependent RNA subfamily of methyltransferase enzymes. Using amino acid conservation across the FmrO family, seven putative key amino acids were selected for mutation to assess their role in forming the SAM cofactor binding pocket or in methyl group transfer. Each mutated residue was found to be essential for Sgm function, as no modified protein could effectively support bacterial growth in liquid media containing gentamicin or methylate 30S subunits in vitro. Using isothermal titration calorimetry, Sgm was found to bind SAM with a $K(D)$ (binding constant) of 17.6 microM , and comparable values were obtained for one functional mutant (N179A) and four proteins modified at amino acids predicted to be involved in catalysis in methyl group transfer. In contrast, none of the G135, D156, or D182 Sgm mutants bound the cofactor, confirming their role in creating the SAM binding pocket. These results represent the first functional characterization of any FmrO methyltransferase and may provide a basis for a further structure-function analysis of these aminoglycoside resistance determinants

Sean Peacock. R., Weljie A. M., Peter H. S., Price F. D., and Vogel H. J. (2005) The solution structure of the C-terminal domain of TonB and interaction studies with TonB box peptides. *J Mol Biol* **345**, 1185-1197.

Abstract: The TonB protein transduces energy from the proton gradient across the cytoplasmic membrane of Gram-negative bacteria to TonB-dependent outer membrane receptors. It is a critically important protein in iron uptake, and deletion of this protein is known to decrease virulence of bacteria in animal models. This system has been used for Trojan horse antibiotic delivery. Here, we describe the high-resolution solution structure of *Escherichia coli* TonB residues 103-239 (TonB-CTD). TonB-CTD is monomeric with an unstructured N terminus (103-151) and a well structured C terminus (152-239). The structure contains a four-stranded antiparallel beta-sheet packed against two alpha-helices and an extended strand in a configuration homologous to the C-terminal domain of the TolA protein. Chemical shift perturbations to

the TonB-CTD (1)H-(15)N HSCQ spectrum titrated with TonB box peptides modeled from the E.coli FhuA, FepA and BtuB proteins were all equivalent, indicating that all three peptides bind to the same region of TonB. Isothermal titration calorimetry measurements demonstrate that TonB-CTD interacts with the FhuA-derived peptide with a $K(D)=36(+/-7)$ microM. On the basis of chemical shift data, the position of Gln160, and comparison to the TolA gp3 N1 complex crystal structure, we propose that the TonB box binds to TonB-CTD along the beta3-strand.

Seelig J. (2004) Thermodynamics of lipid-peptide interactions. *Biochim Biophys Acta* **1666**, 40-50.

Abstract: This review is focused on peptide molecules which exhibit a limited solubility in the aqueous phase and bind to the lipid membrane from the aqueous medium. Surface adsorption, membrane insertion, and specific binding are usually accompanied by changes in the heat content of the system and can be measured conveniently with isothermal titration calorimetry, avoiding the necessity of peptide labeling. The driving forces for peptide adsorption and binding are hydrophobicity, electrostatics, and hydrogen bonding. An exclusively hydrophobic interaction is exemplified by the immunosuppressant drug cyclosporine A. Its insertion into the membrane can be described by a simple partition equilibrium $X_b=K_0C_{eq}$. If peptide and membrane are both charged, electrostatic interactions are dominant leading to nonlinear binding curves. The concentration of the peptide near the membrane interface can then be much larger than its bulk concentration. Electrostatic effects must be accounted for by means of the Gouy-Chapman theory before conventional binding models can be applied. A small number of peptides and proteins bind with very high affinity to a specific lipid species only. This is illustrated for the lantibiotic cinnamycin (Ro 09-0198) which forms a 1:1 complex with phosphatidylethanolamine with a binding constant of $10^8 M^{-1}$. Membrane adsorption and insertion can be accompanied by conformational transitions facilitated, in part, by hydrogen bonding mechanisms. The two membrane-induced conformational changes to be discussed are the random coil-to-alpha-helix transition of amphipathic peptides and the random coil-to-beta-structure transition of Alzheimer peptides.

Senkovich O., Cook W. J., Mirza S., Hollingshead S. K., Protasevich I. I., Briles D. E. and Chattopadhyay D. (2007) Structure of a complex of human lactoferrin N-lobe with pneumococcal surface protein A provides insight into microbial defense mechanism. *J Mol Biol* **370**, 701-713.

Abstract: Human lactoferrin, a component of the innate immune system, kills a wide variety of microorganisms including the Gram positive bacteria *Streptococcus pneumoniae*. Pneumococcal surface protein A (PspA) efficiently inhibits this bactericidal action. The crystal structure of a complex of the lactoferrin-binding domain of PspA with the N-lobe of human lactoferrin reveals direct and specific interactions between the negatively charged surface of PspA helices and the highly cationic lactoferricin moiety of lactoferrin. Binding of PspA blocks surface accessibility of this bactericidal peptide preventing it from penetrating the bacterial membrane. Results of site-directed mutagenesis, in vitro protein binding assays and isothermal titration calorimetry measurements corroborate that the specific electrostatic interactions observed in the crystal structure represent major associations between PspA and lactoferrin. The structure provides a snapshot of the protective mechanism utilized by pathogens against the host's first line of defense. PspA represents a major virulence factor and a promising vaccine candidate. Insights from the structure of the complex have implications for designing therapeutic strategies for treatment and prevention of pneumococcal diseases that remain a major public health problem worldwide.

Shalev D. E., Rotem S., Fish A., and Mor A. (2006) Consequences of N-acylation on structure and membrane binding properties of dermaseptin derivative K4-S4-(1-13). *J Biol Chem* **281**, 9432-9438.

Abstract: Acyl conjugation to antimicrobial peptides is known to enhance antimicrobial properties. Here, we investigated the consequences of aminolauryl (NC(12)) conjugation to the dermaseptin derivative K(4)-S4-(1-13) (P) on binding properties to bilayer models mimicking bacterial plasma membrane, which is often cited as the ultimate site of action. Isothermal titration calorimetry revealed that acylation was responsible for enhancing the binding affinity of NC(12)-P compared with P ($K = 13 \times 10^5$ and $1.5 \times 10^5 m^{-1}$, respectively). Surface plasmon resonance measurements confirmed the isothermal titration calorimetry results ($K(app) = 12.6 \times 10^5$ and $1.53 \times 10^5 m^{-1}$, respectively) and further indicated that enhanced adhesion affinity ($K(adhesion) = 3 \times 10^5$ and $1 \times 10^5 m^{-1}$, respectively) was coupled to enhanced tendency to insert within the bilayer ($K(insertion) = 4.5$ and 1.5 , respectively). To gain insight into the molecular basis for these observations, we investigated the three-dimensional structures in the presence of dodecylphosphocholine using NMR. The ensemble of NMR-calculated structures (backbone

root mean square deviation $<0.6 \text{ \AA}$) showed that the acyl moiety was responsible for a significant molecular reorganization, possibly affecting the electrostatic potential distribution in NC(12)-P relative to that of P. The combined data present compelling evidence in support of the hypothesis that N-acylation affects antimicrobial properties by modifying the secondary structure of the peptide in a manner that facilitates contact with the membrane and consequently increases its disruption.

Soykut E. A., Dudak F. C. and Boyaci I. H. (2008) Selection of staphylococcal enterotoxin B (SEB)-binding peptide using phage display technology. *Biochem Biophys Res Commun* **370**, 104-108.

Abstract: In this study, peptides were selected to recognize staphylococcal enterotoxin B (SEB) which cause food intoxication and can be used as a biological war agent. By using commercial M13 phage library, single plaque isolation of 38 phages was done and binding affinities were investigated with phage-ELISA. The specificities of the selected phage clones showing high affinity to SEB were checked by using different protein molecules which can be found in food samples. Furthermore, the affinities of three selected phage clones were determined by using surface plasmon resonance (SPR) sensors. Sequence analysis was realized for three peptides showing high binding affinity to SEB and WWRPLTPESPPA, MNLHDYHRLFWY, and QHPQINQTLYRM amino acid sequences were obtained. The peptide sequence with highest affinity to SEB was synthesized with solid phase peptide synthesis technique and thermodynamic constants of the peptide-SEB interaction were determined by using isothermal titration calorimetry (ITC) and compared with those of antibody-SEB interaction. The binding constant of the peptide was determined as $4.2 \pm 0.7 \times 10^5 \text{ M}^{-1}$ which indicates a strong binding close to that of antibody

Stiff C. M., Zhong M., Sarver R. W., Gao H., Ho A. M., Sweeney M. T., Zurenko G. E. and Romero D. L. (2007) Correlation of carboxylic acid pKa to protein binding and antibacterial activity of a novel class of bacterial translation inhibitors. *Bioorg. Med. Chem Lett* **17**, 5479-5482.

Abstract: Previously we reported the discovery and initial optimization of a novel anthranilic acid derived class of antibacterial agents which suffered from extensive protein binding. This report describes efforts directed toward understanding the relationship of the acidity of the carboxylic acid with the extent of protein binding. The pK(a) of the acid was modified via the synthesis of a number of anthranilic acid analogs which vary the aromatic ring substituent at the 4-position. The pK(a) and HSA binding constants have been determined for each of the analogs. Our results indicate a correlation between pK(a) and HSA K(d). The physical properties and antibacterial activities will be discussed as well as how these results help address the protein binding issue with this series of compounds.

Sun T. and Zhang Y. (2008) Pentamidine binds to tRNA through non-specific hydrophobic interactions and inhibits aminoacylation and translation. *Nucleic Acids Res* **36**, 1654-1664.

Abstract: The selective and potent inhibition of mitochondrial translation in *Saccharomyces cerevisiae* by pentamidine suggests a novel antimicrobial action for this drug. Electrophoresis mobility shift assay, T1 ribonuclease footprinting, hydroxyl radical footprinting and isothermal titration calorimetry collectively demonstrated that pentamidine non-specifically binds to two distinct classes of sites on tRNA. The binding was driven by favorable entropy changes indicative of a large hydrophobic interaction, suggesting that the aromatic rings of pentamidine are inserted into the stacked base pairs of tRNA helices. Pentamidine binding disrupts the tRNA secondary structure and masks the anticodon loop in the tertiary structure. Consistently, we showed that pentamidine specifically inhibits tRNA aminoacylation but not the cognate amino acid adenylation. Pentamidine inhibited protein translation in vitro with an EC(50) equivalent to that binds to tRNA and inhibits tRNA aminoacylation in vitro, but drastically higher than that inhibits translation in vivo, supporting the established notion that the antimicrobial activity of pentamidine is largely due to its selective accumulation by the pathogen rather than by the host cell. Therefore, interrupting tRNA aminoacylation by the entropy-driven non-specific binding is an important mechanism of pentamidine in inhibiting protein translation, providing new insights into the development of antimicrobial drugs

Svenson J., Brandsdal B. O., Stensen W. and Svendsen J. S. (2007) Albumin binding of short cationic antimicrobial micropeptides and its influence on the in vitro bactericidal effect. *J Med. Chem* **50**, 3334-3339.

Abstract: The interactions between a range of small cationic antibacterial tripeptides and bovine and human serum albumin in a buffered aqueous solution at 25 degrees C have been studied using isothermal

titration calorimetry. Results from the binding study indicate a single binding site on albumin with a dissociation constant between 4.3 and 22.2 microM for the different peptides. In a theoretical mouse model, a dissociation constant in this range corresponds to 95% albumin binding. The effect of this albumin interaction on the antibacterial capacity of the peptides against *Staphylococcus aureus*, strain ATCC 25923 was studied by including albumin in the assays at a 0.55 mM concentration. Presence of albumin induced a 10-fold increase of the minimal inhibitory concentration for the bulk of the peptides. Albumin itself has no effect on the bacterial growth and this increase is entirely ascribed to a strong competing protein binding. Collectively these results indicate that these antibacterial peptides do bind to albumin and that this binding strongly reduces the effective concentration of peptides available to combat bacteria.

Svenson J., Stensen W., Brandsdal B. O., Haug B. E., Monrad J. and Svendsen J. S. (2008) Antimicrobial peptides with stability toward tryptic degradation. *Biochemistry* **47**, 3777-3788.

Abstract: The inherent instability of peptides toward metabolic degradation is an obstacle on the way toward bringing potential peptide drugs onto the market. Truncation can be one way to increase the proteolytic stability of peptides, and in the present study the susceptibility against trypsin, which is one of the major proteolytic enzymes in the gastrointestinal tract, was investigated for several short and diverse libraries of promising cationic antimicrobial tripeptides. Quite surprisingly, trypsin was able to cleave very small cationic antimicrobial peptides at a substantial rate. Isothermal titration calorimetry studies revealed stoichiometric interactions between selected peptides and trypsin, with dissociation constants ranging from 1 to 20 microM. Introduction of hydrophobic C-terminal amide modifications and likewise bulky synthetic side chains on the central amino acid offered an effective way to increased half-life in our assays. Analysis of the degradation products revealed that the location of cleavage changed when different end-capping strategies were employed to increase the stability and the antimicrobial potency. This suggests that trypsin prefers a bulky hydrophobic element in S1' in addition to a positively charged side chain in S1 and that this binding dictates the mode of cleavage for these substrates. Molecular modeling studies supported this hypothesis, and it is shown that small alterations of the tripeptide result in two very different modes of trypsin binding and degradation. The data presented allows for the design of stable cationic antibacterial peptides and/or peptidomimetics based on several novel design principles

Swaminathan C. P., Brown P. H., Roychowdhury A., Wang Q., Guan R., Silverman N., Goldman W. E., Boons G. J., and Mariuzza R. A. (2006) Dual strategies for peptidoglycan discrimination by peptidoglycan recognition proteins (PGRPs). *Proc Natl Acad Sci U S A* **103**, 684-689.

Abstract: The innate immune system constitutes the first line of defense against microorganisms in both vertebrates and invertebrates. Although much progress has been made toward identifying key receptors and understanding their role in host defense, far less is known about how these receptors recognize microbial ligands. Such studies have been severely hampered by the need to purify ligands from microbial sources and a reliance on biological assays, rather than direct binding, to monitor recognition. We used synthetic peptidoglycan (PGN) derivatives, combined with microcalorimetry, to define the binding specificities of human and insect peptidoglycan recognition proteins (PGRPs). We demonstrate that these innate immune receptors use dual strategies to distinguish between PGNs from different bacteria: one based on the composition of the PGN peptide stem and another that senses the peptide bridge crosslinking the stems. To pinpoint the site of PGRPs that mediates discrimination, we engineered structure-based variants having altered PGN-binding properties. The plasticity of the PGRP-binding site revealed by these mutants suggests an intrinsic capacity of the innate immune system to rapidly evolve specificities to meet new microbial challenges.

te Welscher Y. M., ten Napel H. H., Balague M. M., Souza C. M., Riezman H., de K. B. and Breukink E. (2008) Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *J Biol Chem* **283**, 6393-6401.

Abstract: Natamycin is a polyene antibiotic that is commonly used as an antifungal agent because of its broad spectrum of activity and the lack of development of resistance. Other polyene antibiotics, like nystatin and filipin are known to interact with sterols, with some specificity for ergosterol thereby causing leakage of essential components and cell death. The mode of action of natamycin is unknown and is investigated in this study using different in vitro and in vivo approaches. Isothermal titration calorimetry and direct binding studies revealed that natamycin binds specifically to ergosterol present in model membranes. Yeast sterol biosynthetic mutants revealed the importance of the double bonds in the B-ring of

ergosterol for the natamycin-ergosterol interaction and the consecutive block of fungal growth. Surprisingly, in strong contrast to nystatin and filipin, natamycin did not change the permeability of the yeast plasma membrane under conditions that growth was blocked. Also, in ergosterol containing model membranes, natamycin did not cause a change in bilayer permeability. This demonstrates that natamycin acts via a novel mode of action and blocks fungal growth by binding specifically to ergosterol

Thennarasu S., Lee D. K., Tan A., Prasad K. U., and Ramamoorthy A. (2005) Antimicrobial activity and membrane selective interactions of a synthetic lipopeptide MSI-843. *Biochim Biophys Acta* **1711**, 49-58. **Abstract:** Lipopeptide MSI-843 consisting of the nonstandard amino acid ornithine (Oct-OOLLOOLOOL-NH₂) was designed with an objective towards generating non-lytic short antimicrobial peptides, which can have significant pharmaceutical applications. Octanoic acid was coupled to the N-terminus of the peptide to increase the overall hydrophobicity of the peptide. MSI-843 shows activity against bacteria and fungi at micromolar concentrations. It permeabilizes the outer membrane of Gram-negative bacterium and a model membrane mimicking bacterial inner membrane. Circular dichroism investigations demonstrate that the peptide adopts alpha-helical conformation upon binding to lipid membranes. Isothermal titration calorimetry studies suggest that the peptide binding to membranes results in exothermic heat of reaction, which arises from helix formation and membrane insertion of the peptide. ²H NMR of deuterated-POPC multilamellar vesicles shows the peptide-induced disorder in the hydrophobic core of bilayers. ³¹P NMR data indicate changes in the lipid head group orientation of POPC, PPG and Escherichia coli total lipid bilayers upon peptide binding. Results from ³¹P NMR and dye leakage experiments suggest that the peptide selectively interacts with anionic bilayers at low concentrations (up to 5 mol%). Differential scanning calorimetry experiments on DiPOPE bilayers and ³¹P NMR data from E.coli total lipid multilamellar vesicles indicate that MSI-843 increases the fluid lamellar to inverted hexagonal phase transition temperature of bilayers by inducing positive curvature strain. Combination of all these data suggests the formation of a lipid-peptide complex resulting in a transient pore as a plausible mechanism for the membrane permeabilization and antimicrobial activity of the lipopeptide MSI-843.

Thomas C. J., Surolia N., and Surolia A. (1999) Surface plasmon resonance studies resolve the enigmatic endotoxin neutralizing activity of polymyxin B. *J Biol Chem* **274**, 29624-29627. **Abstract:** Polymyxin B (PMB), a cyclic cationic peptide antibiotic, despite its severe side effects continues to occupy a premiere position for treating endotoxemia. Its mode of neutralization of endotoxin has remained elusive for the last three decades. Several synthetic peptide mimics of PMB, capable of binding endotoxin, have been made. However, the binding ability alone appears to be a deceptive indicator of endotoxin neutralizing activity as molecules with similar binding propensities could either sequester or opsonize the toxin. Hence identification of additional physical parameters which describe adequately the outcome of PMB-endotoxin interaction become imperative. Surface plasmon resonance (SPR) studies reported here show that several mimics of PMB despite exhibiting lipopolysaccharide binding affinities comparable with it but, unlike it, do not sequester the endotoxin. These studies thus provide a striking illustration of the difference in the behavior of PMB, vis a vis its mimics toward the endotoxin lamellae, and define further, in chemical terms, mechanism of the action of PMB and allow us to posit that the design of molecules as effective antidotes for sepsis should incorporate the ability to sequester endotoxin specifically.

Thoppil A. A., Sharma R. and Kishore N. (2008) Complexation of beta-lactam antibiotic drug carbenicillin to bovine serum albumin: energetics and conformational studies. *Biopolymers* **89**, 831-840. **Abstract:** Binding of the antibiotic drug carbenicillin to bovine serum albumin (BSA) has been studied using isothermal titration calorimetry (ITC) in combination with fluorescence and circular dichroism (CD) spectroscopies. The thermodynamic parameters of binding have been evaluated as a function of temperature, ionic strength, and in the presence of anionic, cationic and nonionic surfactants, tetrabutylammonium bromide, and sucrose. The values of van't Hoff enthalpy do not agree with the calorimetric enthalpy indicating conformational changes in the protein upon drug binding. These observations are supported by the intrinsic fluorescence and CD spectroscopic measurements. A reduction in the binding affinity of carbenicillin to BSA is observed with increase in ionic strength of the solution, thereby suggesting, prevailing of electrostatic interactions in the binding process. The involvement of hydrophobic interactions in the binding of the drug to the protein is also indicated by a slight reduction in binding constant in the presence of tetrabutylammonium bromide. The experiments in the presence of

sucrose suggest that hydrogen bonding is perhaps not dominant in the binding. The anionic surfactant sodium dodecyl sulphate (SDS) is observed to completely interfere in the ionic interactions in addition to its partial denaturing capacity. However, the presence of cationic surfactant hexadecyl trimethylammonium bromide (HTAB) and nonionic surfactant Triton-X 100 induce a slight reduction in the values of binding affinity. These calorimetric and spectroscopic results, provide quantitative information on the binding of carbenicillin to BSA and suggests that the binding is dominated by electrostatic interactions with contribution from hydrophobic interactions

Verly R. M., Rodrigues M. A., Daghasanli K. R., Denadai A. M., Cuccovia I. M., Bloch C., Jr., Frezard F., Santoro M. M., Pilo-Veloso D. and Bemquerer M. P. (2008) Effect of cholesterol on the interaction of the amphibian antimicrobial peptide DD K with liposomes. *Peptides* **29**, 15-24.

Abstract: DD K is an antimicrobial peptide previously isolated from the skin of the amphibian *Phyllomedusa distincta*. The effect of cholesterol on synthetic DD K binding to egg lecithin liposomes was investigated by intrinsic fluorescence of tryptophan residue, measurements of kinetics of 5(6)-carboxyfluorescein (CF) leakage, dynamic light scattering and isothermal titration microcalorimetry. An 8 nm blue shift of tryptophan maximum emission fluorescence was observed when DD K was in the presence of lecithin liposomes compared to the value observed for liposomes containing 43 mol% cholesterol. The rate and the extent of CF release were also significantly reduced by the presence of cholesterol. Dynamic light scattering showed that lecithin liposome size increase from 115 to 140 nm when titrated with DD K but addition of cholesterol reduces the liposome size increments. Isothermal titration microcalorimetry studies showed that DD K binding both to liposomes containing cholesterol as to liposomes devoid of it is more entropically than enthalpically favored. Nevertheless, the peptide concentration necessary to furnish an adjustable titration curve is much higher for liposomes containing cholesterol at 43 mol% (2 mmol L⁻¹) than in its absence (93 micromol L⁻¹). Apparent binding constant values were 2160 and 10,000 L mol⁻¹, respectively. The whole data indicate that DD K binding to phosphatidylcholine liposomes is significantly affected by cholesterol, which contributes to explain the low hemolytic activity of the peptide

Wei S. Y., Wu J. M., Kuo Y. Y., Chen H. L., Yip B. S., Tzeng S. R., and Cheng J. W. (2006) Solution structure of a novel tryptophan-rich peptide with bidirectional antimicrobial activity. *J Bacteriol* **188**, 328-334.

Abstract: Trp-rich antimicrobial peptides play important roles in the host innate defense mechanisms of many plants, insects, and mammals. A new type of Trp-rich peptide, Ac-KWRRWVRWI-NH(2), designated Pac-525, was found to possess improved activity against both gram-positive and -negative bacteria. We have determined that the solution structures of Pac-525 bound to membrane-mimetic sodium dodecyl sulfate (SDS) micelles. The SDS micelle-bound structure of Pac-525 adopts an alpha-helical segment at residues Trp2, Arg3, and Arg4. The positively charged residues are clustered together to form a hydrophilic patch. The three hydrophobic residues Trp2, Val6, and Ile9 form a hydrophobic core. The surface electrostatic potential map indicates the three tryptophan indole rings are packed against the peptide backbone and form an amphipathic structure. Moreover, the reverse sequence of Pac-525, Ac-IWRVWRRWK-NH(2), designated Pac-525(rev), also demonstrates similar antimicrobial activity and structure in membrane-mimetic micelles and vesicles. A variety of biophysical and biochemical methods, including circular dichroism, fluorescence spectroscopy, and microcalorimetry, were used to show that Pac-525 interacted strongly with negatively charged phospholipid vesicles and induced efficient dye release from these vesicles, suggesting that the antimicrobial activity of Pac-525 may be due to interactions with bacterial membranes.

Wen S., Majerowicz M., Waring A. and Bringezu F. (2007) Dicynthaurin (ala) monomer interaction with phospholipid bilayers studied by fluorescence leakage and isothermal titration calorimetry. *J Phys. Chem B* **111**, 6280-6287.

Abstract: The interaction of the antimicrobial peptide dicynthaurin (ala) monomer with model membranes of zwitterionic and negatively charged lipids and mixtures thereof was studied by means of isothermal titration calorimetry (ITC), fluorescent leakage, and dynamic light scattering (DLS) measurements. For the ITC analysis, we have applied the surface partitioning equilibrium model which shows that the interaction is predominately driven by hydrophobic effects (K_b between 2×10^4 and 1×10^5 M⁻¹). Under low salt conditions, the enhanced electrostatic interaction leads to larger peptide concentrations immediately above the vesicle surface, which initiates the insertion of the peptide into the bilayer more effectively.

Fluorescent leakage measurements have shown a fast leakage of the fluorescent dye within seconds after peptide addition. The analysis of the leakage kinetics was performed in terms of an initial pore formation model (up to $t = 1000$ s) that takes the reversible surface aggregation of bound peptide monomers into account. From this analysis, a minimum aggregation number of $n = 7 \pm 2$ per pore is obtained.

Wenk M. R. and Seelig J. (1998) Magainin 2 amide interaction with lipid membranes: calorimetric detection of peptide binding and pore formation. *Biochemistry* **37**, 3909-3916.

Abstract: The interaction of the antibiotic magainin 2 amide (M2a) with lipid bilayers was investigated with high-sensitivity titration calorimetry. The enthalpy of transfer of the cationic M2a to negatively charged small unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) (75:25, mol/mol) was measured as $\Delta H = -17.0 \pm 1$ kcal/mol of peptide. The adsorption isotherm was determined by injecting lipid vesicles into peptide solutions at low peptide concentrations ($c_{Po} < 7 \mu\text{M}$). The apparent partition coefficient was K_{app} approximately $1.2 \times 10^4 \text{ M}^{-1}$ at a peptide equilibrium concentration of $1 \mu\text{M}$ but decreased with increasing peptide concentration. The hydrophobic partitioning of M2a into the lipid membrane is modulated by electrostatic effects that arise from the attraction of the positively charged peptide to the negatively charged membrane. Using the Gouy-Chapman theory to correct for electrostatic attraction, the experimental binding isotherms can be explained with an intrinsic (hydrophobic) partition coefficient of $K = 55 \pm 5 \text{ M}^{-1}$ and an effective peptide charge of $z = 3.7-3.8$. The free energy of binding is $\Delta G = -4.8$ kcal/mol. At peptide concentrations $c_{Po} > \text{approximately } 7 \mu\text{M}$, a second effect comes into play, and the titration enthalpies can no longer be explained exclusively by peptide partitioning. The first few injections produce enthalpies of reaction which are distinctly smaller than expected from a pure partition equilibrium, followed by a series of injections with reaction heats larger than expected. After subtracting the enthalpic contribution due to partitioning, the residual enthalpies are endothermic for the first few injections, and exothermic for the consecutive steps. Furthermore, the endothermic excess heat is compensated exactly by the exothermic excess heat; i.e., the excess heat consumed in the first part of the titration experiment is returned during the second part. Endothermic excess enthalpies are observed for total molar peptide-to-lipid ratios of $P/L > \text{approximately } 3.0\%$, whereas exothermic excess heats were seen for $0.7\% < P/L < 3.0\%$. Below $P/L < \text{approximately } 0.7\%$, the binding follows the partition equilibrium. Based on earlier spectroscopic evidence, it is suggested that magainin 2 amide binds to the lipid membrane and forms pores at high peptide-to-lipid ratio, this process being characterized by an endothermic reaction enthalpy. Pore formation is reversed with increasing lipid concentration, and the peptide pores disintegrate. The limiting peptide-to-lipid ratio deduced from titration calorimetry for M2a pore formation is in excellent agreement with spectroscopic methods. The enthalpy of pore formation amounts to $\Delta H = +6.2 \pm 1.6$ kcal/mol peptide or ΔH approximately 25-45 kcal/mol pore if the pore is comprised of 4-7 peptide molecules.

Touze T., Eswaran J., Bokma E., Koronakis E., Hughes C., and Koronakis V. (2004) Interactions underlying assembly of the Escherichia coli AcrAB-TolC multidrug efflux system. *Mol Microbiol* **53**, 697-706.

Abstract: The major Escherichia coli multidrug efflux pump AcrAB-TolC expels a wide range of antibacterial agents. Using in vivo cross-linking, we show for the first time that the antiporter AcrB and the adaptor AcrA, which form a translocase in the inner membrane, interact with the outer membrane TolC exit duct to form a contiguous proteinaceous complex spanning the bacterial cell envelope. Assembly of the pump appeared to be constitutive, occurring in the presence and absence of drug efflux substrate. This contrasts with substrate-induced assembly of the closely related TolC-dependent protein export machinery, possibly reflecting different assembly dynamics and degrees of substrate responsiveness in the two systems. TolC could be cross-linked independently to AcrB, showing that their large periplasmic domains are in close proximity. However, isothermal titration calorimetry detected no interaction between the purified AcrB and TolC proteins, suggesting that the adaptor protein is required for their stable association in vivo. Confirming this view, AcrA could be cross-linked independently to AcrB and TolC in vivo, and calorimetry demonstrated energetically favourable interactions of AcrA with both AcrB and TolC proteins. AcrB was bound by a polypeptide spanning the C-terminal half of AcrA, but binding to TolC required interaction of N- and C-terminal polypeptides spanning the lipoyl-like domains predicted to present the intervening coiled-coil to the periplasmic coils of TolC. These in vivo and in vitro analyses establish the central role of the AcrA adaptor in drug-independent assembly of the tripartite drug efflux pump, specifically in coupling the inner membrane transporter and the outer membrane exit duct.

Tsai F. T., Singh O. M., Skarzynski T., Wonacott A. J., Weston S., Tucker A., Pauptit R. A., Breeze A. L., Poyser J. P., O'Brien R., Ladbury J. E., and Wigley D. B. (1997) The high-resolution crystal structure of a 24-kDa gyrase B fragment from *E. coli* complexed with one of the most potent coumarin inhibitors, clorobiocin. *Proteins* **28**, 41-52.

Abstract: Coumarin antibiotics, such as clorobiocin, novobiocin, and coumermycin A1, inhibit the supercoiling activity of gyrase by binding to the gyrase B (GyrB) subunit. Previous crystallographic studies of a 24-kDa N-terminal domain of GyrB from *E. coli* complexed with novobiocin and a cyclothialidine analogue have shown that both ligands act by binding at the ATP-binding site. Clorobiocin is a natural antibiotic isolated from several *Streptomyces* strains and differs from novobiocin in that the methyl group at the 8 position in the coumarin ring of novobiocin is replaced by a chlorine atom, and the carbamoyl at the 3' position of the noviose sugar is substituted by a 5-methyl-2-pyrrolylcarbonyl group. To understand the difference in affinity, in order that this information might be exploited in rational drug design, the crystal structure of the 24-kDa GyrB fragment in complex with clorobiocin was determined to high resolution. This structure was determined independently in two laboratories, which allowed the validation of equivalent interpretations. The clorobiocin complex structure is compared with the crystal structures of gyrase complexes with novobiocin and 5'-adenylyl-beta, gamma-imidodiphosphate, and with information on the bound conformation of novobiocin in the p24-novobiocin complex obtained by heteronuclear isotope-filtered NMR experiments in solution. Moreover, to understand the differences in energetics of binding of clorobiocin and novobiocin to the protein, the results from isothermal titration calorimetry are also presented.

Walker D., Moore G. R., James R., and Kleanthous C. (2003) Thermodynamic consequences of bipartite immunity protein binding to the ribosomal ribonuclease colicin E3. *Biochemistry* **42**, 4161-4171.

Abstract: Colicin E3 is a 60 kDa, multidomain protein antibiotic that targets its ribonuclease activity to an essential region of the 16S ribosomal RNA of *Escherichia coli*. To prevent suicide of the producing cell, synthesis of the toxin is accompanied by the production of a 10 kDa immunity protein (Im3) that binds strongly to the toxin and abolishes its enzymatic activity. In the present work, we study the interaction of Im3 with the isolated cytotoxic domain (E3 rRNase) and intact colicin E3 through presteady-state kinetics and thermodynamic measurements. The isolated E3 rRNase domain forms a high affinity complex with Im3 ($K_d = 10^{-12}$ M, in 200 mM NaCl at pH 7.0 and 25 degrees C). The interaction of Im3 with full-length colicin E3 under the same conditions is however significantly stronger ($K_d = 10^{-14}$ M). The difference in affinity arises almost wholly from a marked decrease in the dissociation rate constant for the full-length complex ($8 \times 10^{-7} \text{ s}^{-1}$) relative to the E3 rRNase-Im3 complex ($1 \times 10^{-4} \text{ s}^{-1}$), with their association rates comparable (approximately $10^8 \text{ M}^{-1} \text{ s}^{-1}$). Thermodynamic measurements show that complex formation is largely enthalpy driven. In light of the recently published crystal structure of the colicin E3-Im3 complex, the additional stabilization of the wild-type complex can be ascribed to the interaction of Im3 with the N-terminal translocation domain of the toxin. These observations suggest a mechanism whereby dissociation of the immunity protein prior to translocation into the target cell is facilitated by the loss of the Im3-translocation domain interaction.

Wang T. and Wade R. C. (2002) Comparative binding energy (COMBINE) analysis of OppA-peptide complexes to relate structure to binding thermodynamics. *J Med Chem* **45**, 4828-4837.

Abstract: The periplasmic oligopeptide binding component (OppA) of the oligopeptide permease found in Gram-negative bacteria acts as a receptor for peptide transport across the cell membrane and is a potential target for antibacterial drug design. OppA exhibits broad specificity, binding to diverse peptides of 2-5 amino acid residues length. Crystallographic and calorimetric measurements have been carried out by Tame et al. of the binding of 28 peptides of sequence K-X-K to OppA, where X is a natural or nonnatural amino acid. Despite this extensive experimental characterization, a clear relationship between structural and thermodynamic parameters could not be readily identified, with a complicating factor being the observation of varying numbers of water molecules at the binding interface in the different complexes. Consequently, we have applied COMparative BINDing Energy (COMBINE) analysis to derive quantitative structure-activity relationships (QSARs) for these 28 OppA-tripeptide complexes. This is the first application of COMBINE analysis to predict binding enthalpies and entropies, and predictive QSAR models were obtained for these quantities as well as for binding free energies. These QSAR models highlight several protein residues and bound water molecules in the binding site, as well as the electrostatic desolvation energies of the protein and the peptides, as responsible for most of the differences in binding

thermodynamics between the peptides studied. The QSAR models aid rationalization of the determinants of binding affinity of the OppA:peptide complexes and provide guides for further ligand design. This study also points to the general applicability of COMBINE analysis to estimating thermodynamic parameters for protein-peptide complexes.

Wei S. Y., Wu J. M., Kuo Y. Y., Chen H. L., Yip B. S., Tzeng S. R., and Cheng J. W. (2006) Solution structure of a novel tryptophan-rich peptide with bidirectional antimicrobial activity. *J Bacteriol* **188**, 328-334.

Abstract: Trp-rich antimicrobial peptides play important roles in the host innate defense mechanisms of many plants, insects, and mammals. A new type of Trp-rich peptide, Ac-KWRRWVRWI-NH(2), designated Pac-525, was found to possess improved activity against both gram-positive and -negative bacteria. We have determined that the solution structures of Pac-525 bound to membrane-mimetic sodium dodecyl sulfate (SDS) micelles. The SDS micelle-bound structure of Pac-525 adopts an alpha-helical segment at residues Trp2, Arg3, and Arg4. The positively charged residues are clustered together to form a hydrophilic patch. The three hydrophobic residues Trp2, Val6, and Ile9 form a hydrophobic core. The surface electrostatic potential map indicates the three tryptophan indole rings are packed against the peptide backbone and form an amphipathic structure. Moreover, the reverse sequence of Pac-525, Ac-IWRVWRRWK-NH(2), designated Pac-525(rev), also demonstrates similar antimicrobial activity and structure in membrane-mimetic micelles and vesicles. A variety of biophysical and biochemical methods, including circular dichroism, fluorescence spectroscopy, and microcalorimetry, were used to show that Pac-525 interacted strongly with negatively charged phospholipid vesicles and induced efficient dye release from these vesicles, suggesting that the antimicrobial activity of Pac-525 may be due to interactions with bacterial membranes.

Welch K. T., Virga K. G., Whittemore N. A., Ozen C., Wright E., Brown C. L., Lee R. E., and Serpersu E. H. (2005) Discovery of non-carbohydrate inhibitors of aminoglycoside-modifying enzymes. *Bioorg Med Chem* **13**, 6252-6263.

Abstract: Chemical modification and inactivation of aminoglycosides by many different enzymes expressed in pathogenic bacteria are the main mechanisms of bacterial resistance to these antibiotics. In this work, we designed inhibitors that contain the 1,3-diamine pharmacophore shared by all aminoglycoside antibiotics that contain the 2-deoxystreptamine ring. A discovery library of molecules was prepared by attaching different side chains to both sides of the 1,3-diamine motif. Several of these diamines showed inhibitory activity toward two or three different representative aminoglycoside-modifying enzymes (AGMEs). These studies yielded the first non-carbohydrate inhibitor N-cyclohexyl-N'-(3-dimethylamino-propyl)-propane-1,3-diamine (Compound G,H) that is competitive with respect to the aminoglycoside binding to the enzyme aminoglycoside-2"-nucleotidyltransferase-Ia (ANT2"). Another diamine molecule N-[2-(3,4-dimethoxyphenyl)-ethyl]-N'-(3-dimethylamino-propyl)-propane-1,3-diamine (Compound H,I) was shown to be a competitive inhibitor of two separate enzymes (aminoglycoside-3'-phosphotransferase-IIIa (APH3') and ANT2") with respect to metal-ATP. Thermodynamic and structural-binding properties of the complexes of APH3' with substrates and inhibitor were shown to be similar to each other, as determined by isothermal titration calorimetry and NMR spectroscopy.

Wieprecht T., Apostolov O., Beyermann M., and Seelig J. (1999) Thermodynamics of the alpha-helix-coil transition of amphipathic peptides in a membrane environment: implications for the peptide-membrane binding equilibrium. *J Mol Biol* **294**, 785-794.

Abstract: Amphipathic alpha-helices are the membrane binding motif in many proteins. The corresponding peptides are often random coil in solution but are folded into an alpha-helix upon interaction with the membrane. The energetics of this ubiquitous folding process are still a matter of conjecture. Here, we present a new method to quantitatively analyze the thermodynamics of peptide folding at the membrane interface. We have systematically varied the helix content of a given amphipathic peptide when bound to the membrane and have correlated the thermodynamic binding parameters determined by isothermal titration calorimetry with the alpha-helix content obtained by circular dichroism spectroscopy. The peptides investigated were the antibiotic magainin 2 amide and three analogs in which two adjacent amino acid residues were substituted by their d-enantiomers. The thermodynamic parameters controlling the alpha-helix formation were found to be linearly related to the helicity of the membrane-bound peptides. Helix formation at the membrane surface is characterized by an enthalpy change of $\Delta H(\text{helix})$ approximately -0.7

kcal/mol per residue, an entropy change of $\Delta S(\text{helix})$ approximately -1.9 cal/molK residue and a free energy change of $\Delta G(\text{helix})=-0.14$ kcal/mol residue. Helix formation is a strong driving force of peptide insertion into the membrane and accounts for about 50 % of the free energy of binding. An increase in temperature entails an unfolding of the membrane-bound helix. The temperature dependence can be described with the Zimm-Bragg theory and the enthalpy of unfolding agrees with that deduced from isothermal titration calorimetry.

Wieprecht T., Dathe M., Epanand R. M., Beyermann M., Krause E., Maloy W. L., MacDonald D. L., and Bienert M. (1997) Influence of the angle subtended by the positively charged helix face on the membrane activity of amphipathic, antibacterial peptides. *Biochemistry* **36**, 12869-12880.

Abstract: To investigate the influence of the angle subtended by the positively charged helix face on membrane activity, six amphipathic alpha-helical peptides with angles between 80 degrees and 180 degrees, but with retained hydrophobicity, hydrophobic moment, and positive overall charge, were designed starting from the sequence of the antibacterial peptide magainin 2. CD investigations revealed that all analogs are in an alpha-helical conformation in vesicle suspension. The ability of the peptides to induce dye release from negatively charged phosphatidylglycerol (PG) vesicles decreased with increasing angle. However, peptides with a large angle of positively charged residues (140-180 degrees) exhibited a considerably higher permeabilizing activity at zwitterionic phosphatidylcholine (PC) and mixed PC/PG (3:1) vesicles than analogs with a small angle (80-120 degrees). In addition, analogs with large angles were more active in antibacterial and hemolytic assays. The antibacterial specificity of these analogs was decreased. Binding investigations showed that peptide binding is favored by a large angle and a high content of negatively charged phospholipid. In contrast, a small angle and a low negative membrane charge enhanced the membrane-permeabilizing efficiency of the bound peptide fraction. All analogs stabilized the bilayer phase of phosphatidylethanolamine over the inverted hexagonal phase. Therefore, a class L mechanism of permeabilization can be excluded. Furthermore, the analogs do not act by the induction of positive curvature strain or by a "carpet-like" mechanism. Our results are in accordance with a pore mechanism: The membrane-permeabilizing efficiency of analogs with enhanced angle of positively charged residues is reduced due to electrostatic repulsion between adjacent helices within the pore, thus resulting in a decreased pore-forming probability and/or pore destabilization.

Wieprecht T., Beyermann M., and Seelig J. (1999) Binding of antibacterial magainin peptides to electrically neutral membranes: thermodynamics and structure. *Biochemistry* **38**, 10377-10387.

Abstract: Magainins are positively charged amphipathic peptides which permeabilize cell membranes and display antimicrobial activity. They are usually thought to bind specifically to anionic lipids, and binding studies have been performed almost exclusively with negatively charged membranes. Here we demonstrate that binding of magainins to neutral membranes, a reaction which is difficult to assess with spectroscopic means, can be followed with high accuracy using isothermal titration calorimetry. The binding mechanism can be described by a surface partition equilibrium after correcting for electrostatic repulsion by means of the Gouy-Chapman theory. Unusual thermodynamic parameters are observed for the binding process. (i) The three magainin analogues that were investigated bind to neutral membranes with large exothermic reaction enthalpies ΔH of -15 to -18 kcal/mol (at 30 degrees C). (ii) The reaction enthalpies increase with increasing temperature, leading to a large positive heat capacity ΔC_p of approximately 130 cal mol⁻¹K⁻¹ (at 25 degrees C). (iii) The Gibbs free energies of binding ΔG are between -6.4 and -8.6 kcal/mol, resulting in a large negative binding entropy ΔS . The binding of magainin to small unilamellar vesicles is hence an enthalpy-driven reaction. The negative ΔH and ΔS and the large positive ΔC_p contradict the conventional understanding of the hydrophobic effect. CD experiments reveal that the membrane-bound fraction of magainin is approximately 80% helical at 8 degrees C, decreasing to approximately 60% at 45 degrees C. Since the random coil --> alpha-helix transition in aqueous solution is known to be an exothermic process, the same process occurring at the membrane surface is shown to account for up to 65% of the measured reaction enthalpy. In addition to membrane-facilitated helix formation, the second main driving force for membrane binding is the insertion of the nonpolar amino acid side chains into the lipid bilayer. It also contributes a negative ΔH and follows the pattern for the nonclassical hydrophobic effect. Addition of cholesterol drastically reduces the extent of peptide binding and reveals an enthalpy-entropy compensation mechanism. Membrane permeability was measured with a dye assay and correlated with the extent of peptide binding. The level of dye efflux is linearly related to the amount of surface-bound peptide and can be traced back to a membrane perturbation effect.

Wieprecht T., Apostolov O., and Seelig J. (2000) Binding of the antibacterial peptide magainin 2 amide to small and large unilamellar vesicles. *Biophys Chem* **85**, 187-198.

Abstract: The thermodynamics of binding of the antibacterial peptide magainin 2 amide (M2a) to negatively charged small (SUVs) and large (LUVs) unilamellar vesicles has been studied with isothermal titration calorimetry (ITC) and CD spectroscopy at 45 degrees C. The binding isotherms as well as the ability of the peptide to permeabilize membranes were found to be qualitatively and quantitatively similar for both model membranes. The binding isotherms could be described with a surface partition equilibrium where the surface concentration of the peptide immediately above the plane of binding was calculated with the Gouy-Chapman theory. The standard free energy of binding was ΔG_0 approximately -22 kJ/mol and was almost identical for LUVs and SUVs. However, the standard enthalpy and entropy of binding were distinctly higher for LUVs ($\Delta H_0 = -15.1$ kJ/mol, $\Delta S_0 = 24.7$ J/molK) than for SUVs ($\Delta H_0 = -38.5$ kJ/mol, $\Delta S_0 = -55.3$ J/molK). This enthalpy-entropy compensation mechanism is explained by differences in the lipid packing. The cohesive forces between lipid molecules are larger in well-packed LUVs and incorporation of M2a leads to a stronger disruption of cohesive forces and to a larger increase in the lipid flexibility than peptide incorporation into the more disordered SUVs. At 45 degrees C the peptide easily translocates from the outer to the inner monolayer as judged from the simulation of the ITC curves.

Wieprecht T., Apostolov O., Beyermann M., and Seelig J. (2000) Membrane binding and pore formation of the antibacterial peptide PGLa: thermodynamic and mechanistic aspects. *Biochemistry* **39**, 442-452.

Abstract: The antibacterial peptide PGLa exerts its activity by permeabilizing bacterial membranes whereas eukaryotic membranes are not affected. To provide insight into the selectivity and the permeabilization mechanism, the binding of PGLa to neutral and negatively charged model membranes was studied with high-sensitivity isothermal titration calorimetry (ITC), circular dichroism (CD), and solid-state deuterium nuclear magnetic resonance ((2)H NMR). The binding of PGLa to negatively charged phosphatidylcholine (PC)/phosphatidylglycerol (PG) (3:1) vesicles was by a factor of approximately 50 larger than that to neutral PC vesicles. The negatively charged membrane accumulates the cationic peptide at the lipid-water interface, thus facilitating the binding to the membrane. However, if bulk concentrations are replaced by surface concentrations, very similar binding constants are obtained for neutral and charged membranes (K approximately 800-1500 M^{-1}). Membrane selectivity is thus caused almost exclusively by electrostatic attraction to the membrane surface and not by hydrophobic insertion. Membrane insertion is driven by an exothermic enthalpy (ΔH approximately -11 to -15 kcal/mol) but opposed by entropy. An important contribution to the binding process is the membrane-induced random coil --> alpha-helix transition of PGLa. The peptide is random coil in solution but adopts an approximately 80% alpha-helical conformation when bound to the membrane. Helix formation is an exothermic process, contributing approximately 70% to the binding enthalpy and approximately 30% to the free energy of binding. The (2)H NMR measurements with selectively deuterated lipids revealed small structural changes in the lipid headgroups and in the hydrocarbon interior upon peptide binding which were continuous over the whole concentration range. In contrast, isothermal titration calorimetry of PGLa solutions with PC/PG(3:1) vesicles gave rise to two processes: (i) an exothermic binding of PGLa to the membrane followed by (ii) a slower endothermic process. The latter is only detected at peptide-to-lipid ratios >17 mmol/mol and is paralleled by the induction of membrane leakiness. Dye efflux measurements are consistent with the critical limit derived from ITC measurements. The endothermic process is assigned to peptide pore formation and/or lipid perturbation. The enthalpy of pore formation is 9.7 kcal/mol of peptide. If the same excess enthalpy is assigned to the lipid phase, the lipid perturbation enthalpy is 180 cal/mol of lipid. The functional synergism between PGLa and magainin 2 amide could also be followed by ITC and dye release experiments and is traced back to an enhanced pore formation activity of a peptide mixture.

Williams D. H., Searle M. S., Mackay J. P., Gerhard U., and Maplestone R. A. (1993) Toward an estimation of binding constants in aqueous solution: studies of associations of vancomycin group antibiotics. *Proc Natl Acad Sci U S A* **90**, 1172-1178.

Abstract: An approach toward the estimation of binding constants for organic molecules in aqueous solution is presented, based upon a partitioning of the free energy of binding. Consideration is given to polar and hydrophobic contributions and to the entropic cost of rotor restrictions and bimolecular associations. Several parameters (derived from an analysis of entropy changes upon the melting of crystals and from the binding of cell wall peptide analogues to the antibiotic ristocetin A) which may be useful guides to a crude understanding of binding phenomena are presented: (i) amide-amide hydrogen bond

strengths of $-(1 \text{ to } 7) \pm 2 \text{ kJ}\cdot\text{mol}^{-1}$, (ii) a hydrophobic effect of $-0.2 \pm 0.05 \text{ kJ}\cdot\text{mol}^{-1}$. A-2 of hydrocarbon removed from exposure to water in the binding process, and (iii) free energy costs for rotor restrictions of $3.5\text{-}5.0 \text{ kJ}\cdot\text{mol}^{-1}$. The validity of the parameters for hydrogen bond strengths is dependent on the validity of the other two parameters. The phenomenon of entropy/enthalpy compensation is considered, with the conclusion that enthalpic barriers to dissociations will result in larger losses in translational and rotational entropy in the association step. The dimerization of some vancomycin group antibiotics is strongly exothermic ($-36 \text{ to } -51 \text{ kJ}\cdot\text{mol}^{-1}$) and is promoted by a factor of 50-100 by a disaccharide attached to ring 4 (in vancomycin and eremomycin) and by a factor of ca. 1000 by an amino-sugar attached to the benzylic position of ring 6 in eremomycin. The dimerization process (which, as required for an exothermic association, appears to be costly in entropy) may be relevant to the mode of action of the antibiotics.

Wright E. and Serpersu E. H. (2005) Enzyme-substrate interactions with an antibiotic resistance enzyme: aminoglycoside nucleotidyltransferase(2'')-Ia characterized by kinetic and thermodynamic methods. *Biochemistry* **44**, 11581-11591.

Abstract: Aminoglycoside nucleotidyltransferase(2'')-Ia is one of the most often detected enzymes in aminoglycoside-resistant bacteria. Despite its prevalence, little biochemical and biophysical work has been reported for this enzyme. In the current study, substrate specificity and temperature dependence of $k(\text{cat})$ are determined by kinetic assays. Dissociation constants and thermodynamic properties of enzyme-substrate complexes are determined by isothermal titration calorimetry, electron paramagnetic resonance, and fluorescence spectroscopy. Kinetic studies show that aminoglycosides with 2'-NH(2) are better substrates (higher $k(\text{cat})/K(\text{m})$) than ones with 2'-OH when magnesium(II) is used as the catalytically required divalent cation. The activity is reduced 10-fold for substrates with 2'-NH(2) when manganese(II) replaces magnesium as the required metal. However, kanamycin A, which has a 2'-OH, shows a much smaller decrease in activity when manganese substitutes for magnesium as the divalent cation. Temperature dependence studies show the activation energy of catalysis to be 19.2 kcal/mol and the temperature optimum between 30 and 32 degrees C. The binding of the aminoglycoside substrate tobramycin to the enzyme occurs with a favorable enthalpy which compensates for a large entropic penalty to yield a negative ΔG value for the complex formation. Enthalpy of binding is less exothermic in the presence of metal-nucleotide. However, due to the more favorable entropy, a more favorable ΔG is observed for the formation of the enzyme-metal-nucleotide:aminoglycoside complex. Tobramycin binds to ANT(2'') with a dissociation constant of 0.6 μM , which is further reduced by 3-fold when metal-nucleotide is present. Binding of ATP to the enzyme is determined to be very weak in the absence of a divalent cation, and becomes 2 orders of magnitude tighter when magnesium or manganese is present. Binding studies also show that, in addition to binding to the enzyme in the form of metal-nucleotide complex, a second catalytically required metal binds to an additional site on the enzyme.

Wright E. and Serpersu E. H. (2006) Molecular determinants of affinity for aminoglycoside binding to the aminoglycoside nucleotidyltransferase(2'')-Ia. *Biochemistry* **45**, 10243-10250.

Abstract: One of the most commonly occurring aminoglycoside resistance enzymes is aminoglycoside 2''-O-nucleotidyltransferase [ANT(2'')]. In the present study molecular determinants of affinity and specificity for aminoglycoside binding to this enzyme are investigated using isothermal titration calorimetry (ITC). Binding of aminoglycosides is enthalpically driven accompanied by negative entropy changes. The presence of metal-nucleotide increases the affinity for all but one of the aminoglycosides studied but has no effect on specificity. The substituents at positions 1, 2', and 6' are important determinants of substrate specificity. An amino group at these positions leads to greater affinity. No correlation is observed between the change in affinity and enthalpy. At the 2' position greater affinity results from a more negative enthalpy for an aminoglycoside containing an amino rather than a hydroxyl at that position. At the 6' position the greater affinity for an aminoglycoside containing an amino substituent results from a less unfavorable entropic contribution. The thermodynamic basis for the change in affinity at position 1 could not be determined because of the weak binding of one of the aminoglycoside substrates, amikacin. The effect of increasing osmotic stress on affinity was used to determine that a net release of approximately four water molecules occurs when tobramycin binds to ANT(2''). No measurable net change in the number of bound water molecules is observed when neomycin binds the enzyme. Data acquired in this work provide the rationale for the ability of ANT(2'') to confer resistance against kanamycins but not neomycins.

Wu H. Y., Zhang X. L., Pan Q., and Wu J. (2005) Functional selection of a type IV pili-binding peptide that specifically inhibits Salmonella Typhi adhesion to/invasion of human monocytic cells. *Peptides* **26**, 2057-2063.

Abstract: Salmonella enterica serovar Typhi (S. Typhi) is an important pathogen which infects humans exclusively and causes typhoid or enteric fever. Recently it has been discovered that type IVB pili, encoded by the S. Typhi pil operon located in the major pathogenicity island, may be important in the pathogenesis of epidemic enteric fever. To further investigate the roles of type IVB pili of S. Typhi, a 12-mer peptide (RQERSSLSKPVV), binding to the structural protein PilS of the type IVB pili of S. Typhi, was isolated with a ribosome display system. This peptide was designated as peptide R. We found that peptide R inhibited adhesion to/invasion of human monocytic THP-1 cells by piliated S. Typhi bacteria, but had no effects on nonpiliated S. Typhi bacteria. A random 12-mer peptide, of size and solubility equal to peptide R, served as a control on the specificity of peptide R. The specific interaction and binding equilibrium between the 12-mer peptide R and PilS protein was determined by isothermal titration calorimetry (ITC) and a binding constant K_a determined to be between 0.4×10^5 and $2.2 \times 10^5 \text{ L mol}^{-1}$. Our findings suggest that the type IV pili-binding peptide R holds potential as an antibacterial peptide effective against S. Typhi infections, both in terms of prevention and therapeutic treatment. The data further provide insights into the understanding of the pathogenic roles of the type IVB pili of S. Typhi.

Yin N., Marshall R. L., Matheson S., and Savage P. B. (2003) Synthesis of lipid A derivatives and their interactions with polymyxin B and polymyxin B nonapeptide. *J Am Chem Soc* **125**, 2426-2435.

Abstract: Lipid A is the causative agent of Gram-negative sepsis, a leading cause of mortality among hospitalized patients. Compounds that bind lipid A can limit its detrimental effects. Polymyxin B, a cationic peptide antibiotic, is one of the simplest molecules capable of selectively binding lipid A and may serve as a model for further development of lipid A binding agents. However, association of polymyxin B with lipid A is not fully understood, primarily due to the low solubility of lipid A in water and inhomogeneity of lipid A preparations. To better understand lipid A-polymyxin B interaction, pure lipid A derivatives were prepared with incrementally varied lipid chain lengths. These compounds proved to be more soluble in water than lipid A, with higher aggregation concentrations. Isothermal titration calorimetric studies of these lipid A derivatives with polymyxin B and polymyxin B nonapeptide indicate that binding stoichiometries (peptide to lipid A derivative) are less than 1 and that affinities of these binding partners correlate with the aggregation states of the lipid A derivatives. These studies also suggest that cooperative ionic interactions dominate association of polymyxin B and polymyxin B nonapeptide with lipid A.

Yu S., Giroto S., Lee C., and Magliozzo R. S. (2003) Reduced affinity for Isoniazid in the S315T mutant of Mycobacterium tuberculosis KatG is a key factor in antibiotic resistance. *J Biol Chem* **278**, 14769-14775.

Abstract: Catalase-peroxidase (KatG) from Mycobacterium tuberculosis is responsible for the activation of the antitubercular drug isonicotinic acid hydrazide (INH) and is important for survival of M. tuberculosis in macrophages. Characterization of the structure and catalytic mechanism of KatG is being pursued to provide insights into drug (INH) resistance in M. tuberculosis. Site-directed mutagenesis was used to prepare the INH-resistant mutant KatG[S315T], and the overexpressed enzyme was characterized and compared with wild-type KatG. KatG[S315T] exhibits a reduced tendency to form six-coordinate heme, because of coordination of water to iron during purification and storage, and also forms a highly unstable Compound III (oxyferric enzyme). Catalase activity and peroxidase activity measured using t-butylhydroperoxide and o-dianisidine were moderately reduced in the mutant compared with wild-type KatG. Stopped-flow spectrophotometric experiments revealed a rate of Compound I formation similar to wild-type KatG using peroxyacetic acid to initiate the catalytic cycle, but no Compound I was detected when bulkier peroxides (chloroperoxybenzoic acid, t-butylhydroperoxide) were used. The affinity of resting (ferric) KatG[S315T] for INH, measured using isothermal titration calorimetry, was greatly reduced compared with wild-type KatG, as were rates of reaction of Compound I with the drug. These observations reveal that although KatG[S315T] maintains reasonably good steady state catalytic rates, poor binding of the drug to the enzyme limits drug activation and brings about INH resistance.

Zhang Y., Roy S., Jones L. S., Krishnan S., Kerwin B. A., Chang B. S., Manning M. C., Randolph T. W., and Carpenter J. F. (2004) Mechanism for benzyl alcohol-induced aggregation of recombinant human interleukin-1 receptor antagonist in aqueous solution. *J Pharm Sci* **93**, 3076-3089.

Abstract: Benzyl alcohol, an antimicrobial preservative, accelerates aggregation and precipitation of recombinant human interleukin-1 receptor antagonist (rhIL-1ra) in aqueous solution. The loss of native monomer during incubation at 37 degrees C was determined by analysis of sample aliquots with size exclusion high performance liquid chromatography (SE-HPLC). Benzyl alcohol caused minor perturbation of the tertiary structure of the protein without changing its secondary structure, documenting that the preservative caused a minor shift in the protein molecular population toward partially unfolded species. Consistent with this conclusion, in the presence of benzyl alcohol the rate of H-D exchange was accelerated and the fluorescence of 1-anilinonaphthalene-8-sulfonic acid in the presence of rhIL1ra was increased. Benzyl alcohol did not alter the free energy of unfolding based on unfolding experiments in urea or guanidine HCl. With differential scanning calorimetry it was determined that benzyl alcohol reduced the apparent T_m of rhIL-1ra, but this effect occurred because the preservative lowered the temperature at which the protein aggregated during heating. Isothermal calorimetry documented that the interaction of benzyl alcohol with rhIL-1ra is relatively weak and hydrophobically driven. Thus, benzyl alcohol accelerates protein aggregation by binding to the protein and favoring an increase in the level of partially unfolded, aggregation-competent species. Sucrose partially inhibited benzyl alcohol-induced aggregation and tertiary structural change. Sucrose is preferentially excluded from the surface of the protein, favoring most compact native state species over expanded aggregation-prone forms.

Zhao X., Yu S. and Magliozzo R. S. (2007) Characterization of the binding of isoniazid and analogues to Mycobacterium tuberculosis catalase-peroxidase. *Biochemistry* **46**, 3161-3170.

Abstract: The first-line antituberculosis drug isonicotinic hydrazide (INH) is a prodrug whose bactericidal function requires activation by Mycobacterium tuberculosis catalase-peroxidase (KatG) to produce an acyl-NAD adduct. Peroxidation of INH is considered a required catalytic process for drug action. The binding of INH and a series of hydrazide analogues to resting KatG was examined using optical and calorimetric techniques to provide thermodynamic parameters, binding stoichiometries, and kinetic constants (on and off rates). This work revealed high-affinity binding of these substrates to a small fraction of ferric enzyme in a six-coordinate heme iron form, a species most likely containing a weakly bound water molecule, which accumulates during storage of the enzyme. The binding of hydrazides is associated with a large enthalpy loss (>100 kcal/mol); dissociation constants are in the range of 0.05-1.6 microM, and optical stopped-flow measurements demonstrated kon values in the range of 0.5-27 x 10(3) M-1 s-1 with very small koff rates. Binding parameters did not depend on pH in the range 5-8. High-affinity binding of INH is disrupted in two mutant enzymes bearing replacements of key distal side residues, KatG[W107F] and KatG[Y229F]. The rates of reduction of KatG Compound I by hydrazides parallel the on rates for association with the resting enzyme. In a KatG-mediated biomimetic activation assay, only isoniazid generated in good yield the acyl-NAD adduct which is considered a key molecule in INH action, providing a better understanding of the action mechanism of INH.

Zhao Y., White M. A., Muralidhara B. K., Sun L., Halpert J. R., and Stout C. D. (2006) Structure of microsomal cytochrome P450 2B4 complexed with the antifungal drug bifonazole: insight into P450 conformational plasticity and membrane interaction. *J Biol Chem* **281**, 5973-5981.

Abstract: To better understand ligand-induced structural transitions in cytochrome P450 2B4, protein-ligand interactions were investigated using a bulky inhibitor. Bifonazole, a broad spectrum antifungal agent, inhibits monooxygenase activity and induces a type II binding spectrum in 2B4dH(H226Y), a modified enzyme previously crystallized in the presence of 4-(4-chlorophenyl)imidazole (CPI). Isothermal titration calorimetry and tryptophan fluorescence quenching indicate no significant burial of protein apolar surface nor altered accessibility of Trp-121 upon bifonazole binding, in contrast to recent results with CPI. A 2.3 Å crystal structure of 2B4-bifonazole reveals a novel open conformation with ligand bound in the active site, which is significantly different from either the U-shaped cleft of ligand-free 2B4 or the small active site pocket of 2B4-CPI. The O-shaped active site cleft of 2B4-bifonazole is widely open in the middle but narrow at the top. A bifonazole molecule occupies the bottom of the active site cleft, where helix I is bent approximately 15 degrees to accommodate the bulky ligand. The structure also defines unanticipated interactions between helix C residues and bifonazole, suggesting an important role of helix C in azole recognition by mammalian P450s. Comparison of the ligand-free 2B4 structure, the 2B4-CPI structure, and the 2B4-bifonazole structure identifies structurally plastic regions that undergo correlated conformational changes in response to ligand binding. The most plastic regions are putative membrane-binding motifs involved in substrate access or substrate binding. The results allow us to model the

membrane-associated state of P450 and provide insight into how lipophilic substrates access the buried active site.

Zorko M. and Jerala R. (2008) Alexidine and chlorhexidine bind to lipopolysaccharide and lipoteichoic acid and prevent cell activation by antibiotics. *J Antimicrob. Chemother.* **62**, 730-737.

Abstract: OBJECTIVES: Many antibiotics used to treat infections cause release of immunostimulatory cell wall components from bacteria. Therefore, a combination of antimicrobial and endotoxin-neutralizing activity is desired to prevent inflammation induced by destroyed bacteria. Chlorhexidine and alexidine are amphipathic bisbiguanides and could neutralize bacterial membrane components as stimulators of Toll-like receptors (TLRs). METHODS: Binding of chlorhexidine and alexidine to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) was determined by fluorescence displacement assay and isothermal calorimetric titration. Neutralization of the biological effect of LPS and LTA on TLR-activated cellular activation was determined by NF-kappaB reporter luciferase activation on cells transfected with specific TLRs and NO production of murine macrophages in the presence of isolated agonists and antibiotic-treated bacteria. RESULTS: Alexidine and chlorhexidine bind not only to LPS but also to LTA from Gram-positive bacteria. Alexidine has a higher affinity than chlorhexidine for both compounds. Calorimetric titration shows an initial endothermic contribution indicating participation of hydrophobic interactions in LPS binding, while binding to LTA displayed initial exothermic contribution. Both compounds prevent cell activation of TLR4 and TLR2 by LPS and LTA, respectively. The addition of both compounds suppressed NO production by macrophages in the presence of bacteria treated with different types of antibiotics. CONCLUSIONS: Chlorhexidine and alexidine suppress bacterial membrane-induced cell activation at concentrations two orders of magnitude lower than that used in topical applications. Combining biocides with different types of antibiotics prevented macrophage activation in the presence of bacteria and demonstrated the potential of chlorhexidine and alexidine to suppress inflammatory responses caused by activation of TLRs